PART I

NATIONAL INSTITUTE OF DENTAL RESEARCH

ANNUAL REPORT

OFFICE OF THE DIRECTOR

OCTOBER 1, 1977 to September 30, 1978

Compiled by:
Dental Research Data Officer
National Institute of Dental Research
National Institutes of Health

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National Institute of Dental Research

National Institutes of Health

Bethesda, Maryland

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REPORT OF THE DIRECTOR

THE NATIONAL INSTITUTE OF DENTAL RESEARCH

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The NIDR, the primary source of support for dental research in the country, also serves as an international focal point. As a consequence of the Institute's unique role, the Director is called upon in advisory or representational roles and to address various U.S. Governmental and international organizations. For example, this year the Director Dr. Scott was asked to make formal presentations to the Environmental Protection Agency's National Water Fluoridation meeting in Dallas and to their advisory council in Washington; both presentations dealt with dental fluorosis--its cause and characteristics. The Director also serves on the World Health Organization's Oral Research Advisory Group and, therefore, attends the Group's annual meeting and carries out functions for it throughout the year. He also cooperates with the American Dental Association's Council on Dental Research, the International and the American Associations for Dental Research and others.

This year the NIDR Director was the recipient of three awards:

International Award for the Advancement of Dental Research, of the Massachusetts Dental Society
Fred Birnberg Research Medal, of the Association of Dental Alumni of Columbia University, New York.
Honorary Membership in the Royal Society of Medicine (England).

At these awards' ceremonies, he presented formal speeches. Among other major speeches given by the NIDR Director was one on the "Impact of Government-Sponsored Research on Dental Education" given in Jerusalem at the 25th Anniversary Celebration of the Hebrew University School of Dental Medicine; and another at Harvard University's Symposium on Oral Health Aspects of Aging. Speeches were also presented at meetings of the American Cleft Palate Association, the American College of Dentists' Washington Section, and the Public Health Service Professional Association.

In addition to formal presentations made to professional groups, the Director also appeared on two television programs -- a 30 minute interview on NBC's Health Field program, and as a panel member on Metromedia T.V.'s David Susskind Show. His interview with U.S. News and World Report was published in the magazine's "From an Expert" column in June, 1978.

The NIDR this year established an intra-Institute committee on fluoride research initiatives to identiy areas requiring additional study. The committee has set priorities in the field, identified mechanisms to accomplish the research and has assigned responsibilities within NIDR for implementing specific efforts.

A major personnel change in the Office of the Director was the retirement of Mrs. Frances H. Pettinato, Executive Officer, and the selection of her successor Mr. John P. Patterson who is returning to the post after being Deputy Associate Commissioner for Administration, Food and Drug Administration, for the last three years. Also this year the NIDR appointed Ms. Lucille Strickland as its first Assistant Personnel Officer, a position created because the Personnel Officer has dual responsibility for the NIDR and the NINCDS. In June 1978, the planning officer, Mrs. Helen M. Riches also retired.

SPECIAL ASSISTANT TO THE DIRECTOR

Among the duties of the Special Assistant is the responsibility for advising the Director in matters relating to support of the social and behavioral sciences. In that capacity, the Special Assistant prepared and delivered the paper, "Behavioral Research in Dentistry: A Federal Strategy" which was highlighted to keynote the first National Conference on Behavioral Dentistry (University of West Virginia). That document was published in the proceedings of the conference and has been circulated to NIDR staff, the National Advisory Dental Research Council and to interested investigators wishing to pursue NIDR support in this area. Program guidance for the Pain and Behavioral Studies Program Area of the Extramural Programs was further provided on a regular basis including the drafting of a Request for Applications, providing referrals of potential grantees and generating expanded contacts for programming purposes. Specific programming assistance in the substantive content areas of the social and behavioral sciences was provided during this fiscal year to a variety of organizations and institutions.

In addition to providing regular staff support to the Director, the Special Assistant handled special projects including the preparation of NIDR contributions on the subject of prevention for the Department and the preparation of NIDR and the DHEW international dental health initiatives.

The Special Assistant chaired and organized several symposia:

"Meeting the Challenge of the Non-Patient,"
American Dental Association, Miami, Florida, October 1977;
"Primary Dental Care; A New Movement?"
American Dental Association, Miami, Florida, October 1977;
"Quality of Life of Dental Professionals"
Federation Dentaire Internationale, Toronto, Canada, October 1977;
"Developing New Technologies: Opportunities for Assessment and Diffusion". Organized for International and American Associations for Dental Research, Washington, D.C. March 1978;
"Health Occupations: Socialization and Delivery of Health Care."
Organized for International Sociological Association, Upsalla, Sweden, August 1978;
"International Collaboration for Better Dental Health".
Organized for Federation Dentaire Internationale, Madrid, Spain, September 1978.

The following seminar and lecture presentations were delivered:

"USPHS/WHO International Collaborative Study of Dental Manpower Systems in Relation to Oral Health Status", annual Scientific Session, Faculty of Dentistry, Royal College of Surgeons, Dublin, Ireland, November 1977.

"Can Oral Health Behaviors Be Changed?" Meeting of Scottish Health Education and Dental Officers, National Health Service, Edinburgh, Scotland, November, 1977;

Discussant for symposium, "Becoming and Being a Dentist," session of the American Association of Dental Schools, Washington, D.C., March 1977;

"The Federal Role in Social and Behavioral Research in Dentistry," Walter Reed Institute for Dental Research, Washington, D.C., April 1978;

"Social Class, Dental Disease and Tooth Loss," (reactor to panel presentations) Australia and New Zealand Division of the International Association for Dental Research, Wellington, New Zealand, August 1978;

"Social Sciences and Dentistry," New Zealand Dental Association, Christchurch, New Zealand, August 1978;

"A Sociologist's View of the New Zealand Results of the International Collaborative Study of Dental Manpower Systems in Relation to Oral Health Status," New Zealand Dental Association, Christchurch, New Zealand, August 1978;

"Characteristics of Dentists in Australia, Federal Republic of Germany, Japan, New Zealand, Norway and the U.S.", Federation Dentaire Internationale, Madrid, Spain, September, 1978.

Additional public information was provided through a TV news interview (Channel 6 - Miami, Florida, October 10, 1977) on the subject of why people do not go to the dentists. Information was provided to Glamour Magazine on the same subject.

Information was prepared and transmitted to the Committee on International Health, Institute of Medicine, National Academy of Sciences and was published in the report of that committee, "Strengthening U.S. Programs to Improve Health in Developing Countries," (April 1978).

Program data were transmitted to staff of the Institute of Medicine's Committee on Health Services Research and that material served as reference background material for their consideration of dental health services research.

The Special Assistant serves as a member of the Institute of Medicine's Committee on Dental Options for National Health Insurance which met several times during this fiscal year.

A substantial portion of time was devoted to monitoring the research contract (HRA), "USPHS/WHO International Collaborative Study of Dental Manpower Systems in Relations to Oral Health Status," as well as the replication project of that study in Poland. Duties associated with those projects now active in nine countries included previously mentioned activities, contract activities, site visits to WHO headquarters in Geneva in January and September 1978, and data transfer and analysis facilitation to U.S. Federal facilities.

Organizational commitments were honored during the year and are listed below:

- Program Committee, 8th International Congress on Oral Biology (1979);
- Planning Committee for 6th International Conference on Social Sciences and Medicine (1979);
- Coordinator, DC-Baltimore Area members of Behavioral Scientists in Dental Research (monthly);
- Chairperson, Dental Public Health, Council on Scientific Session, American Dental Association, (1977-78);
- Consultant to Commission on Classification and Statistics for Oral Conditions and Commission on Public Dental Health Services, and Scientific Assembly Committee, Federation Dentaire Internationale, (1977-78);
- Program Committee for Behavioral Scientists in Dental Research, (FDI-1979 and IADR-1980);
- Councillor, Behavioral Science Group. American Association for Dental Research, 1977-78;
- Chairperson, Committee on Health Promotion, International Association for Dental Research, 1978;
- Chairperson, Membership Committee, American Association for Dental Research, 1978;
- Chairperson, Honorary Membership Committee, Behavioral Science Group, American Association for Dental Research, 1977-78;
- Nominating Committee, Medical Section, American Sociological Association, 1977-78;
- Local Arrangements Committee, International and American Associations for Dental Research, 1977-78.

Reviews of submitted articles were provided to the Journal of Preventive Dentistry and the journal, Sociology of Work and Occupations as were proposals submitted to WHO and to the Social Science Research Council (London, U.K.).

The Special Assistant served on the Director's 'ad-hoc' committee for the evaluation of the NIDR training investment. Because of the retirement of the Planning Officer in June, 1978, the Special Assistant assumed the duties associated with Planning and Evaluation functions of that office, a major activity being the evaluation of the National Caries Program. A contract was awarded at the end of the year for the support of this evaluation.

The following articles were published during the current fiscal year:

"Social Sciences Research: Ethical and Policy Implications," Community Dentistry and Oral Epidemiology, 1977, 5:257-272.

"The International Collaborative Study", International Dental Care Delivery Systems: Proceedings of a Colloquium, John Ingle and Patricia Blair, editors, National Academy of Sciences: Ballinger Books, August 1978.

PLANNING OFFICER

The Planning Officer devoted a major effort to the development of an evaluation strategy for the NIDR National Caries Program. The strategy was critiqued by outside consultants including experts in dental caries research and specialists in evaluation design.

During the year, the Planning Officer prepared the following major reports:

The NIDR Forward Plan, FY 1980-84
Narrative for the Zero Base Budget, FY 1980
Design of an Evaluation Strategy for the National Caries Program
Request for Proposals (RFP) for the Evaluation of the NIDR National Caries Program
NIDR Evaluation Plan, FY 1979

Responses were prepared to ad hoc requests from NIH/OD, emanating from Congress, OMB, HEW, and others.

A briefing on Program Planning was presented to an NIH Grants Associate Seminar and another briefing on Evaluation was presented to an NIDR Workshop on Craniofacial Anomalies Objectives.

In June, 1978, Mrs. Helen M. Riches, the Planning Officer, retired.

OFFICE OF SCIENTIFIC AND HEALTH REPORTS

In addition to administering the information program of the NIDR, the Information Officer spent April 1978 on detail at the White House to provide public affairs help to the President's Commission on Mental Health. She also served on several NIH Information committees including a new work-output liaison committee, which is devising ways to measure productivity of NIH Information Offices; the Publications Study Group, composed of seven Information Officers (elected by peers) who will develop guidelines on NIH publications; the NIH Printing Liaison Committee, which is updating the guidelines for NIH printing activities; and the Information Training Committee, which organizes workshops and seminars for mid- and senior-level public information specialists.

Nutrition

This year the NIDR Information staff has paid special attention to activities in nutrition and nutrition education. Both the scientific and general press expressed great interest in the subject, and the staff provided a great deal of information to reporters throughout the year. Resulting stories were carried in newspapers such as the Los Angeles Times and news and women's magazines. As an example, the Washington Post carried a feature on the controversy over sugary cereals and tooth decay; the story quoted two scientists from the National Caries Program.

The Information staff promoted the publication of two proceedings in the field: Taste and Development: The Genesis of Sweet Preference and Sweeteners and Dental Caries.

As interest in nutrition increased, the Information office began efforts to produce a television public service announcement directed at children to let them know about the dangers of snacking on sweet foods. We hope to complete the first spot in a few months and plan 2 to 3 others to follow up. A leaflet for children and parents will be available to provide more information. Plans for pretesting and some evaluation of the TV efforts are also being made, as are other elements of a broad campaign.

Through contacts made to distribute publications, the staff learned of a nutrition education project planned by the NHLBI high blood pressure education progam and Giant Foods. Both were eager to have NIDR participation in the program, especially with respect to sugary foods. Further discussions are needed to work out details.

The Information office has also prepared reports for the NIH Nutrition Coordinating Committee and the Diabetes National Commission on nutrition education.

Press Activities

This year NIDR was involved with two press briefings, for which the Office either assisted in planning or with press contacts. In November 1977 the National Caries Program announced the postponement of clinical trials of a xylitol-sweetened chewing gum because English testing suggested that the sugar alcohol produced tumors in laboratory animals. The press conference held at the State University of New York in Stony Brook resulted in articles in most of the major newspapers throughout the country. Press interest went beyond the xylitol story and extended into the search for noncariogenic artificial sweeteners and other anti-cariogenic approaches. Papers reporting the xylitol study included the New York Times, the Washington Post, and the Wall Street Journal.

The second press briefing was held at NIH in July 1978 to report that fluoride mouthrinsing programs were ready for implementation nationwide in schools in areas without fluoridated water. The briefing,

called at short notice, attracted key science writers from wire services and newspapers. Coverage on the story was extensive and included the Washington Post, the Evening Star, the New York Daily News, and the Chicago Tribune. In addition, the AP and UPI stories received wide coverage. The New York Times used the news as a basis for a story on progress being made toward dental health for all Americans.

This Office arranged for articles concerning dental research in both professional journals and lay magazines. The Journal of the American Medical Association carried two NIDR items in their "From the NIH" column - one on NIDR research showing that interferon may be responsible for asthma attacks that follow viral infections and the other on the adverse effects antacids have on bone.

The Pharmacy Times carried an article, written by Dr. Philip Swango of the National Caries Program, about topical fluorides. The American Dental Association publications, their journal, newspaper, and leadership bulletin carried numerous NIDR stories. The Journal of the American Osteopathic Association also carried several NIDR articles.

The U.S. News and World Report carried an article on their interview of Dr. Scott, NIDR Director. The question and answer session touched on nearly all fields of dental research.

Lay magazines requesting our assistance on dental articles include Current Health, Apartment Life, Glamour, and Vogue. The Better Homes & Garden Family Medical Guide asked our advice for re-writing and updating their lengthy section on dentistry. We went over the old version and suggested deletions and additions needed to make the section current and correct. The Office also assisted World Book/Child Craft, Prevention and other magazines by providing photographs on various aspects of dental research.

For several international meetings, the Office prepared press summaries of NIDR papers. Meetings included those of the International Association for Dental Research, the Second World Congress on Pain, and the Federation Dentaire Internationale.

Audio-Visual Activities

This year extensive efforts were undertaken to produce audio-visuals in addition to traditional press-oriented activities. Arrangements were made for two television and two radio broadcasts. The NIDR Director was on a panel discussing dentistry on the David Susskind show, and also a one-half hour interview on the Health Field show which was carried on NBC network TV throughout the country. The Field show covered many aspects of dental research as well as oral hygiene and dentistry.

The Office developed for distribution a 30-second TV spot "Sealants for Us Kids," which informs children and parents of the protection against tooth decay that adhesive sealants offer to children's most vulnerable

tooth surfaces. The spot was distributed to over 700 television stations. Response cards from over 30% of the program directors of the TV stations indicated that the spot was well received and was used on a rotation basis in children's prime-time viewing hours. Program directors commented that the announcement was informative, interesting, and that additional announcements on dental subjects and for children would be welcomed.

The director of the American Chemical Society's radio program "Man and Molecules" also came to the NIDR to tape record 30-minute interviews with the Director, Dr. Scott, and with a National Caries Program investigator and administrator, Dr. William Bowen.

Two 30-second spot announcements, one on canker sores and fever blisters and another on dental sealants, were aired on radio stations throughout the country. As a result, many requests for our pamphlets on the subject were received.

The Office worked with the NCP to contract out the production of four films on self-applied fluoride programs in schools. The films will be used to train parents, teachers, school administrators, and dental and medical professionals and students, and others on various aspects of the programs.

Three existing NIDR films on dental sealants, vaccine against caries, and topical fluorides continued to be distributed widely this year by the National Medical Audiovisual Center in Atlanta and by the American Dental Association.

The Institute's Exhibit program was active again this year, with five exhibits shown at 20 different meetings and conventions. One exhibit on early detection of oral cancer received an award from the American Medical Association at its annual meeting.

Two new exhibits were designed, built, and shown this year. One describes new research on the microbiology of periodontal diseases and the other is a fold-up exhibit which describes school-based fluoride programs. Slides were prepared for a variety of uses including some on the organization of HEW, PHS and the NIDR that were prepared for Dr. Frazier, Chief, Soft Tissue Stomatology and Nutrition Program Branch, NIDR. The slides prepared will serve as a base for an NIDR slide show being planned.

The Information Office assisted the NIH Project REACH staff in securing proper speakers for an educational satellite TV program on oral cancer conducted at the U. of Colorado, and another on pain carried out by the U. of South Carolina. Dr. Dubner participated in the latter.

Publications

To give our information materials new identity and a family resemblance, we worked with the NIH's medical arts staff and with their contractor to

design new formats, designs, and a logo. Publications redesigned this year include "NIDR Research News," "NIDR Abstracts," "Key Scientific and Administrative Staff," "Canker Sores and Fever Blisters," "Grant and Contract Research Programs of the NIDR," "NIDR Research Programs," and a hanging mailer used throughout the Institute when publications are sent and when individual letters are not required.

One of the Office's periodicals also has a modified content. "NIDR Research News" is our new periodical which is issued at the time of newsworthy events, instead of the monthly or quarterly basis of its predecessor periodicals--"Research News for NIDR" and "Research Capsules." The new Periodical has sections on research progress, conferences, new publications and films. It is sent to newspaper and magazine science writers, editors of dental journals and others who have expressed an interest in dental research items.

This year we extensively revised the extramural flyer, now entitled, "Grant and Contract Research Programs of the National Institute of Dental Research" in time for distribution at the 1978 meeting of the International Association for Dental Research.

A popular leaflet, "Rx for Sound Teeth," also was redesigned to provide appealing illustrations. The leaflet has been distrubuted widely in supermarkets, through the Consumer Information Exchange in Pueblo, Colorado, and through other outlets.

"Good Teeth For You and Your Baby," a booklet developed as a joint publication with the National Association of Community Health Centers, will be distributed through approximately 200 neighborhood health centers and through supermarket racks in specified neighborhoods during the coming year. The leaflet, which offers practical suggestions for self-help toward the attainment of dental health for children and expectant mothers, will be available in English and in Spanish versions.

Another popular pamphlet "Canker Sores and Fever Blisters" has been updated and redesigned this year. It, too, will receive wide distribution next year.

This Office assisted scientific staff in obtaining concept clearance and in readying the following manuscripts for printing: Taste and Development: The Genesis of Sweet Preference, Alternatives to Gold Alloys, and Rod Anode X-Ray Sources in Dentistry.

The Office has continued to issue its regular publications and contributes to those of the NIH. "NIDR Abstracts" which contains summaries of scientific papers published by NIDR investigators is prepared and distributed each quarter to scientists all over the world who have asked to receive the publication. Each year this Office puts together the NIDR's directory entitled "Key Scientific and Administrative Staff."

NIH publications to which we contribute include the booklet Guide to NIH Programs and Awards, and NIH Scientific Directory/Annual Bibliography, the NIH Almanac, and the NIH Publications List. Also, we prepared material for the PHS Dental Recruitment Manual and other PHS and Department reports. In addition, we contributed NIDR brochures to the NIH Visitor's Center and for use at the DHEW 25th Anniversary celebration.

The Office also has prepared a leaflet on tetracycline-stained teeth to be used in answering public inquiries and, more generally, to inform the public of the danger of permanent staining from the antibiotics.

In addition, this Office has begun developing several new publications. One "Your Teeth and Your Health" explains to Clinical Center patients the importance of dental care to their medical treatment and offers practical suggestions for daily oral hygiene.

Other new publications will outline opportunities for careers in dental research--especially for minorities and women. The Scientific Director and the Office of Personnel and the Equal Employment Opportunity Office have advised us on this endeavor.

Our Office is readying another new publication that will contain abstracts and lists of publications from recent studies in dental behavior and sociological research. This project is being done in close collaboration with and under the direction of Dr. Lois Cohen, Special Assistant to the NIDR Director.

Special efforts were made in editing a 1,000-page book, Fluorine in Stomatology and Hygiene, in readying the manuscript for printing, and in developing an appropriate mailing list for distributing this English translation of a Russian manuscript. The book represents a 20-year study of the subject. The compendium contains information not available in other sources and, therefore, should prove useful to health professionals in promoting research and other activities needed in this field.

Editorial and Public Inquires Activities

The Office handled clearance for some 165 manuscripts and 190 abstracts during the year. Approximately 80 percent of the manuscripts were edited by science writers in this office. On request, we also provided editorial advice to various scientific staff in the preparation of major manuscripts and speeches.

Staff participated in the preparation of various NIDR budget statements throughout the year, as well as reports on various subjects.

The Office of Scientific and Health Reports received more than 3,000 letters and 1,000 telephone calls from the general public. Most could be handled by sending out some 119,000 publications but 425 required individual letter responses.

During several months of 1977 and 1978, the fluoride specialist collected published and unpublished information relevant to a highly publicized controversy about cancer rates in fluoridated and non-fluoridated communities. Study reports, technical correspondence, interim research summaries, hearings testimony, and other information pertinent to the issue were provided to a number of organizations with whom information exchanges had been arranged or who had expressed a need for the documentation service, such as:

USEPA Criteria and Standards Division (Water)
The Center for Disease Control
The Congressional Research Service
The House Committee on Government Operations
The American Dental Association
The Canadian Dental Association
The British Dental Association
The World Health Organization
Swedish Government Committee on Fluorides and Dental Caries
Hadassah Medical School

As Chairman and member of an interagency panel, Mr. Small reviewed several proposals made to the HRA Division of Dentistry. The proposals were for the development of a methodology for research into the effects of long-term community water fluoridation on the dental care demands of older adults. He also reviewed and provided critiques of a number of draft or interim manuscripts on subjects concerning fluorides and health, in response to requests made by other Federal agencies.

A proposal submitted to the Environmental Protection Agency for the development and interpretation of additional epidemiological data on dental fluorosis in humans as related to water-borne fluorides.

Drafts of two papers, prepared by consultants to the Chief Dental Officer, on dental fluorosis and the fluoride concentration limits for drinking water prescribed by the National Interim Drinking Water Regulations.

An editoral, prepared by a committee on NIAMDD advisors and staff for publication in the Journal of the American Medical Association, to inform physicians on use of fluoride compounds in treating bone diseases in which demineralization occurs.

To give assistance to the GAO in the preparation of their NIDR report, specialist assisted in collection of reference material and provided technical and editorial comments on a draft on information about the toxicity and safety of fluorides as used in dentistry and dental public health measures.

Specialist worked with the dental staff of the Office of the Assistant Secretary for Health and other consultants in the preparation of a

legislative proposal and supporting documentation for Federal promotion and funding of preventive dental health measures, including community and school water fluoridation, for consideration and action by the Secretary. The Secretary requested the proposal for inclusion in a grouping of preventive health initiatives to be advocated by the Administration.

Specialist provided background and current information on the effectiveness and safety of community water fluoridation to a Swedish government-appointed committee on caries prevention. Relevant documentation and answers to specific questions were transmitted through the office of the Secretary of the Embassy of Sweden over a period of several months following an initial meeting at the Embassy.

In January 1978, the fluoridation specialist offered assistance to the program chairman of a scheduled international symposium on the biological and medical uses of fluoride compounds. The two-day symposium will be part of the national meeting of the American Chemical Society in Honolulu in April 1979. The specialist provided a listing of researchers and clinicians involved with the health aspects of fluorine compounds, assisted the chairman in making contacts, and provided consultation concerning the relative importance of subjects suggested for the symposium. In May, the chairman met at NIH with staff members from Institutes with an interest in fluorides and health (NIDR, NIAMDD, NIA) to develop the program. The chairman has invited the Director of NIAMDD to chair the symposium session on clinical uses of fluorides. An NIDR researcher (Dr. Edward Eanes) will be one of the fourteen speakers invited from several countries to present research findings.

The fluoridation specialist established a resource file on fluorides and health by consolidating existing files in OSHR, expanding the indexing to cover a broader range of finely specified subjects, obtaining additional literature items from several sources, and establishing individual and institutional contacts to help to keep the information current and complete.

Fluoridation specialist is a member of NIDR fluoride studies committee, which has responsibility for developing, recommending, and seeing to it that research proposals concerning fluorides and health are performed.

FINANCIAL MANAGEMENT OFFICE

The Financial Management Office has the responsibility for the Institute's total budget program. Serving as the Institute's center for budget data and related information, this Office's major activities include the formulation of budget estimates--including the planning, development and review of funds required to support operating programs and future plans; the preparation and presentation of budget estimates; the determination of funds required within authorized limits; the administration of apportionments, allocations and allotments; and the management controls over obligations and the expenditure of funds.

Although budget administration is cyclic, the activities and work operations are continuous. The Financial Management Office provides responses to requests for program data from Congress, OMB, and other federal and non-federal agencies and engages in such diverse activities as budget execution; maintenance of payroll records, including corrections and additions; preparation of routine and special reports, such as reports on special management projects; provision of grant forecasts; and preparation of special reports concerning the NIDR's research relating to that of other Institutes; and on utilization of funds by the intramural program. Monthly status reports on personnel and the rate of expenditures for program activities issued by this office provide administrative program directors with a reliable guide which can assist them in the management of personnel and financial resources.

During this year, the NIDR Financial Management Office experienced its second encounter with Zero Base Budgeting with a new and extensive format named SATT (Science Base, Application, Transfer and Training) to be used for the 1980 budget formulations. Zero Base Budgeting, introduced to the United States Government by President Carter, is designed to encourage collaboration among program managers and administrators, at all levels, in the resource allocation decision-making process. The process is a lengthy one, requiring a great deal of planning and cooperation. In addition to preparation of the fiscal portion of the submission, the Financial Management Office coordinated other materials needed for submission.

The NIDR Office of Financial Management has continued to contribute to the overall programs of NIH by actively participating in the training programs for budget personnel. This Office has worked with three trainees during FY 1978. Experience gained at NIDR should prove beneficial in preparing them for positions in budget offices.

PERSONNEL OFFICE

The Personnel Office serves as the focal point within the Institute for personnel management services. Its activities encompass several major areas including: staffing and placement, position classification, employee relations, employee training and development, and organizational change. During the past year, Personnel Office efforts focused on position classification, communication with Institute personnel, and improved internal operation.

In March 1978, an Assistant Personnel Officer, NIDR, was appointed to serve on a full-time basis since the Personnel Officer's time is divided between two Institutes. Shortly thereafter, a part-time clerk-typist transferred to another agency and her position was filled by a full time Personnel Clerk. In addition to these changes, a senior Personnel Management Specialist from NIAID filled the vacancy created by a staff member who transferred to one of the DPM branches. Staff assignments were also realigned into "teams" consisiting of a

Personnel Management Specialist and a Personnel Clerk, who provide day-to-day service to designated program areas of the Institute.

In a continuing effort to improve the operation of the office, the Senior Personnel Assistant developed a "Standard Operating Procedures Manual" covering all personnel actions processed for the Institute. Also, staff began to update and correct the data base of the Automated Retrieval Management System (ARMS) and, through training sessions conducted by the NIDR Data Processing Systems and the DPM Analysis Section, increased its capability to query the system. These accomplishments have resulted in increased efficiency, a better record of personnel activity, a more timely Biweekly Status Report on Personnel Actions and more useful and reliable information for Institute management officials.

Early in the year a Public Health Service team conducted audits of several positions throughout the Institute. Their final report concurred in the classification of these positions with one exception; decision on a secreterial position was deferred until the Factor Evaluation System (FES) standard for these positions is issued.

Other FES activity included a test application of draft FES standards for the GS-160 EEO series. This test was carried out jointly by the Personnel Office and the NIDR EEO Coordinator and submitted through channels to the CSC. Final GS-681, Dental Assistant FES standards were received and will be implemented during the coming year.

In support of Dr. Nylen's commitment to EEO in the intramural program, this office along with the EEO Office is advising the Information Office in developing a recruitment brochure to be used to attract and hire minorities, women and the handicapped into Institute intramural positions. The brochure will cover various access routes to careers in dental research being carried out and/or supported by the NIDR. Intensive recruitment efforts will be launched during the coming year.

Due to initiatives from DHEW, exemplified by the Three-Year Position Classification Review, intensive effort was directed to the area of position classification. One-third of the positions in the Institute were audited with emphasis on verification and correction of position descriptions and on position management. Personnel office staff worked closely with the staff of the NIH Division of Personnel Management to identify and to solve classification problems. Between 60 and 70 per cent of the staff's time was spent in position classification and related matters.

Factors both internal and external to the Institute will necessitate continued emphasis by the Personnel Office in the areas of position classification, communications, and internal operating procedures. In the coming year, special attention will need to be directed to FES training; to the integration of the DHEW payroll and personnel systems;

and finally, to the Federal Executive Agency Guidelines on employee selection procedures--an activity which has potential for absorbing considerable staff resources.

EEO PROGRAM

An assessment report of equal employment opportunity in NIDR was prepared for the period ending 12/31/77. It covers organization and resources, discrimination complaints, recruitment, utilization of skills and training, upward mobility, supervisory and management commitment, community outreach, program evaluation, NIDR employee profiles by race and sex and a separate report on Hispanic employment. The report is available for review in the NIDR EEO Coordinator's Office. An updated assessment and an accomplishment report of the 1977-1978 NIDR Affirmative Action Plan, completed in September 1978, will be used to identify problem areas, set objectives and develop action items for a new multiyear plan which is consistent with the NIH Plan. A report on age requirements in NIDR-supported programs was prepared by the EEO Coordinator's Office to be used for HEW regulations on implementation of the Age Discrimination Act of 1975.

The NIDR EEO Coordinator was appointed to serve as the Institute's Contracts Compliance Coordinator to work with the NIH Contract Compliance Coordinator and the NIDR Contract Officer on responsibilities under the NIH Civil Rights Program.

A new NIH EEO Counselor from NIDR will be appointed by the Director, NIH, on 10/1/78. Suggested nominations were requested from all employees. Nominations, endorsed by the NIDR EEO Advisory Committee, were submitted by the Director of NIDR to the NIH Division of Equal Opportunity. One c complaint of dicsrimination was made by an NIDR employee during the year and was resolved at the informal stage.

The NIDR EEO Advisory Committee set priorities on issues of concern, worked on internal reorganization and concentrated on communication to and from employees. High priority issues were communication, supervisory training, recruitment mechanisms, evaluation of affirmative action progress, classification reviews, employee training and career development. The Committee established a new subcommittee on women's concerns which is chaired by the NIDR delegate to the NIH Women's Advisory Committee. Committee officers met with a consultant to the NIH Women's Advisory Committee to discuss Committee and Subcomittee operations. The Committee's revised election procedures by building were adopted to provide full employee participation. Following elections, a bylaws provision is used to appoint members who balance the representation of Institute employees by race and sex. The Committee encouraged the establishment of EEO information boards in different buildings for posting of EEO Committee and other announcements which we distribute.

Internal Communication

The NIDR EEO Coordinator and EEO Counselor were contacted by an average of 20 employees and potential applicants each month who sought information on such issues as job applications, classification or training, supervisory-employee relations, and child care.

In October 1977, the NIDR EEO Office distributed a Civil Service Commission questionnaire on equal employment opportunity to all Institute employees. Completed questionnaires from 152 employees were sent to the CSC for tabulation and analysis. The results are available in the NIDR EEO Coordinator's Office.

A meeting on equal employment opportunity was held for all NIDR employees on May 1, 1978. Presentations, based on suggestions solicited from employees, included efforts to increase minority and female intramural scientists, promotion of research support personnel, status of the 3-year classification review, training for employees and supervisors, awards, procedures for grievance and discrimination complaints, the role of the EEO Advisory Committee and affirmative action. Four NIDR employees received EEO achievement awards. Results of a questionnaire sent to Institute employees following this meeting are available in the NIDR EEO Coordinator's Office and will be used to plan future meetings with Institute employees.

The NIDR Secretarial Training Activities Group recommended and coordinated a meeting open to all Institute employees on "Sexual Assault Prevention." Members of the Montgomery County Police Department made the presentation. During National Secretaries Week, the group also planned and coordinated a workshop on "Assertiveness Training" for NIDR secretarial and clerical employees.

An announcement of new and revised procedures for filing discrimination complaints based on age or on physical or mental handicap, and for filing class action complaints was sent to all NIDR employees in September 1978.

The NIDR EEO Coordinator's Office prepared EEO reference notebooks for EEO Advisory Committee members, the Director, Executive Officer, Personnel Officer and Associate Directors. These notebooks include information on EEO laws and regulations, affirmative action, the NIDR EEO Committee, relevant NIH Manual Issuances and employee information pamphlets.

Recruitment and Outside Contacts

The NIH established minority employment goals of 10% for the undergraduate and 5% for the graduate summer programs. The NIDR employed 9 students in each program: 1 student in each program was a minority. The Institute employed a total of 28 employees in 7 summer programs: 14 were female and 3 were minorities.

Among activities engaged in to meet the goals, (1) Representatives of the Minority Biomedical Support Program and the Minority and Women's Staffing Section, DPM, met with the Intramural Laboratory and Branch Chiefs to discuss mechanisms for increasing the numbers of minority and female scientists in NIDR programs. (2) The EEO Coordinator together with the Personnel Office advised the Scientific and Health Reports Office in developing a publication about opportunities in NIDR programs. (3) The NIDR EEO Coordinator's Office prepared a list of minority colleges and universities and contacts at those schools to be used for distribution of information about NIDR programs and for planning NIDR staff visits to those schools.

Also, the EEO Coordinator attended the 1978 Incorporated Mexican American Government Employees (IMAGE) Convention and the 1978 National Dental Association Meeting to establish contacts and gain information from these minority organizations. The NIDR delegate to the NIH Women's Advisory Committee (WAC), the Executive Officer and the Assistant Personnel Officer attended the 1978 Federally Employed Women (FEW) Convention. The Coordinator and the Counselor attended a training session for the NIH EEO Council and a CSC Course on advanced counseling. The WAC delegate attended a WAC training program and the Coordinator attended part of that program.

DENTAL RESEARCH DATA OFFICER

This office is a specialized information center for dental research. It collects, analyzes, processes and reports on the substantive and statistical facts related to dental research. This function of the office is recognized by a variety of information seekers; and while its primary responsibility is to the Director of the Institute and NIDR staff, over half of the queries handled by the office come from outside of the Institute. Its existence as a resource for dental research information has appeared in several directories and most recently in the first edition of the Medical and Health Information Directory (Kruzas, A. T., Detroit, Gale, 1977).

The Dental Research Data Officer is also the Privacy Act and Freedom of Information Act Coordinator for the Institute. Both Acts have required a significant amount of time. The number of requests has continued to increase each year.

Other responsibilities of the DRDO include Institute representation on the NIH Clinical Trials Committee; the NIH Group to Coordinate Cost of Illness Studies and a subcommittee of that Group to develop a Bibliography on the Social and Economic Costs of Illness; the NIH Working Group on Data Policy and Coordination; NIH Coordinating Committee on Maternal and Child Health; and the NIH BAD/SATT Coding Project.

The processing activities of the office are varied. They range from the collection and organization of data; through coding and classification to identification of data elements for compilation and reporting. Files and indexes are maintained for immediate reference. All intramural projects, contracts, interagency agreements, and sub-projects of large parent grants are given coding attention beyond that received in the usual NIH/DRG procedure. Certain documents are collected, edited, coded and forwarded to other data bases. All such activitites are pursued in the interest of developing the best, most comprehensive, and current dental research data available.

Like all information centers, we have users requesting data. Most often, these users are our own staff or other government agencies and the data are used for planning or budget decisions. With the special initiatives and trans-NIH issues, the level of research support related to Arthritis, Cancer, Diabetes, Epidemiology, Genetics, Heart, Lung and Blood, and others have become annual requests. Other subject areas, like Arctic Research, Indian Health, and Marine Biology, have also occurred on an annual basis. But the most challenging requests are those that are made once or infrequently. Some examples of subjects for this year include: Acupuncture, Analgesics, Anthropology, Hypnosis, Implants, Neuroscience, Plaque and Anti-plaque, and Ultrasound.

For our own convenience, for staff use, for archival purposes and to assist in the dissemination and coordination of dental research facts and figures, we compile and print a number of reports on a fiscal year basis. These include the NIDR Annual Report, which will contain Clinical Trials material this year; Dental Research in the United States and Other Countries, with expanded geographic coverage; Dental Research Charts and Tables, with review by the DOD and VA for greater accuracy; Dental Research Institutes; Index to Dental Research Projects; NIDR Programs; Selected List of Technical Reports; and NIDR Trainees and Fellows. With the exception of Dental Research in the United States and Other Countries and NIDR Programs, which are printed by the Government Printing Office, all of these reports are compiled and prepared for printing at NIH by the DRDO.

On a very limited scale, an SDI service (Selective Dissemination of Information) is provided to NIDR staff. It consists of forwarding copies of new research projects, not supported by NIDR, to the appropriate extramural program chief and a selected list of technical reports to those who are interested and have requested copies. Both of these distributions occur about once a month and help to expedite the transfer of scientific information related to dentistry.

Operating in the manner of a "switching station," the office places a great deal of dependence on the data collecting and processing activities of other sources. Concomitant with our need, the Data Processing Systems and Analysis Section of NIDR is probably the number one supplier of data. This, of course, is closely coupled with the Statistics and Analysis Branch and the Data Processing Section of the

Division of Research Grants. Our number two supplier of ongoing dental research data is the Smithsonian Science Information Exchange. For completed and published research activities we rely on the National Technical Information Service, the National Library of Medicine, and the American Dental Association Bureau of Library and Indexing Services. Most of the epidemiological and general statistical data comes from the National Center for Health Statistics or the ADA's Bureau of Economic Research and Statistics. A large number of other sources are available for specialized requests.

The working tools of the office include a small reference collection of directories, encyclopedias, indexes and related monographs; bound reports and computer printouts; vertical files; a microfiche reader/printer; and an IBM mag Card Selectric typewriter that serves as a computer terminal. We do use the terminal for our own data base and can use it for others but most of the computer searching is done for us by the Data Processing Systems and Analysis Section of NIDR, the Statistics and Analysis Branch of DRG, or the Smithsonian Science Information Exchange. A new reference tool, a KWIC index of NIDR research project titles, was developed for us by the Data Processing Systems and Analysis Section this year and has proven to be useful. Space to house this material continues to be a problem and plans to weed out the older and less frequently used documents are being considered.

OFFICE OF COLLABORATIVE RESEARCH, NIDR

FY 78 represented a year of siege on the collaborative research fleet at NIH and on the contracting activities of the Department in general. Unfortunately, NIDR has been swept into the fray, and our collaborative activities have been and will continue to be affected by the results. Inspectors have swept across NIH, studying all contracting components and imposing increasingly severe restrictions and reporting requirements. Such an unsettled atmosphere has not been conducive to encouraging increased use of the contract mechanism. We are confident, however, that the contracting policies and procedures established within NIDR over the past several years have been sufficient to provide adequate ballast to steady our ship through these stormy waters.

We have had fewer new contract actions this year partly due, to reorganization plans and activities within the National Caries Program. We anticipate that new contract initiation will return to normal levels next fiscal year. During FY 78, the Institute had a total of 85 active contracts and interagency agreements, some of which were initiated during the period and others of which were completed. On an average we had approximately 60 to 65 active contracts and agreements at any given time. Approximately $4.5 million was expended for the conduct of these activities. Review and monitoring activities also continued at a high level. During the year the Office held ten review meetings and three mail/conference-call reviews. In addition to annual project officer site visits our Office conducted twelve project

site visits with outside consultants for the purpose of monitoring ongoing contracts. Approximately 90 different consultants were used in the conduct of these review and monitoring activities. For several years, a major problem in obtaining and utilizing consultants for these functions has been our inability to reimburse them in a timely manner. Through negotiations with the Division of Administrative Services, NIDR was able to obtain decentralized authority for utilization of personal services contracts. We hopefully, this will overcome the difficulties we have had.

A major accomplishment this year has been the alleviation of previous year-end workload problems. During the year a planning cycle was established which is designed to tie in with the schedule for developing the NIDR Forward Plan on one end and with the procurement cycles on the other. Initial steps have been taken to implement this planning cycle, and RFP's have been issued to accomodate the award of some of next year's new contracts during the early quarters of the fiscal year. If the planning cycle is followed by the Institute's staff, we estimate that in another year or two the contracting workload can be distributed evenly throughout the fiscal year.

DATA PROCESSING OFFICE

The Data Processing Office is involved in the application of computer science methodology to NIDR research and administrative activities. This endeavour requires not only the effective use of computer technology but also the ability to meet the information requirements of the scientific and administrative staff. The Data Processing Office acts as a central focus for data processing by providing:

- laboratory automation systems
- time sharing computer services
- design, development and operation of information systems
- statistical analysis and programming services
- administrative reports
- computer facilities
- specialized training
- coordination of the annual ADP Plan

The Scientific Applications Unit of this Office has been involved in the project of making our new central system, a PDP 11/70 with 192K words of memory, more fully operational and increasing its availability to research personnel. This system substantially improves the computational resources of the Institute. Projects to improve and extend the graphical and plotting capability of the system have been initiated and include interactive graphics terminals, a multi-color plotter, and a raster-oriented plotter. Software availability has been improved by making standard statistical software packages (like BMDP) a supported item of the system.

A project which recently has been initiated is the development of a special minicomputer (PDP 11/34) dedicated to research in neurophysiology and behavioral psychology. This new system will replace and extend the capability of an existing Honeywell system. An important area of responsibility, and one that has not previously received the attention it needs due to manpower limitations, is the data processing work load of the dental clinic. Meeting and defining the needs for clinical studies is, and will be, a challenging task since consideration must be given not only to the scientific aspects of the research projects but also to concerns for the privacy rights of the patients.

During this fiscal year, a special report was prepared on the entry of former NIDR trainees into research. An Information Handbook also was prepared which shows in tabular and in graphical form the distribution of FY 77 research projects supported by grants, contracts and direct operations (intramural). The handbook, which included many of the regular reports provided by the Administrative Applications Unit of this Office, also displays the NIDR financial and personnel resources utilized. A series of reports were produced for the NIDR Planning Office to facilitate the evaluation of the National Caries Program. Another set of reports was produced which shows the NCP level of effort over time in relation to the NCP strategy areas.

Through coordination with the Grants Management office, we have been able to refine the budget balance reports so that expenditures made and balance remaining for funding grants are kept current. The result is that the grant reports now reflect, on a current basis, both actual and projected grants funding. By combining current allocations and grant obligations, these reports provide very useful information on the status of a major portion of the NIDR budget. The implementation of the Materials Management System (MMS) by NIH in FY 78 introduced changes into the procurement process. These changes have delayed our development of a system to provide better monitoring of NIDR commitments for the "other object" budget categories. However, we still prepare monthly financial reports for the Budget Office that show the overall, year-to-date obligations of the Institute.

We are currently testing an on-line interactive system which will allow users to directly extract information from a data file without programming assistance. Initially, the system is being implemented for the grants data file. Data is now being collected to form a bibliographic reference system. This system will contain data on reported publications resulting from NIDR support. The system will provide the quantification necessary for the analysis of the output of different mechanisms of support. Research Manpower Pool data on FY 77 projects is also being collected. This data provides information on the professional manpower working on NIDR grant projects. Efforts are underway to improve the usage of the centralized personnel data system (ARMS) that is maintained by the NIDR Personnel Office for monintoring a number of positions filled within our ceiling.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DE 00030-11 OD (a)

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (45 characters or less)
Laboratory Automation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	Terry P. Mellin	Supv. Computer Specialist	OD	NIDR
Other:	John J. Wilson	Computer Systems Analyst	OD	NIDR
	Eugene R. Caley	Computer Operator	OD	NIDR
	Leldy D. Jern, Jr.	Elec. Engineering Tech.	OD	NIDR
	M. Frances Rubick	Clerk (Typing)	OD	NIDR
	Janes Horton	Computer Aid	OD	NIDR
	Ava Kushner	Computer Aid	OD	NIDR
	Patricia Friedman	Computer Programmer	OD	NIDR
	Sharon Ortiz	Clerk Typist	OD	NIDR

COOPERATING UNITS (if any)
Computer Systems Laboratory, Division of Computer Research and Technology, NIH

LAB/BRANCH
Office of the Director

SECTION
Data Processing Systems and Analysis Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.60	0.80	3.80

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project involves the concurrent online data acquisition and realtime processing of data from a variety of instrument types which are geographically dispersed in various laboratories. Emphasis has been placed on experimental control as well. The project has evolved by using distributed processing techniques via separate computers in order to meet the ever growing utilization of computers in this research area. Image processing of electron micrographs is a growing demand that is being met via a large minicomputer while the satellite CPUs are (or will be) connected to this large CPU.

Special Descriptors: None
Classification . . : 10250

PHS-NIH-2
(Rev. 10-75)

The orientation of this project is the development of skills and techniques to develop a system or hierarchy of computers that will allow a variety of instruments or experiments to be fully or partially automated and will allow a researcher the opportunity to fully and easily analyze the data collected from such items.

Presently, a large minicomputer and several smaller minicomputers are being developed to meet the above goals. It is hoped that these systems will be fully operational by the end of 1979 so that work can then be directed toward the next goal of interfacing the remote systems to the large system. This will allow researchers to distribute their work on a variety of machines which will provide a more responsive system of computers that will meet his needs.

Additional capabilities have been recently added. Researchers can more fully utilize the capability provided by interactive graphics terminals due to the acquisition of a higher resolution graphics terminal. Color plots can now be obtained and three dimensional graphics capability will soon be added. A digitizer was also purchased to allow users to measure a variety of shape descriptors of molecular bodies on electron micrographs or other media. Finally, an optical mark reader will soon be used as a data entry device for forms used in epidemiological studies.

The immediate future of this project is to make all existing computers operational. The long range goal is the development of a computer network that allows researchers to move from one layer of computing power to the next layer in an easy and transparent way.

SUBMITTAL SCIENCE INFORMATION (SCHEDULE PROJECT NUMBER (DO NOT use this space)

... DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE [illegible]6-07 OD (a)

PERIOD COVERED

October 1, 1977 - September 30, 1978

TITLE OF PROJECT (40 characters or less)

Neurophysiology Software

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	Terry P. Meilia	Supv. Computer Specialist	OD	NIDR
OTHPI:	Sheila A. Taylor	Computer Programmer	OD	NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH

Office of the Director

SECTION

Data Processing Systems and Analysis Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.80	0.20	0.60

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The orientation of this project at present is the development of a software system to allow the simultaneous acquisition of behavioral events, neural spike data, and EMG waveforms. Analysis of this data will be accomplished in real-time, i.e. as the data is being collected. The analysis software will allow all three types of data to be analyzed collectively so that correllations between the data types can be found, examined, and studied in an exhaustive fashion.

Increased requirements for the amount of data and the correllation requirements necessitated a redesign of the hardware used to collect the data and the acquisition of additional disk storage units.

Special Descriptors: None
Classification * : 60210

PHS-6031
(Rev. 10-77)

The current emphasis of this project is designing a completely new hardware interface system that will allow the various data types to be gathered and correllated to a resolution of 500 usec. This effort was necessitated by the fact that the research efforts finally exceeded the capacity of the computer system that had been in use for almost ten years. In addition, a new software system is being designed to expand the capability of the analysis phase of the work. This software will allow three independent sets of data, i.e. behavioral, neural, and EMG, to be correllated. A backup system using analog tapes is also being investigated.

The efforts of the past year have been directed toward determining the types of and amount of hardware required for the system, planning the changeover from the old hardware to the new hardware, and designing the overall system after the system specifications had been developed.

The immediate future of this project is the implementation of the system which has been designed. The first goal is to allow data collected by the old hardware system to be analyzed by the new system. This phase is now in progress. The next phase will be to make a single laboratory operational on the new system. Following this, an extensive review will take place before the second laboratory and the analog tape backup system are developed.

PART II

NATIONAL INSTITUTE OF DENTAL RESEARCH ANNUAL REPORT

NATIONAL CARIES PROGRAM

OCTOBER 1, 1977 to SEPTEMBER 30, 1978

Compiled by:
Dental Research Data Officer
National Institute of Dental Research
National Institutes of Health

PART II

NATIONAL INSTITUTE OF DENTAL RESEARCH

ANNUAL REPORT

NATIONAL CARIES PROGRAM

October 1, 1977 - September 30, 1978

This document was prepared for administrative use at NIH. The comments and declarations of its contributors are their own and do not necessarily represent an official statement of the Institute.

Compiled By

Dental Research Data Officer

National Institute of Dental Research

National Institutes of Health

Bethesda, Maryland

CONTENTS

National Caries Program

Report of Associate Director

The year can best be summarized as one of steady progress (except as noted later) in each of the four National Caries Program Strategy Areas, rather than by noteworthy, discrete developments. This, in fact, is characteristic of current research on dental caries as most studies, especially human clinical trials, require 3 to 4 years for completion.

As dictated by NCP strategy, applied laboratory and clinical research on preventive methods was expanded, while heavy emphasis continued on the acquisition of better information regarding caries etiology, especially on the mechanisms by which cariogenic microorganisms colonize tooth surfaces to form plaque. It is becoming clear that the glucosyltransferases play a major role in adhesion as these enzymes determine the type and amount of extracellular polyglucan formed from sucrose. The demonstration that phospholipids may stimulate glucosyltransferase activity opens a new approach to caries prevention of considerable potential. In addition, efforts to induce secretory antibodies against GTF, which had begun earlier in primate experiments, have now assumed new importance in our research on anticaries vaccines, especially in view of the finding this year that GTF derived from one serotype of S. mutans induces antibodies active against at least two other serotypes as well. Immunization studies continue to yield positive results in terms of partial caries prevention in rats and monkeys, but neither the antigen of choice nor the optimum route and frequency of vaccination is yet established.

The often discussed possibility that the cariogenic oral flora could be replaced by a non-virulent competitor was shown, by a grantee, to be feasible, at least in laboratory rats. Mutants of S. mutans made deficient in lactic dehydrogenase readily colonized the animals' teeth, were only weakly cariogenic and prevented cross-infection by virulent organisms from other animals.

Research continues on the use of topical fluorides for caries prevention, but emphasis has shifted from evaluation of new fluoride compounds to development of optimized delivery methods for already available neutral and acidulated fluoride preparations. Slow release delivery systems are of particular interest. During FY 78, all necessary toxicity studies were completed on an intraoral polymer laminate device and an IND for a clinical trial is nearing completion.

An intriguing report from a grantee that decalcified enamel which is remineralized with solutions of calcium, phosphorus and low concentrations of fluoride has markedly increased resistance to subsequent acid attack, has suggested a new possibility for optimal topical fluoride therapy.

The NCP has increased efforts to identify and develop noncariogenic sweeteners for use as dietary sugar substitutes. This Strategy Area assumed added importance as the regulatory status of saccharin remained in doubt, and as the Federal Trade Commission announced its intention to consider restrictions on advertising of sugar-containing foods on childrens' television programs. Based upon reports from Finnish researchers and a series of studies in our laboratories, the polyol, xylitol, appeared to have considerable potential as a sugar substitute. However, a large scale clinical trial of a xylitol-sweetened chewing gum was suspended early in the year pending clarification of reports of bladder malignancies in rats fed very large amounts of this sweetener. These data remain under analysis by the FDA.

Research with several other new sweeteners including thaumatin, trichlorsucrose and several analogs of neohesperidine dihydrochalcone has reached the stage where full toxicity evaluation is required prior to clinical testing. The expense of such testing is such that the NCP can make only a token effort in this area, although stimulation of commercial development of these compounds is clearly needed.

The NCP-supported publication "Sweeteners and Dental Caries" reported the proceedings of an international symposium on that subject. The volume has been widely distributed and favorably received, both in the U.S. and in Europe.

No doubt a high point of the year was the successful completion of two years of demonstration of weekly fluoride mouthrinsing in elementary schools at seventeen sites across the country. Reports from contractors showed substantial protection against caries from this procedure and documented the low cost (as little as $0.50 per child per year), and the enthusiastic acceptance of the procedure by children, parents and school personnel. As a result, the NCP has intensified efforts to promote implementation of fluoride mouthrinsing in schools in all nonfluoridated communities. Success of these efforts would provide substantial protection against caries (roughly 35%) to an estimated 23 million children.

A major problem arose toward the end of the year when all National Caries Program laboratories were moved from the Auburn to the Park Building in Rockville. The latter facilities were, unfortunately, not in functional condition and, as a result, very little direct operations research has been active during the last quarter of the year. Inability to conduct animal experiments has been (and continues to be) one extremely serious consequence, as animal screening of antimicrobial compounds and tests to develop an acceptable method for identifying cariogenic food items have both been interrupted for an indefinite period.

In the following pages, major activities in each National Program Strategy Area are briefly summarized.

Strategy Area I: Combatting the Microbial Agent

Information is being sought on the identity and special characteristics of the oral microorganisms responsible for root and coronal caries and on epidemiological aspects of the disease. This knowledge is providing a basis for development of chemical and immunological or other biological methods for the prevention of dental caries by combatting cariogenic organisms. This report illustrates the types of studies being supported and highlights some of the information acquired during FY 1978.

Biology of Plaque Formation

Several studies seek to define the factors determining the sequence in which bacteria colonize teeth and the nature of interactions involving adherence between such organisms and salivary components which result in plaque formation. The synergistic and antagonistic interactions among members of the oral flora, which determine the levels of cariogenic bacteria in plaque are also being investigated.

The importance of the streptococcus serotype and the diet of the host was confirmed by NCP scientists recently. They found that of five serotypes of S. mutans tested for their ability to colonize teeth of germ-free rats, type b was the most successful regardless of whether sucrose, glucose or starch was ingested. However, dietary sucrose was essential for colonization by serotype d organisms. The influence of dietary sucrose on the flora of human plaque was demonstrated by a contractor working with fructose-intolerant patients. These individuals are unable to metabolize fructose and consequently avoid sweet foods, especially those containing fructose or sucrose. Whereas no significant differences were noted in the isolation frequency of the plaque organism S. sanguis between patients and control siblings, the known cariogens S. mutans and lactobacilli sp. were isolated more often from plaque of normal siblings than from that of fructose-intolerant individuals.

Grantees have shown that there are different salivary aggregating factors for S. mutans, S. sanguis and S. mitis. The glycoproteins in monkey or human saliva responsible for aggregation of S. sanguis and S. mutans have been partially purified. These factors contain less than fifty percent protein and high levels of hexoses, N-acetyl-glucosamine, sialic acid and sulfate. These investigators reported that the purified aggregating factors were devoid of IgA activity, indicating that immunoglobulins are not necessary for aggregation. However, NCP scientists found that although lectin precipitation separated most of the sIgA from salivary aggregating factor, the aggregating activity of the precipitated material was lost on treatment with anti-sIgA. This indicates that one of the aggregating factors for S. mutans may be an sIgA which constitutes a minor fraction of the total IgA in saliva or that sIgA is bound to the

aggregating factor. Attempts to isolate water soluble protein receptors for salivary aggregating factors from cell walls of S. sanguis have proved successful.

Bacterial glucans produced from sucrose are thought to facilitate adherence of plaque bacteria. Characterization of these polymers and purification of the enzymes responsible for their synthesis (glucosyltransferases, GTF) is proceeding in several laboratories. A grantee reported that the dextran from S. sanguis consists almost entirely of glucose residues of four types: non-reducing termini, branch points substituted at C-3 and C-6, and α-1-3 and α-1-6 linked residues. Approximately 30% of residues are α-1-3 linked and 70% are α-1-6 linked. The grantee has presented evidence that polymer formation proceeds via an insertion mechanism. Another grantee considers that dextran synthesis proceeds by addition of glucosyl residues to non-reducing termini of the polymer. These mechanisms are not exclusive, but further studies will be required to determine their relative importance. Apparently, several molecules of GTF can bind simultaneously to a single high molecular weight dextran molecule permitting rapid growth of the polymer. The close association of the degradative enzyme, dextranase, with the synthetic GTF in extensively purified enzyme preparations may partially explain the heterogeneity in polysaccharide structure. It has been proposed that the dextranase provides small acceptor molecules for the GTF, resulting in the formation of a very compact and highly branched dextran product. Another grantee has isolated a mutant of S. mutans with elevated dextranase activity. In contrast to the wild-type, this mutant is unable to adhere to glass in vitro in the presence of sucrose. A survey of several strains of S. mutans indicated an inverse relationship between dextranase levels and sucrose dependent adherence. Thus, the role of endogenous dextranase in adherence of cariogens remains to be defined. Data were also presented showing that dextranase of other indigenous plaque bacteria, such as S. mitis, blocks adherence of S. mutans and may thus modify colonization by the latter organism.

It was reported that GTF mediated synthesis of water-insoluble and soluble glucans is stimulated, to different extents, by lysophosphatidylcholine (LPC) and other phosphoglycerides. LPC derived from human saliva was active in this respect. This raises the possibility that host phospholipids may normally affect the adherence of cariogenic bacteria. It was shown that the phosphoglyceride binds to the enzyme at sites other than those for the glucosyl donors and acceptors. The inhibition of S. mutans plaque formation in vitro by L-lysine and L-threonine was commented upon in last year's report. Now it has been shown that this resulted from reduction in the specific activity of GTF and cell-free polysaccharide production when the organism was grown in media rich in these amino acids. The sensitivity of GTF to these naturally occurring factors suggests the feasibility of plaque control measures targeted on this enzyme.

Studies with Bacterial Mutants

Several studies with mutants of cariogenic organisms are being supported by grants and contracts. By defining virulence characteristics these investigations are providing information on the molecular basis of cariogenicity. They have suggested specific immunogens for eliciting immunity to caries and they may provide benign mutants which could be used as whole cell vaccines.

Comparison of four mutants of S. mutans, which differed in their GTF activity, adherence to glass, and cariogenicity in gnotobiotic animals, led to the conclusion that plaque formation and caries production are directly related to GTF activity. Previously, grantees have shown that intracellular GTF can be separated into two fractions, synthesizing water-soluble and -insoluble glucan, respectively. Using a mutant of S. mutans, in which both caries inducing activity and the total GTF activity were greater than in the parent strain, purification of the GTF on polyacrylamide gel revealed the same ratio of these enzyme activities in mutant and wild-type. Because it is unlikely that mutations were induced at more than one genetic locus, this suggests that production of the different forms of GTF is under coordinate control. In contrast, other investigators studied mutants of S. mutans in which there were quantitative differences in the enzyme fractions associated with water-soluble and water-insoluble dextran production. The decreased virulence of another mutant was attributable to a failure to form α-1-3-rich glucan. Biochemical analysis of certain non-virulent mutants of S. mutans indicated that a defect in ADP-glucose pyrophosphorylase was responsible for its inability to synthesize intracellular glycogen-type polysaccharide. Investigators have shown that mutants, which have lost the ability to agglutinate on exposure to glucan, retain the ability to form plaque in vitro and in vivo and are as virulent as the wild type. Besides establishing that adherence and agglutination are separate traits, these studies suggest that glucan-induced agglutination is unrelated to virulence.

One NCP-supported scientist has explored the potential of "replacement therapy" to control dental caries. In replacement therapy a non-pathogenic effector strain is introduced, which is able to compete for the ecological niche in susceptible host tissues normally occupied by its pathogenic counterpart. Lactic dehyrogenase-deficient mutants of S. mutans were isolated, which produced significantly less acid from glucose, in vitro, than the parent strain. The ability of the mutants to induce early carious white spots on human enamel in vitro was also impaired. The mutant and parent strains were equally competent in colonizing teeth of gnotobiotic and conventional rats but the incidence of caries was significantly less in the mutant infected animals. Once established in the oral cavity, the lactic dehydrogenase deficient mutant prevented cross-infection by the cariogenic wild-type from other rats. These studies showed that the lactic dehydrogenasedeficient mutants were less virulent and yet they were able to compete with their wildtype parent in the

oral cavity of rats. As such, they offer obvious potential as effector strains for replacement therapy of dental caries. The demonstration of the requirement for lactic dehydrogenase for caries production also provides decisive evidence in support of the acidogenic theory of caries etiology.

Immunological Studies

There is cause for optimism that immunization will be an effective public health method of preventing dental caries. The NCP continues to support research on development of a safe, effective and inexpensive vaccine against this ubiquitous disease. Initial studies in laboratory animal models demonstrated an association between decreased caries and increased salivary IgA reaction in response to antigens of S. mutans, a principal etiologic agent of caries in man. These studies have been extended to determine the chemical nature of effective antigens, and their relation to microbial virulence. Immunization techniques are being optimized with respect to route, dosage, regimen, use of different adjuvants, duration of response, and questions of immunological memory. Improvements are being sought in detection and quantitation of antibodies in saliva and plaque. The relationship between secretory and systemic immunity is receiving increasing attention. Studies are being supported on the mechanism of antibacterial effects of secretory antibodies and the extent of cross reaction between antibodies to microbial and mammalian antigens. Additional information is being sought on cellular aspects of immunity, including the sites and mechanism of antigenic stimulation, origin of the T cells in salivary glands and the regulation of homing of IgA precursors from mucosal associated lymphoid tissue to the salivary glands.

Previous annual reports have mentioned the work of NCP-supported scientists who successfully immunized rodents and non-human primates with S. mutans whole cells and cell wall fractions. They observed diminished colonization by infecting organisms and reductions in clinically evident disease. During the past year grantees have reported that ingestion of killed cells of S. mutans by human volunteers induced salivary and lacrymal but not serum, IgA antibodies. To induce a more specific immune response, many investigators have concentrated on the virulence factor, glucosyl transferase, as an antigen. GTF is the enzyme responsible for production of adherent polysaccharide. However, as at least seven serotypes of S. mutans have been identified, it is important to induce the most specific response, while retaining adequate cross-reactivity to combat the various serotypes of cariogenic organisms. Fortunately, the immune response in hamsters to purified, soluble, GTF antigen from any of the individual serotypes, a, c, or g of S. mutans, confers protection against infection by the other serotypes of S. mutans. Similarly, local immunization of rats with GTF gave rise to serum and salivary antibodies, which bound to heterologous GTF preparations. In another study with rats, animals immunized with S. mutans serotype a whole cells were protected against challenging serotype g organisms, although the protection was greatest against

the homologous organisms. These data indicate that antigen preparations from one serotype may protect against many or possibly all serotypes.

While a broad spectrum of activity against antigens from the principal cariogenic organisms is a desirable feature in an antiserum, if the activity extends to mammalian antigens, the lack of specificity becomes unacceptable. Using immunofluorescence techniques, grantees and contractors have shown that rabit antisera prepared against S. mutans serotypes a-e react with human heart and skeletal tissue fractions. Examination of membranes from S. pyogenes, S. sanguis, and S. salivarius indicated the presence of common immunodeterminants with antigens from human sarcolemmal sheaths. Reaction of antisera to S. mutans serotype e with preparations of monkey kidney glomeruli have also been observed. Identification of these common antigens and development of methods for their elimination are necessary prerequisites for production of a safe vaccine against dental caries.

Reports of correlations between protection against infection by cariogenic organisms and immunization with specific bacterial antigens have not been consistent from laboratory to laboratory. NCP scientists compared the results of using different routes of vaccination (subcutaneous, intramucosal, parenteral, intraductal or by gastric intubation), and different antigens (live cells, formalinized cells, GTF, fructosyltransferase, lipoteichoic acid and dextranase) in rodents and monkeys. They concluded that these and other variables could account for the different results reported. For example, significant but not readily detectable dextranase activity present in "purified" glucosyltransferase antigen preparations may have accounted for apparent differences in response. Antisera from primates immunized by gastric intubation with live cells of S. mutans had antilipoteichoic acid activity byt not anti-GTF activity; subsequent subcutaneous immunization resulted in antibodies to GTF. Grantees found that induction of secretory antibody in rats to S. mutans whole cells is dose dependent, and excessive doses of antigen resulted in a state of unresponsiveness. These data emphasize the need for well-defined antigen preparations and standardized protocols in vaccination studies.

Determination of caries incidence in patients with immune dysfunction is shedding light on the importance of natural immunity to caries. Investigators have found that patients with selective serum IgA dysfunction can be divided into three groups on the basis of salivary immunoglobulin levels: those with sIgA, those with sIgM, and those with no immunoglobulin. The presence of sIgA and sIgM appeared to correlate inversely with caries incidence, but an increase in caries incidence was not associated with the complete absence of secretory antibody. The levels of the nonspecific immune factors, lactoferrin, lactoperoxidase, and lysozyme were normal or elevated in saliva from immune deficient patients, suggesting that they may compensate for the lack of specific immunity.

Other NCP Initiatives

During the past year the NCP has supported conferences at academic institutions on Methods of Caries Prediction, The Secretory Immune System and Caries Immunity and Saliva and Dental Caries. Each workshop was attended by approximately fifty internationally-recognized experts drawn from a variety of disciplines. They spent two and one-half days critically evaluating the current status of research. Areas where new or intensified research is warranted were identified. Their recommendations from these workshops are being disseminated widely, and it is anticipated that investigatorinitiated research will be stimulated greatly in these areas.

During the year 65 grants, 14 contracts and 20 direct operations projects were active in Strategy Area I, representing 53 percent of National Caries Program research projects.

Strategy Area II: Increasing Tooth Resistance

Of the various strategies identified for the prevention of dental caries, increasing tooth resistance through the use of fluorides has thus far shown the greatest potential for prevention on a public health basis. One method, the use of weekly topical fluoride mouthrinses in nonfluoridated communities, has been researched and developed over the past decade and has now moved into the public health demonstration phase. (This development is detailed in the Strategy Area IV report.)

Clinical trials of various methods of delivering fluoride and the use of adhesive sealants have demonstrated that these methods of caries prevention are safe and effective. In fact, studies have shown that the coupling of optimum fluoride and sealant therapies can virtually eliminate caries in study populations of children. However, such combined sealant and fluoride regimens appear too expensive for widespread public health application. Thus, the immediate challenge is to improve and refine currently available caries preventive agents and technics to improve their cost-effectiveness.

Despite the research of the last thirty years establishing the effectiveness of fluoride therapy in caries prevention, the exact mechanism of fluoride activity has not been established. However, in vitro and animal model studies have shown that fluoride decreases the acid solubility of enamel, can inhibit the metabolism of various caries-inducing streptococcal microorganisms and promotes the remineralization of decalcified apatite structures. The relative contribution of each of these mechanisms to the overall caries preventive activity of the fluoride ion remains to be clarified.

Results reported during the past year confirm the activity of certain heavy metals, most notably strontium, in the enhancement of fluoride uptake by enamel when these metals are present in trace amounts. Further, the concentration of these heavy metals appears to be critical. In in vitro systems 4 to 6 ppm strontium potentiated the effect of 1 ppm F in reducing acid demineralization, but concentrations greater than 8 ppm resulted in increased acid solubility of tooth enamel. Such findings may help to explain the inconsistency in published reports of the effects of various trace elements on caries prevalence in humans.

Remineralization studies have also provided interesting findings on the mechanism of action of fluoride. Hydroxyapatite that has been remineralized by a solution containing low concentrations of calcium (1 mM) and fluoride (1 ppm) tends to be more resistant to subsequent acid demineralization than the original apatite. Within limits the mineral uptake is proportional to the mineral removed by prior acid etching. The decreased acid solubility resulting from this controlled demineralization/ remineralization cycle suggests that a two-step treatment might provide a basis for improving caries resistance of teeth through fluoride therapy.

An important question with regard to the use of fluorides is the duration of the preventive effect after the procedure is stopped as, for example, when a child leaves a school-based program. Because data are very limited on the presence of retained benefits, steps were taken to gain this information in a long-term evaluation of fluoride tablets. In this study one group of children, initially in the first and second grades, used an acidulated phosphate-fluoride chewable tablet (1 mg.F) once a day in school for six years, and another group took 2 tablets daily. Dental examinations conducted two years after treatments were discontinued indicated that most of the benefit derived during the treatment period was still evident. The final examination is planned for May 1979, four years after the end of treatment.

Because community water fluoridation is not possible for a large segment of the population who lack central water systems, alternative methods for the delivery of fluoride have been studied. Special attention continues to be given to the development of methods for the self-application of topical fluorides because these methods can be implemented with little professional manpower, thus reducing costs, and can reach large numbers of children.

Of the several procedures that have been investigated, fluoride mouthrinsing appears to have the greatest public health potential. Weekly rinsing with a 0.2% sodium fluoride (NaF) solution and daily rinsing with a 0.05% NaF solution have each been shown to be effective in reducing the incidence of dental decay among school-age children in nonfluoridated communities. However, there is insufficient evidence to determine if one regimen is more effective than the other and if either regimen offers substantial additional benefits to children reared in optimally fluoridated areas.

To help answer these questions, a three-year, double-blind clinical trial was initiated in an optimally fluoridated community. Children in the study follow either the weekly fluoride mouthrinsing procedure, the daily fluoride mouthrinsing procedure or use a weekly placebo mouthrinse. All rinsings are carried out in school under the classroom teacher's supervision. Information on the comparative benefits of the two procedures will be helpful to school and health officials currently operating or contemplating the implementation of a school fluoride mouthrinsing program in optimally fluoridated areas.

In vitro and animal testing have been completed on a device that is capable of releasing a small amount of fluoride in the mouth at a fixed, steady rate for a 6-month period. The device is small enough to be attached to one or two of the posterior teeth, and consists of a reservoir core of granular sodium fluoride, dispersed in an acrylic copolymer matrix, and a coating made of another acrylic copolymer, which functions as a semipermeable membrane to control the diffusional release of the fluoride. Toxicity studies in animals are complete and no untoward effects were observed. Steps are now being taken to apply for Phase I clinical trials.

Several other contract-supported studies were initiated during FY 1978. Among these is a 3-year clinical trial in which the effect upon dental caries of weekly mouthrinsing with a 0.2% NaF solution without prior cleaning of the teeth is being compared with the effect of rinsing preceded by cleaning of the teeth either with a toothbrush alone or with a toothbrush and dental floss. This study will help determine whether it is necessary (or desirable) to remove dental plaque prior to self-applied fluoride treatments.

Under another contract, a 3-year clinical trial is being conducted to evaluate the caries-preventive effects of regular, supervised use of a NaF mouthrinse in four different combinations of concentration and frequency. These are: daily use at 225 ppm F, daily use at 900 ppm F, weekly use at 225 ppm F and weekly use at 900 ppm F.

Another contractor has begun study of the caries-preventive effect of neutral sodium fluoride and acidulated phosphate fluoride, when each is used as a tablet and as a solution. This study will provide much needed information on the relative effects of neutral and acidulated fluoride, as well as information on whether the type of vehicle (tablet or solution) makes a difference in terms of caries protection.

During the year 26 grants, 8 contracts and 16 direct operations projects were active in Strategy Area II, representing 27 percent of National Caries Program research projects.

STRATEGY AREA III: Alter the Cariogenic Properties of the Diet

Dietary Sugar Substitutes

Recently a clinical trial was carried out in Finland in which over 90 percent of caries was prevented. This outstanding effect was achieved by simply replacing the sucrose in foods with a sweetener which is not fermented in the mouth. Xylitol, the sweetener used in the study, does not possess all the features that one would desire in a sucrose substitute. However, if other non-sucrose sweeteners can be found and used to provide an array of attractive non-cariogenic snacks, beverages and dessert foods the total benefit to the U.S. population would be large. Indeed, one would expect that savings in dental bills alone would be over $2 billion dollars per year.

There are many advantages to a dietary approach to caries prevention. One is that problems of delivery of preventive agents, often a factor that increases the cost and complexity of public health programs, would not arise. A second is that the school-age population, in which the need for caries prevention is acute, would be specifically and almost uniformly provided with at least some level of caries reduction.

Today, the competitive marketing of prepared foods, whether for mealtime or between-meal consumption, is based increasingly on sweetness. Food stores, dispensing machines, pantry and refrigerator shelves, cafeteria displays and lunch boxes are dominated by sugar-loaded foods. With use of these foods becoming more frequent, one would expect that caries prevalence would be increasing, and the most current national statistics suggest that this is the case.

The National Caries Program, from its inception, has sought substitutes for sucrose that would provide food manufacturers with ways to achieve sweet products without risk to the teeth. It also has sought reliable methods that could be employed by manufacturers and scientists to predict whether a food would contribute to the dietary cariogenic load. In each of the last four years the NCP has allocated more program resources to research on, and development of, non-cariogenic sweeteners. Detailed research strategies in the area have been designed and opportunities for making substantial progress in this area are excellent.

There are a number of current candidates for an ideal food sweetener. Among them are the proteinaceous sweeteners monellin and thaumatin; the dihydrochalcones such as neohesperidine; trichlorosucrose, stevioside,

SRI oxime 5, and the sugar alcohols sorbitol, mannitol and xylitol. The National Caries Program is conducting preliminary screening (that which can be done inexpensively and would clearly disqualify poor candidates) on all of these compounds. The next step is to conduct acute and chronic toxicity tests on the most promising compounds but here cost is a major impediment to progress.

For several years the Program also has been supporting more fundamental research on several new sweeteners. In a project at the University of Pennsylvania scientists have elucidated much of the structure and composition of a unique sweet protein, monellin. The fundamental interest of these scientists is in the interaction of this molecule with the taste bud receptors. Simultaneously, the protein has been found by food technologists to be an attractive sweetener for certain food uses. A related sweet protein, thaumatin or "Talin," is being evaluated for use in Europe. Probably the commercial development of either of these sweeteners hinges on whether a supply of the plant source from tropical countries can be made sufficiently large.

The NCP also is continuing support of research to synthesize new sweeteners in a family of compounds known as dihydrochalcones that are derived from citrus rind. Though several of these compounds are intensely sweet, there is a menthol-type cooling flavor and lingering quality to their sweetness that detracts from commercial utilizability. The Program currently is supporting work to synthesize dihydrochalcones analogs with improved taste characteristics. More than 28 new analogs have been prepared. Most have been evaluated by sensory panels and five appear commercially attractive. Though the potential for profit is considerable, full-scale evaluation of a sweetener probably would cost in excess of $10 million, certainly a major consideration.

It was reported last year that the National Caries Program was initiating a clinical trial to evaluate and compare the low cariogenicity that has been claimed for the sugar alcohols, sorbitol and xylitol. Both sugar alcohols have been extensively studied and sorbitol, the less expensive of the two, is commonly used as a sweetener for chewing gum. Xylitol is an intermediary metabolite in man, has been used as a calorie source for intravenous feeding, and employed in foods at levels of severl hundred grams per day in feeding studies. In a two-year trial conducted in Finland the caries incidence in children chewing between four and five sticks of xylitol-sweetened gum per day was reduced to approximately the same level as in a previous experiment in which xylitol replaced nearly all sucrose in sweet foods. Our clinical trial was designed to obtain further information on the degree of caries prevention. Hardly had the study begun, however, when a report was received from England of tumorogenic effects in animals fed high levels of xylitol. Though the reported effect was believed to be a non-specific response to the high level at which the sugar alcohol was fed, the clinical trial was necessarily terminated, pending evaluation of the animal data by the FDA.

Increasingly, public interest groups have voiced concern about the large amounts of sugar being added to snacks and processed foods and about the effect of these additions on caries and obesity and possibly on major chronic diseases. Also, there has been a substantial exchange of opinion concerning the risks and benefits of saccharin usage which led during the year to a postponement of its prohibition in food usage. Saccharin, of course, is the last non-nutritive sweetener still allowed for use as a food additive. Finally, there has been an expression of public concern over the extent of advertising of high-sugar foods to young children, primarily on TV but through other media as well. Much of this concern reflects the apprehension that lifetime food consumption habits are established at this formative age.

The importance of fermentable sugars in caries etiology and NCP's almost sole involvement at NIH in research and development of non-sugar sweeteners has given the Program a responsibility to balance and appropriately responsibility to balance and appropriately respond to this multitude of concerns. In early 1977, therefore, the Program awarded a grant to Harvard University to organize a meeting to explore the major issues in the area. The meeting, held in October 1977, brought together experts from many involved groups including academic researchers, government research and regulatory agencies, commercial firms and consumer organizations.

The symposium allowed scientific information and public issues to be summarized and brought up-to-date in the disparate areas of sweetener research and provided a forum for open interchange of opinion. The proceedings of the symposium, published under the title "Sweeteners and Dental Caries," has already become the definitive reference in this area. The discussions by symposium participants of priority research needs have sharply defined for staff the urgency and potential of sweetener research and have led to accelerated Program activities in toxicity and anticariogenicity testing, development of assays with which the cariogenicity of foods can be predicted, and determination of American snacking patterns, to name a few.

Dietary Trace Elements

It is well known that addition of fluoride to municipal water supplies is the most known effective method of caries prevention. The approach is simple and inexpensive. The National Caries Program has been supporting a search for other trace elements that conceivably could potentiate the effect of fluoride or exert an independent cariostatic effect. Epidemiological studies have been supported for several years in which caries experience in various regions of the country has been compared to trace element concentrations in the soil and water and in tooth enamel of residents of these areas. The

search appears to be reaching its initial objective in that much evidence now points to dietary- and water-borne strontium as having a marked caries-preventive effect in humans. Research efforts are now concentrated on developing an animal model with which the mechanism of the effects of strontium hopefully can be elucidated. Recent results show that caries in laboratory rats is markedly reduced when strontium is provided in an intermediate range but not when the element is present at higher or lower levels. As is the case for fluoride, the caries inhibitory level for strontium in animals appears to be approximately ten times that which causes maximal caries prevention in the human. In in vitro studies on the remineralization of "white spots" artificially formed on enamel it has been shown that strontium and fluoride have marked synergistic effects. Though the objective of identifying an additional anticaries trace element appears achieved, many questions concerning safety and optimal level remain to be answered before strontium can be considered for applied caries prevention.

Epidemiological studies have also suggested that the trace element, selenium, appears to cause a significant increase in human caries. Selenium is common in soils and water supplies in the northwestern United States and may reduce the benefits obtained from programs of water fluoridation. Research on this trace element, now in progress at the University of Oregon, also suggests that hazards to mineralized tissues other than enamel may result from consumption of this element.

During the year 6 grants, 4 contracts and 2 direct operations projects were active in Strategy Area III, representing 7 percent of the National Caries Program research projects.

STRATEGY AREA IV: Improved Delivery and Acceptance of Caries Preventive Methods

The National Caries Program has as one of its objectives increased community awareness and implementation of preventive programs that include the use of agents and methods known to reduce dental caries. School-based programs of self-applied fluorides (fluoride tablets and fluoride mouthrinses or a combination of these regimens) are the major emphasis of this effort. The implementation and continuation by communities of a proven caries preventive regimen is the ultimate test of acceptance. The NCP has continued to expand its efforts to gain widespread acceptance by the public and the profession of these preventive programs.

Seventeen community demonstration projects involving the use of weekly fluoride mouthrinsing are in their final year. Although the treatment regimen is the same at each site, (a 0.2 percent neutral sodium fluoride is rinsed weekly by elementary school children) each community staffed and implemented the preventive program in a different way. These projects are located throughout the country and on the Island of Guam, thus providing a mix of urban and rural, social, ethnic and other characteristics of the involved population. Thus, at the completion of the projects, a great deal of information will be available on cost/benefit of utilizing different kinds of personnel to operate the preventive programs as well as on community acceptance in a variety of settings. In June 1978, the NCP convened a conference attended by the principal investigator, co-principal investigator, the community program coordinator and a community official such as school principal or superintendent from each site. The purpose of the conference was to share interim findings and general experiences with all 17 communities and to help the participants plan for continuation of the program once the formal part of the contracts are completed.

After two years, participation rates range from 60 percent to 95 percent and in many of the sites participation has increased during the program. The contractors reported that caries protection was as great as 46 percent, and the cost as low as 32¢ per child per school year, although the level of prevention and the cost varied widely.

In view of these encouraging interim findings from the demonstration projects, a press conference was held to report the findings to the general public. Press coverage was carried by all major television and radio networks and by all major newspapers. Wide interest in self-applied fluoride programs has been generated as a result.

One study that demonstrates long-term efficacy of a regimen of self-applied fluoride in a fluoride-deficient community is in progress in Nelson County, Virginia. The purpose of the study is to determine

the total effectiveness of a combination of: 1) daily ingestion of a 1 mg. fluoride tablet, 2) weekly rinsing with a 0.2 percent neutral sodium fluoride solution, and 3) ad libitum use of a fluoride dentifrice at home. The program has completed its sixth year of operation with 96 percent of elementary school children in kindergarten through 6th grade participating. The program will be expanded into the 7th and 8th grades on an incremental basis beginning in the fall of 1978. Students, parents, and school personnel have accepted the regimen as a routine part of school activities, which is a necessity for communities in which water fluoridation is not possible.

In an effort to increase dissemination of information on caries prevention to the profession and to the public, an NCP exhibit on school-based self-applied fluoride programs was shown at the annual sessions of the American Dental Association, American Association of School Administrators, National School Boards Association, the National Congress of the PTA, the American Academy of Pediatrics, the National Dental Association and the California PTA. During most of these meetings an NCP staff member made a presentation to further increase awareness of the merits of school-based, self-applied fluorides. The exhibit received honorable mention in the scientific category at the American Dental Association annual session in 1977.

In an effort to assist state and local health and educational groups in promoting the use of self-applied fluorides in their respective communities, the NCP has developed a second and smaller exhibit for free loan. The exhibit has been extremely popular with health department and dental and dental and dental hygiene groups, and is well subscribed for 1978.

The NCP's publication, "Preventing Tooth Decay: a guide to implementing self-applied fluoride programs in schools," has been extremely well received. To date, over 10,000 copies of the guide have been distributed upon request. The NCP has begun a survey of a sample of those who have requested a copy of the publication. The objectives of the survey are to determine the extent to which communities requesting the guide have implemented a self-applied fluoride program in schools and whether the publication is adequate in providing guidance to begin a program.

The television film, Reading, Writing and Rinsing has been aired over 90 times across the country in the past year. Because the film is in such demand for use in in-service training in communities, 25 copies of the film have been placed with the American Dental Association's film library to facilitate access to it.

NCP staff have been invited to conduct workshops on implementing school-based fluoride programs for school boards, school districts and

health departments and societies. In addition, staff have given lectures and papers to undergraduate and graduate health professionals at numerous international, national, state and local meetings.

A newly-funded grant in Strategy Area IV is designed to determine which factors discriminate between the adoption and non-adoption of a self-applied fluoride program in U.S. school systems. The data collected will include information about who makes and influences decisions about health programs, aspects of preventive health measures that make them attractive or unattractive to communities, and barriers and problems in implementing self-applied fluoride regimens in schools. Data will be available in 3 years.

In addition, over 44,000 copies of the leaflet, "Fluoride Mouthrinsing in Schools...protection for children's teeth," and over 29,000 copies of the leaflet, "Fluoride Tablets...A Healthier Smile for School Children" have been sent to communities to be used to inform parents when implementing self-applied fluoride programs.

During the year 4 grants, 19 contracts and 1 direct operations studies were active in Strategy Area IV, representing 13 percent of the National Caries Program research projects.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00029-11-CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978 CT 06Q0057

TITLE OF PROJECT (80 characters or less)

The Effect of School Water Fluoridation on Dental Caries

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. B. Heifetz	Clinical Investigator	NCP, NIDR
Other:	H. S. Horowitz	Chief, CPS	NCP, NIDR
	W. S. Driscoll	Clinical Investigator	NCP, NIDR
	J. A. Brunelle	Chief, Biometry Section	NCP, NIDR
	R. Meyers	Public Health Analyst	NCP, NIDR

COOPERATING UNITS (if any)
Dental Health Division, North Carolina State Board of Health, Division of Water Hygiene, Environmental Protection Agency

LAB/BRANCH
Caries Prevention and Research

SECTION
Community Programs

INSTITUTE AND LOCATION
National Institute of Dental Research, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.03	0.02	0.01

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

luorides were added to the water supply of a school in Seagrove, North Carolina. he concentration of fluoride used was higher than the level considered optimal or community water fluoridation in the geographic area. Children attending the eagrove school live in an area without a central water supply and where the arious sources of well water contain negligible levels of fluoride. Children are xposed to the higher fluoride level only while at school in an attempt to approxmate the total fluoride intake of children who drink optimally fluoridated water n a full-time basis. Baseline dental examinations for dental caries were made rior to the installation of fluoridation equipment. Follow-up examinations are onducted after four, eight, and twelve years to determine the extent of caries rotection as increasingly larger segments of the study population become continously exposed to fluoridated water at school since entering the first grade. esults of the four-year examinations showed appreciable decreases in caries prevlence compared with baseline findings. On the eight-year examinations, an assessment of the possible prevalence of dental fluorosis was made along with the egular examinations for dental caries. No children showed any definite signs of he condition. NIDR Classification: 10550

Objective

The purpose of the study is to determine the decay preventive benefits derived by children who when at home drink water that is essentially devoid of fluoride but when at school consume water fluoridated at 7 times the level considered optimal for community water fluoridation in the same geographic area.

Methods

In 1968, fluorides were added to the water supply of a consolidated school (grades 1-12) in Seagrove, North Carolina. The target level of fluoride, 6.3 ppm, is seven times the level considered optimal for community fluoridation in the same geographic area. Children are exposed to the higher fluoride level in an attempt to approximate the total fluoride intake of children who drink optimally fluoridated water on a full-time basis. Prior to the installation of fluoridation equipment dental examinations using the DMF tooth and surface index were conducted on approximately 1100 children to determine baseline caries prevalence. Surveillance of the fluoride levels maintained is provided by school personnel under the supervision of the North Carolina State Board of Health. Follow-up examinations are conducted at four-year intervals to measure the extent of caries protection as increasingly larger segments of the study population become continuously exposed to fluoridated water at school since entering in the first grade.

Findings

Eight-year follow-up examinations were conducted in 1976. The interim data showed that children 6 through 14 years of age, the full beneficiaries of the procedure after eight years of exposure, had an overall 40% difference in age-specific DMF surface scores compared with those of their counterparts of the baseline. Examinations for dental fluorosis were also conducted on the 1976 examinations. None of the children examined exhibited any signs of the conditon.

Significance:

Currently about 23% of the U.S. population reside in areas which lack central water systems. These persons are deprived of the benefits of community water fluoridation. School fluoridation is an alternative method of preventing dental caries in children living in such areas. The present study will help determine the optimal concentration of fluoride for school water fluoridation. Currently a level of 4.5 times the optimum is used.

Proposed Course

Fluoride will be maintained at the target level until the final examinations in 1980, at which time children in all grades (1-12) will have been continuously exposed to the higher fluoride level at school since the first grade. Comparison of findings after 12 years of school water fluoridation at 7 and at 4.5 times the optimum will be made to determine if greater anticaries protection is conferred at the higher fluoride concentration.

Publications

Heifetz, S.B. and Horowitz, H.S.: Effect of School Fluoridation on Dental Caries: Interim Results in Seagrove, N.C. After Four Years. JADA 88:352-355, 1974.

Heifetz, S.B., Horowitz, H.S. and Driscoll, W.S. Effect of School Fluoridation on Dental Caries: Results in Seagrove, N.C. After Eight Years. JADA, In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00032-10-CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978 CT 0600042

TITLE OF PROJECT (80 characters or less)

Effects of chewable fluoride tablets on dental caries in school children

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. S. Driscoll	Clinical Investigator	NCP	NIDR
Other:	S. B. Heifetz	Clinical Investigator	NCP	NIDR
	H. S. Horowitz	Chief, Community Programs	NCP	NIDR
	J. Brunelle	Chief, Biometry, CPRS	NCP	NIDR

COOPERATING UNITS (if any)

Wayne County, North Carolina, Public School System

LAB/BRANCH
Caries Prevention and Research

SECTION
Community Programs

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.26	0.19	0.07

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The study was initiated in October 1969 with 1034 children in the first and second grades of nine schools located in Wayne County, North Carolina, an area that has negligible amounts of fluoride (F) in its supplies of drinking water. Following baseline dental examinations, in which the DMF surface index was used, the children were stratified according to certain variables and then randomly assigned to one of the following three study groups: Group A (controls) chewed a placebo tablet, rinsed their teeth for 30 seconds with the resulting salivary solution, and then swallowed the material; Group B followed an identical procedure using an acidulated phosphate-fluoride (APF) tablet that contained 1 mg. F; Group C followed the same procedure as Group B except that, after at least 3 hours, the procedure was repeated with a second APF tablet that also contained 1 mg. F. The procedures were carried out each day in school under the classroom teacher's supervision for a period of six years. Follow-up examinations were conducted in April 1972, May 1974, September 1975 and May 1977. The next follow-up examinations are planned for May 1979, four years after discontinuation of treatments.

NIDR Classification: 10550

Objective

To evaluate the caries-preventive effect of the daily use in school of acidulated phosphate-fluoride (APF) chewable tablets.

Methods Employed

The study, a longitudinal double-blind clinical trial, was initiated in October 1969 on 1034 children attending the first and second grades of nine public schools located in Wayne County, North Carolina, an area that has negligible amounts of fluoride in its sources of drinking water. Children were stratified according to selected variables and then were randomly assigned to one of the following three study groups: Group A (controls) chewed a placebo tablet for 25 seconds, rinsed their teeth for 30 seconds with the resulting salivary solution and then swallowed the material; Group B followed an identical procedure using an APF tablet that contained 1 mg. F; Group C followed the same procedure as Group B except that, after at least 3 hours, the procedure was repeated with a second APF tablet that also contained 1 mg. F. The procedures were carried out each day in school under the classroom teacher's supervision for a period of six school years. Baseline dental examinations, using the DMF surface index, were conducted just prior to treatment initiation. Follow-up examinations were carried out in April 1972, May 1974, September 1975 and in May 1977, two years after discontinuation of treatments.

Findings

Examinations conducted in May 1977, two years after treatments had been discontinued, showed statistically significant reductions in DMFS increment for both treatment groups (B and C), compared with the controls (Group A). The two treatment procedures were about equal in effectiveness. Percentage reductions were approximately 25% for early erupting teeth (incisors and first molars), 45% for late erupting teeth (cuspids, bicuspids and second molars) and 32% for all teeth combined. These percentage figures are of the same magnitude as those found in September 1975, shortly after treatments were terminated, indicating that benefits derived during the treatment period are retained thereafter for at least a two year period. It may be concluded from the findings that the once-a-day procedure followed by Group B can be recommended highly as a public health measure for the prevention of dental caries.

Significance

Approximately 50% of the U.S. population resides either in areas that have no central water supplies or in areas that do have central water supplies but have not implemented community water fluoridation. Because these persons are deprived of the benefits afforded by community water fluoridation, other methods of caries prevention must be developed and utilized. One method that appears to offer considerable potential

is administration of fluoride tablets to children in school. The present study is important in determining both the efficacy and the feasibility of the procedure. By continuing to do dental examinations after treatments have been terminated, valuable information with regard to retained benefits will be gained.

Proposed Course

The next follow-up examinations are planned for May 1979, four years after discontinuation of treatments, if sufficient numbers of participants remain.

Publications

Driscoll, W.S., Heifetz, S.B. and Korts, D.C.: Effect of acidulated Phosphate-Fluoride Chewable Tablets on Dental Caries in Schoolchildren: Results After 30 Months. JADA 89:115-120, 1974.

Driscoll, W.S., et al.: Effect of Acidulated Phosphate-Fluoride Chewable Tablets in Schoolchildren. Results after 55 Months. JADA 94:537-543, 1977.

Driscoll, W.S., Heifetz, S.B. and Korts, D.C.: Effect of Chewable Fluoride Tablets on Dental Caries in Schoolchildren: Results After Six Years of Use. JADA In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00039-09 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Study of the mode of action of ambient fluoride on the development of dental caries

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Roald J. Shern	Clinical Investigator	CP PR NIDR
OTHER:	Albert Kingman	Statistician - Health	CP PR NIDR
	William H. Bowen	Acting Chief	CP PR NIDR
	Kathleen M. Couet	Biologist	CP PR NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Preventive Methods Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.38	0.23	0.15

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ultimate goal of this work is to determine the minimum fluoride exposure associated with maximum caries protection in humans. Recent studies agree with those of Bowen that hard water helps protect the teeth against dental caries.

NIDR Classification: 10220;10230

1. Project Description

Objectives

The goal of this work is to study the relationship of F and other components of water with caries in humans.

Methods

Prevalence and incidence studies are being conducted in adolescents consuming high and low concentrations of fluoride and hardness in their drinking water. The studies measure dental caries and plaque extent as well as various measurements of the microbiological and biochemical aspects of dental plaque including plaque fluoride.

Major Findings

Hard water might be protective. Animal studies suggest a synergistic interaction of fluoride and $CaPO_4$. Additional population studies are needed testing the effects of fluoride at different levels of water hardness.

Significance

If hard water is beneficial in combatting dental caries, it might be supplied in different ways, e.g., as a rinse or as a water additive.

Proposed Course

The prevalence and incidence of dental caries will be measured in optimally fluoridated communities that have differing levels of water hardness. Other variables that could obscure the association will be measured.

Publications

Shern, R.J., Driscoll, W.S., and Korts, D.C.: Enamel Biopsy Results of Children Receiving Fluoride Tablets. JADA 95:310-314, 1977.

Shern, R.J.: Discussion - Aspects of Design and Data Evaluation in Population Studies. Proceedings 'Methods of Caries Prediction' Eds. Bibby and Shern. Sp. Supp. Microbiology Abstracts. pp 305-309, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00070-06-CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978 CT 0600045

TITLE OF PROJECT (80 characters or less)
Combined self-applied fluorides for caries prevention in a non-fluoridated area

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. S. Horowitz	Chief, CPS	NCP, NIDR
Other:	S. B. Heifetz	Clinical Investigator	NCP, NIDR
	R. Meyers	Clinical Investigator	NCP, NIDR
	W. S. Driscoll	Clinical Investigator	NCP, NIDR

COOPERATING UNITS (if any)
Nelson County, Virginia, Public School System

LAB/BRANCH
Caries Prevention and Research

SECTION
Community Programs

INSTITUTE AND LOCATION
National Institute of Dental Research, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.45	0.34	0.11

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Baseline dental examinations were conducted in October 1972, on approximately 200 first through twelfth grade children in Nelson County, Virginia. All study participants in grades K-6 chew daily in school under supervision of the classroom teacher an acidulated phosphate-fluoride (APF) tablet containing 1 mg. F, rinse for 30 seconds with the resulting solution and then swallow the material. Once a week in school the same children also swish 10 milliliters of a 0.2 percent sodium fluoride solution for 60 seconds and then empty the contents of the mouth into a cup. A fluoride-containing dentifrice is distributed to the same children for use at home, and they receive toothbrushes periodically to take home. These combined preventive procedures will continue in the elementary schools for a minimum of ten years. Follow-up dental examinations are carried out biennially in all schools. Follow-up surveys were done in early fall of 1974, 1976 and 1978. Final examinations will be made in the Fall of 1982 when all senior high school students will have participated continuously in the elementary school program since entering first grade.

IDR Classification: 10540

Objective

The purpose of the study is to determine the total effectiveness of a combination of some of the most feasible methods of self-administering fluorides in a non-fluoride area.

Methods

In October 1972, a self-administered dental health program was started in Nelson County, Va., a fluoride-deficient community. Children in the County's 7 elementary schools, under teacher supervision, ingest daily a 1 mg. F tablet and rinse weekly with a 0.2% NaF solution; F dentifrice is provided for ad libitum use at home. Baseline DMFS examinations were made of 2135 children in the County's elementary (grades 1-6), junior (grades 7 and 8) and senior high schools (grades 9-12). Follow-up examinations are conducted at two-to-three year intervals to determine the effectiveness of the program as increasingly larger segments of the participants become exposed to the F treatments since entering school in the first grade.

Findings

The most recent follow-up examinations that have been analyzed are those done in 1976. Those findings showed that continuous participants in grades 2-7 (ages 7-12) had 35 percent fewer DMF surfaces in 1976 than their cohorts in 1972. These children in 1976 had 70 percent fewer DMF mesiodistal surfaces than comparable children in 1972. Children in 1976 who had participated in the preventive program at any time had 29 percent fewer DMFS than their cohorts. As assessed by four-year incremental caries scores, the estimated mean increment was 46.2% lower during the period of the program than prior to its initiation.

Significance

Self-administered procedures, unlike traditional, professionally administered fluoride applications, can be implemented extensively with few demands on dental manpower, school personnel, facilities and financial resources. From the standpoint of optimizing dental health programs in areas where community water fluoridation is not possible, there is a compelling need to determine the impact of various combinations of feasible, self-administered methods of fluoride delivery.

Proposed Course

Treatment procedures will continue in the elementary school for a minimum of ten years, or until the Spring of 1982. Continuing the treatments for this length of time will enable a final evaluation in 1983 of a senior high school population that has received the full benefits of the program. Interim 2-year findings have been presented at a scientific meeting and have been published.

Publications

Horowitz, H.S., Heifetz, S.B., Meyers, R.J., Driscoll, W.S. and Korts, D.C.: Effect of a Combination of Self-Administered Fluoride Measures on Dental Caries. Abstracted, IADR Program and Abstracts of Papers. J. Dent. Res. 55:88, 1976.

Horowitz, H.S., Heifetz, S.B., Meyers, R.J., Driscoll, W.S. and Korts, D.C. Evaluation of a combination of self-administered fluoride procedures for control of dental caries in a non-fluoride area: findings after 2 years. Caries Res. 11:178-185, 1977.

Horowitz, H.S., Heifetz, S.B., Meyers, R.J., Driscoll, W.S. and Korts, D.C. Evaluation of a combination of self-administered fluoride procedures for the control of dental caries in a non-fluoride area: findings after 4 years. In press, J. Amer. Dent. Assoc.

Horowitz, H.S., Heifetz, S.B., Meyers, R.J. and Driscoll, W.S.: Evaluation after 4 years of a combination of self-applied fluoride procedures in a non-fluoride area. Program and abstracts, 25th Congress, European Organization for Caries Research, Turku, Finland, June 29-July 1, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00081-05 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Development of an Anticaries Vaccine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. H. Bowen	Acting Chief, CPR	CPR, NIDR
OTHER:	H. M. Kuzmiak-Jones	Biologist	CPR, NIDR
	I. M. Gomez	Microbiologist	CPR, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research Branch

SECTION
--

INSTITUTE AND LOCATION
NIH, NIDR, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	.3	.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A colony of primates, Macaca fascicularis, which has been infected with S. mutans and is being fed a diet rich in sucrose, is being used to develop a vaccine against dental caries. A variety of antigens with and without adjuvant has been administered either intramucosally or intraductally. The purpose of the investigation is to determine the most appropriate antigen, the optimum route of administration, and the type of antibody response required to protect animals against caries attack.

NIDR Classification: 10570

The possibility of developing a caries vaccine continues to attract an increasing level of attention. The advantages of using this approach are manifold. The vaccine would be easy to administer, could be delivered on a public health basis and would probably be inexpensive. Results from a number of studies have shown that animals can be successfully vaccinated against dental caries. The purpose of the present investigation is to determine whether a variety of antigens, some of which are accepted for use in humans and which are biochemically related to the extracellular polysaccharide produced by S. mutans, would confer protection in primates and rats vaccinated through a variety of routes. Antibody responses are being monitored in saliva and serum; the cellular immune response is also being examined. The influence of vaccination on the biochemistry of plaque and its microbial composition is being determined using standard biochemical techniques.

The prospects of developing a vaccine against dental caries are becoming increasingly attractive. Such an approach to caries prevention offers several advantages and could be used to supplement existing methods. Rats, hamsters and monkeys have been successfully vaccinated using a variety of immunogens in different stages of purity. The purpose of the present investigation is to determine whether polysaccharides derived from organisms other than mutans and which are acceptable for use in humans will confer protection in primates. The bacterial composition of plaque from animals which have been vaccinated differs from that of control animals. Animals which have been vaccinated introductally appear to have developed more caries than other animals. Preliminary results show that monkeys vaccinated with a crude preparation of glycosyl transferase have less caries than controls.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00098-05 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

An Evaluation of Knutson's Formula for Estimating Age Specific DMFT

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David C. Korts	Statistician - Health	CPR, NIDR
COPI:	Sven Poulsen	Guest Worker	CPR, NIDR
	Albert Kingman	Statistician - Health	CPR, NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH
Caries Prevention and Research Branch

SECTION
Biometry Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.10	.10	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In 1944 Knutson described a formula for estimating age specific DMFT means from the percent of individuals in the age groups having one or more decayed, missing or filled teeth. This formula was evaluated for both different age ranges, and for different allowable maximum percentages using 14 NCP data sets. The method of weighted least squares was used to fit the formula. Results indicate that restrictions of age range, and maximum percent of individuals with one or more DMF teeth, are necessary before applying the formula. It was determined that for K=100, and B=0.542 the formula could be applied to age groups in the 5-11 year old range which also had not more than 70% of the children with one or more DMFT.

NIDR Classification: 10500

Objective:

In 1944 Knutson described a formula for estimating age specific DMFT (X) from the percent of individuals in an age group having one or more decayed, missing, or filled teeth (Y). This study was designed to evaluate Knutson's formula for estimating age specific DMFT using 14 NCP data sets. More recently several manuscripts on this topic have suggested that such an estimation procedure might be more applicable for a restricted range of ages, and/or for age groups with not too large a percentage of individuals having one or more DMFT. This study was designed to investigate these questions.

Methods:

The method of weighted least squares was applied to 149 age specific means and corresponding Y percentages. This method was subsequently reapplied to data sets restricted by either age range, Y percentage or both simultaneously.

Major Findings:

Restricting applicability of the estimating formulas to specific age ranges and maximum Y percentage age groups produced significant improvement in the accuracy of the estimation. The age groups giving best results were the 5-11 year olds, while the most appropriate maximum Y percentage cut-off was 70%.

Significance;

This procedure could be used to estimate DMFT prevalence with much simpler and shorter examinations. The resulting cost of such a study could consequently be reduced, or many more children could be examined for the same cost.

Proposed Course:

A revision of the manuscript has been completed and submitted for publication in an internationally-circulated dental journal.

SCIENCE INFORMATION EXCHANGE (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00103-05 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Synthesis of 8-Hydroxyquinolines with potential for long-term anticaries activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dale B. Mirth	Staff Fellow	CP PR NIDR
OTHER:	Carol J. Miller	Biologist	CP PR NIDR
	Albert Kingman	Statistician - Health	CP PR NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Preventive Methods Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.24	.14	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In an effort to develop new antiplaque-anticaries agents, substituted 8-hydroxyquinolines are being prepared which have the potential for binding irreversibly to hydroxyapatite or proteins on the surface of a tooth. These compounds contain acid hydrolyzable bonds to allow for the slow release of the 8-hydroxyquinoline portion of the molecule at the time of acid challenge, which should result in the suppression of bacterial growth and subsequent plaque formation and caries development. Compounds will be evaluated for in vitro antibacterial and antiplaque effects. Promising compounds will be evaluated for their ability to control plaque and experimental dental caries in the rat model.

NIDR Classification: 10510

1. Project Description

Objective

The objective of this project is to synthesize and evaluate new antibacterial agents with the potential for long-term antiplaque and anticaries activity.

Our general approach to this problem involves starting with an antibacterial agent which has three desirable properties: (1) active against Streptococcus mutans and other oral organisms implicated in the caries process, (2) relatively nontoxic, and (3) has affinity for the tooth surface or for pellicle or plaque. To this antibacterial molecule one then attaches a side chain which has at its end a group capable of covalent bond formation with proteins in pellicle or plaque. The side chain is designed so that it contains a link which is susceptible to acid hydrolysis.

On administration of this compound, the antibacterial portion of the molecule undergoes its normal reversible interaction with the tooth surface. This interaction or reversible binding brings the group on the side chain which is capable of covalent bond formation into close proximity with nucleophilic groups present in pellicle or plaque protein. This could result in covalent bond formation and irreversible binding of the compound to the tooth surface. Then in the presence of bacterial activity and subsequent acid production, the acid hydrolyzable link would be broken, freeing the antibacterial portion of the molecule and allowing it to exert its antibacterial effect. This approach should have two advantages over the use of standard antibacterial agents: (1) it should prolong the duration of action of a given dose, and (2) it should increase the effectiveness of a given dose as the antibacterial molecule would be released right at the tooth surface or in the plaque.

For our initial model compounds, we have chosen 8-hydroxyquinoline and substituted 8-hydroxyquinolines as the antibacterial portion of the molecule. The 8-hydroxyquinolines are known to have activity against Strep. mutans and they have affinity for the tooth surface. We plan to attach side chains containing fluorosulfonyl groups via ester or acetyl linkages at the 8- or 5- position of the 8-hydroxyquinoline molecule. The fluorosulfonyl group will serve as the covalent bonding portion of the molecule.

Methods

8-Hydroxyquinolines were obtained from commercial sources or were prepared using literature procedures. Side chains were attached via

ester linkages by reacting acid chloride derivatives of the side chain with the parent 8-hydroxyquinoline molecules. Antibacterial screening was carried out using the tube dilution technique. Additional *in vitro* testing was carried out under the auspices of the National Caries Program screening program. *In vivo* antiplaque and anticaries screening was performed using the rat model.

Major Findings

1. *In vitro* studies have shown that 8-hydroxyquinolyl benzoate and 8-hydroxyquinolyl p-(fluorosulfonyl) benzoate have greater residual antiplaque activity than 8-hydroxyquinoline (8-HQ) itself, indicating that it is possible to enhance the adsorption of an 8-HQ molecule on the tooth surface and thereby increase *in vitro* antiplaque activity by attaching the appropriate side chain in the 8-position. Both compounds appeared to be worthy of evaluation in animals as antiplaque and anticaries agents.

2. Daily mouthrinsing of a group of rats with a 95% ethanol solution containing 10^{-2} M 8-hydroxyquinolyl p-(fluorosulfonyl) benzoate produced a 64% reduction in smooth surface caries in these animals compared to an untreated control group. This was equivalent to the caries reduction seen in animals treated with the positive control alexidine. However, neither the alexidine or the 8-HQ p-(fluorosulfonyl) benzoate group's caries score was significantly different from the smooth surface caries score for the solvent control group.

3. In a second study where the test compounds were dissolved in a solution consisting of 10% acetone, 20% ethanol, and 70% glycerol, no significant differences were found between mean plaque or smooth surface caries scores for groups treated with the 8-HQ derivatives and the controls. Unfortunately the caries level was higher in this study, than in the previous study, and many of the sulcal lesions had advanced to the point that they were difficult to tell from smooth surface lesions. As a result the smooth surface lesion picture in this study was probably distorted, making the data somewhat unreliable.

4. A third rat mouthrinse study was run in which 8-hydroxyquinolyl p-(fluorosulfonyl) benzoate was evaluated using 24 animals per group instead of the normal 12. The length of the study was decreased from 9 weeks to 4 weeks to minimize the chance of extensive lesions distorting the caries picture. The caries results are being evaluated at the present time.

Significance

The successful application of the principles outlined in this report should lead to the development of antiplaque and anticaries agents with enhanced efficacy and a longer duration of action.

Proposed Course

The results of the latest animal trial will be evaluated. No further studies in this area are planned at this time.

2. Publications

Mirth, D.B., Chite, A.F., and Schuster, G.S.: 8-Hydroxyquinolines with the potential for long-term anticaries activity. Design, synthesis, and *in vitro* evaluation. *J. Dent. Res.* *57* (No 1) 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00112-05 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Screening of Anticaries Agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Roald J. Shern	Clinical Investigator	CP PR NIDR
OTHER:	Albert Kingman	Statistician - Health	CP PR NIDR
	Kathleen M. Couet	Biologist	CP PR NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Preventive Methods Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.93	.33	.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to identify anticaries agents suitable for short term clinical trials. Screening is conducted both in vitro and in animals. The in vitro studies measure adsorption, minimal inhibitory concentration, contact angle and modification of enamel dissolution rate. The animal phase measures caries and plaque control and also monitors untoward effects such as acute toxicity staining.

NIDR Classification: 10510; 10520

1. Project Description

Objective

The objective of this project is to identify anticaries agents suitable for clinical testing and to improve screening methods.

Methods

The methods of evaluation vary according to the postulate properties of the agents and the therapeutic objectives sought. The testing is conducted in vivo and, if indicated, in rats or subhuman primates. The in vivo methods assess the effects of the agent on restricting bacterial challenge and enamel dissolution. The ability of the agent to resist oral clearance is estimated also. Animal studies routinely measure presumptive safety and restriction of plaque and its ability to produce acid. Electively, stain of the enamel and fluoride uptake by the enamel is measured.

Major Findings

Certain cationic detergents and fluorides can restrict dental caries when used at low dosages. Of the cationic antiseptics, the bisbiguanides proved most effective. Stannous fluoride and the organic fluorides of alexidine and the long chain fatty amine seemed most promising, markedly restricting caries and plaque while being used. The antiplaque effects noted in the rat plaque studies were similar to those noted in coordinated clinical studies that used the same regimens. Pretreatment of rat teeth with a saturated solution of $CaHPO_4$-$2H_2O$ (pH2) markedly enhanced the uptake of fluoride and seemed to enhance the caries restrictive effects of fluoride.

Significance

The systematic use of in vitro tests permits an efficient method of identifying agents which possess adequate potential for limiting caries and plaque in humans. The animal model provides useful information regarding the safety and regimen for a given formulation. The sequence of testing establishes a rational basis for continued testing of an agent and reduces the number of studies which need to be done in humans, resulting in greater safety, economy and speed of evaluation. If pretreatment with $CaHPO_4$-$2H_2O$ provides synergistic activity it might be possible to augment current fluoridation procedures.

Proposed Course

Four principal objectives are sought for improving the preclinical phase: (1) increase reliability of caries, plaque and pH scoring, (2) simplify all scoring methods without decreasing reliability, (3) validate existing estimates of incipient dental caries and of plaque extent using the appropriate validating criteria, and 4) test agents in subhuman primates when human studies were not yet authorized.

Major Findings

In vitro studies showed that SnF_2 prolonged the substantivity of amine fluoride. SnF_2 and APF functioned synergistically in reducing the susceptibility of enamel to acid attack. However, the SnF_2-amine fluoride combination and SnF_2-APF combination were about as effective in reducing caries in rats as were equimolar (F^-) solutions of the individual fluoride salts. Interim results suggest that the efficacy of fluorides and other anticaries agents is significantly affected by the choice of vehicle.

2. Publications

Shern, R.J., Amsbaugh, S.M., and Reynolds, G.R.: Effects of Daily Rinses with Sodium fluoride and Two Organic Fluoride on Rat Caries. J. Dent. Res., 56:1063-1066, 1977.

Shern, R.J., Couet, K.M., Chow, L.C., and Brown, W.E.: Effects of Sequential Calcium Phosphate on Fluoride Uptake in Rats. J. Dent. Res. (Supplemental Issue) in press.

Shern, R.J. and Couet, K.M.: Effect of Stannous Fluoride and Tiodonium Chloride on Dental Plaque in Rats. J. Dent. Res. submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00113-05 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

Contract #N01-DE-52484

TITLE OF PROJECT (80 characters or less)

Clinical Trial #0600075

Short-term clinical trials of antiplaque agents

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Roald J. Shern	Clinical Investigator	CP PR NIDR
OTHER:	Janet A. Brunelle	Statistician - Health	CP PR NIDR
	Kathleen M. Couet	Biologist	CP PR NIDR
	S. L. Yankell	Dental Reseracher	Univ. of Pennsylvania

COOPERATING UNITS (if any)
Department of Periodontology, School of Dentistry
University of Pennsylvania, Philadelphia, Pennsylvania

LAB/BRANCH
Caries Prevention and Research

SECTION
Preventive Methods Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.42	.32	.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are: (1) to identify, adapt and pretest in the laboratory, measurements of response variables including plaque bacteria speciation, cytologic changes and pH modification; (2) to conduct short-term clinical studies of agents which might be capable of restricting plaque and caries. These studies would measure various safety and efficacy parameters of antiplaque agents.

NIDR Classification: 10520

1. Project Description

Objectives

The objectives are twofold: to identify, adapt and pretest methods of measurements of oral responses, and to conduct short-term clinical studies of agents which might be capable of restricting dental plaque and caries.

Methods & Findings

The methods of evaluation vary according to the postulated properties of the agent and the therapeutic objectives sought. The measurements are both standard and elective permitting standardization yet flexibility in evaluation of the agents. Response to both safety and efficacy are measured. Specifically, the effects of the agent on plaque extent, mass, composition and metabolism are measured.

New methods are being developed for measuring the fluoride and hydrogen ion concentrations in plaque. Interim findings suggest that the microdiffusion technique is more appropriate than the Birkeland method of measuring fluoride. The chemically sensitive field effect transistor (CHEMFET) might measure the hydrogen ion concentration more effectively than does the electrode.

Collaborative studies with the U. of Pennsylvania have shown SnF_2 to be the most promising antiplaque agent. Collaborative studies conducted at Hazelton on subhuman primates have shown similar results.

Significance

The development of new measurements permits more information to be derived in a clinical trial. The variety of measurements provides insights into the mechanism of action of a given agent. Such information can be used for designing appropriate additional studies. Initial studies suggest that the subhuman primates can be used for evaluating agents when conventional clinical testing is deemed inappropriate. If the CHEMFET proves to be stable it will supplement conventional sensors for detection of various ions.

Proposed Course

Studies will emphasize measurements of the chemical composition of dental plaque. We will continue to develop a microdiffusion method for measuring nanogram amounts of fluoride in biological samples such as dental plaque. Various sensors will be tested intraorally for detecting H^+, F^-, Ca^{++} and K^+.

MITHSONIAN SCIENCE INFORMATION EXCHANGE ROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00117 05 CPR

ERIOD COVERED
October 1, 1977 to September 30, 1978

ITLE OF PROJECT (80 characters or less)
Fluoride Effect on incorporation of radioactive ^{45}Ca into hard tissues of developing rat teeth

AMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER ROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Stiles, H. M. Microbiologist CPR, NIDR

OOPERATING UNITS (if any)

University of Maryland Dental School, Department of Anatomy

AB/BRANCH
Caries Prevention and Research Branch

ECTION
Etiology

NSTITUTE AND LOCATION
NIDR

OTAL MANYEARS:	PROFESSIONAL:	OTHER:
.32	.12	.20

HECK APPROPRIATE BOX(ES)

] (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

] (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rat pups are given intraperitoneal injections containing various concentrations of fluoride and radiolabeled ^{45}Ca on days of age 5, 9, and 12-20. The animals are sacrificed just prior to eruption of first molars, the jaws resected and the teeth examined for radioactivity. A group of rat pups treated in the same manner using non-radioactive Ca are used to study the development of dental caries.

NIDR Classification: 10550--75%; 10210--25%

1. Project Description

Objectives:

To examine the effect of physiologically injected fluorine on the uptake and dissolution of radiolabeled calcium from the teeth of rats.

Methods:

The teeth of rat pups are labeled via two methods with radiolabeled Calcium (^{45}Ca). One group of pregnant female rats (Osborne-Mendel) are given IP injections of radiolabeled calcium two days prior to expected delivery. Another group of rat pups are given IP injections of ^{45}Ca on days of age 5, 10, 12-20. Both groups of rats receive IP injections of various concentrations of fluoride. The animals are sacrificed just prior to eruption of first molars, the jaws resected and the teeth examined for radioactivity. A group of rat pups treated in the same manner using non-radioactive Ca are used to study the development of dental caries.

Major Findings:

Results suggest that as the concentration of fluoride injected post-natally increases, there is a decrease in radioactive calcium incorporated into the tooth structure. However, the concentration of fluoride injected appears to have little effect, if any, on the uptake or loss of ^{45}Ca from the teeth when incubated in solutions having a terminal pH of 4.5.

These experiments have resulted in a highly reproducible method for labeling of rat teeth with ^{45}Ca. Continuation of the study will include investigation of fluoride content of the enamel, electron microscope use to examine the difference (s) in crystal formation and structure, and the introduction of calcium blocking agents into the regimen.

Results from the study on caries development is inconclusive and studies are being repeated.

Results from the other facets of the study are incomplete.

Significance to Program:

The protective effect of fluoride against dental caries is well established; however, the mechanisms(s) by which this is accomplished is unclear. The research will provide information relative to this problem.

Proposed Course:

This research is a collaborative effort with Dr. Sue-ning C. Barry, University of Maryland Dental School. The injections and the radio-assays of the teeth are done by Dr. Barry while the animal caries studies are conducted by the PI, NCP, NIDR.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00147-04 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Lectins in the study of plaque and caries development

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dale B. Mirth	Staff Fellow	CP PR NIDR
OTHER:	Carol J. Miller	Biologist	CP PR NIDR
	William H. Bowen	Acting Chief, CP&RB	CP PR NIDR
	Albert Kingman	Statistician - Health	CP PR NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Preventive Methods Development Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.57	0.77	0.80

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lectins, which are proteins capable of interacting with certain macromolecules and/or cell types via specific sugar moieties, are being used to investigate the interactions between saliva and/or bacteria in order to better elucidate the role these interactions play in plaque and caries development. Findings to date support the conclusion that 4 lectins, wheat germ agglutinin, concanavalin A, fucose binding protein and soybean agglutinin, can reversibly bind to and inactivate by complexation and/or precipitation the aggregating factor in saliva that is responsible for inducing the aggregation of Streptococcus mutans cells. These results provide evidence that the salivary aggregating factor contains N-acetyl-D-glucosamine, D-mannose and/or D-glucose, L-fucose and N-acetyl-D-galactosamine and/or D-galactose. Chromatographic and immunochemical techniques are being investigated for the isolation and characterization of the salivary aggregating factor contained in the lectin-induced precipitates.

NIDR Classification: 10260

1. Project Description

Objective

Lectins, which are naturally occurring proteins capable of interacting with certain macromolecules and/or cell types via specific sugar moieties, are being used to investigate the interactions between saliva and oral bacteria in order to elucidate the nature of the substances responsible for these interactions and the role they play in plaque formation and caries development.

The feature that originally attracted us to lectins was their ability to bind to and precipitate specific types of glycoproteins. This property was of special interest since reports by investigators such as Ericson, Gibbons, Kashket, and Hay have provided evidence that the aggregating factor in saliva is a glycoprotein. Because lectins can react with glycoproteins, we reasoned that one or more lectins would most likely react with the aggregating substance and thereby modify the process of saliva-induced bacterial aggregation. In accomplishing this, we should also learn something about the sugar moieties present in the aggregating substances, because a given lectin will only react with glycoproteins containing specific sugar moieties and the sugar specificity is different for different lectins.

Methods

The ability of saliva to induce bacterial aggregation and the effect of lectins on this process was determined using a spectrophotometric assay procedure. Fresh whole saliva was diluted 1:1 with pH 6.8 phosphate buffered saline and centrifuged at 5000 x g for 15 minutes. Concurrently, an overnight culture of bacteria, e.g., strains of Streptococcus mutans grown in Todd Hewitt media with 5% glucose or Jordans Streptococcus media with 0.2% glucose, was washed and resuspended in the pH 6.8 PBS so the absorbance of the suspension at 700nm was approximately 1.5. One part of the saliva supernatant was then mixed with two parts of the bacterial suspension and the change in A_{700} followed. Aggregation was detected by the resulting large decrease in the A_{700} reading after 2 hours of incubation at 37^{0}. When lectins were used, the saliva was pretreated with a given concentration of the lectin for 30 minutes and then centrifuged at 10,000 x g for 30 minutes to remove the resulting precipitate. The aggregating activity in the supernatant, lectin-induced precipitate, and precipitate washings was determined.

Major Findings

1. Soybean agglutinin (SBA), a lectin capable of binding to N-acetyl-D-galactosamine and D-galactose moieties in glycoproteins, can precipitate the aggregating factor for S. mutans strain 1B.

2. Concanavalin A (ConA) and fucose binding protein (FBP) are both more effective than SBA in precipitating material with aggregating activity from saliva.

3. ConA-, FBP-, and SBA-induced precipitates contained less secretory IgA (S-IgA) per unit of aggregating activity than corresponding untreated saliva and lectin-treated saliva supernatants, suggesting that no more than a small fraction of the total S-IgA in saliva could be involved in inducing aggregation.

4. Treatment of dissolved lectin-induced precipitates with antiserum to human α-chain abolished aggregating activity, suggesting that the S-IgA present in the lectin-induced precipitates may either be directly involved in inducing aggregation or is complexed with the actual aggregating factor.

5. Anti-aggregating factor antiserum has been produced in rabbits by using a FBP-induced precipitate from saliva as the source of antigen.

6. Treatment of saliva with the anti-aggregating factor antiserum can completely block the subsequent aggregation of S. mutans by the saliva.

Significance

This investigation has shown that lectins are useful for elucidating the structure of salivary aggregating factors. The findings that a least 4 lectins can precipitate the salivary aggregating factor for S. mutans and the observation that the lectin-aggregating factor interactions can be reversed by the sugar for which the lectins are specific, suggest that these lectins could be useful for purifying salivary aggregating factors by precipitation and/or affinity chromatography. The development of a purified anti-aggregating factor antiserum could lead to a convenient method of assaying saliva for aggregating activity by rocket immunoelectrophoresis or similar techniques.

These studies will help to elucidate the role of saliva induced bacterial aggregation in plaque and caries development. This knowledge should aid the development of new caries preventive measures.

Proposed Course

Various chromatographic, immunochemical and electrophoretic techniques will be used in an attempt to isolate and identify the substance with aggregating activity that lectins precipitate from saliva. The purificati of the antiserum with anti-aggregating factor activity will be investigate

2. Publications

Mirth, D.B., Miller, C.J., Kingman, A., and Bowen, W.H.: Inhibition of saliva-induced aggregation of Streptococcus mutans by wheat germ agglutinin. Caries Res., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00154-04 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biochemical Products and Energy Requirements of Plaque

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robrish, Stanley A.	Research Microbiologist	CPRB, NIDR
Other:	Bowen, W. H.	Acting Chief, CPRB	CPRB, NIDR
	Kemp, C. W.	Microbiologist	CPRB, NIDR
	Dennis, Donna	Physical Science Asst.	CPRB, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Etiology

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.22	0.52	0.7

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have established the direct relationship between the analysis of the protein content of dental plaque samples and the dry weight of the samples. We have quantitated very small dental plaque samples by applying methods of analysis which have been developed in this laboratory. We have also quantitated dental plaque samples by the extraction and analysis of flavin mononucleotide (FMN). The FMN analysis to quantitate plaque can be performed in the presence of protein or amine containing materials. The specific FMN content of dental plaque samples was constant for both pooled plaque samples and for small samples obtained from individual teeth. The minimum sample size for a constant specific FMN content of dental plaque was 30 micrograms dry weight. Attempts have continued to normalize the plaque samples using unique bacterial constituents such as D-alanine and muramic acid. The analysis of the viable fraction of the dental plaque mass has continued with attempts to establish the relationship of "adenylate energy charge" (AEC) to the percent viable cells in a population.

NIDR Classification: 10230; 10250

PHS-6040
(Rev. 10-76)

Normalization of dental plaque:

Last year we reported the analysis for viable cell mass of plaque samples obtained from monkeys by extractable ATP. We calculated the protein content of the plaque and expressed the total cell mass in terms of the equivalent protein content of S. sanguis. At the time, there was no published evaluation of the relationship of the protein content of dental plaque to the dry weight of the sample. We had developed a very sensitive and simple assay for protein using o-phthalaldehyde (OPT) and it seemed to be the only reasonable method available to do small dental plaque samples. We tested the relationship of this reaction to the dry weight of dental plaque. Pooled plaque samples were obtained from the mouths of 19 individual monkeys and the samples suspended in distilled water. The dry weight and protein content of each sample was plotted and the scatter of the points fitted by linear regression. These data showed a linear relationship and the factors were correlated with a coefficient of 0.97. We therefore conclude that the protein content of a plaque sample is a direct measure of the dry weight of the sample.

Determination of protein with OPT has been used by other members of this program. We have assisted both Drs. Shern and Stiles in using this reaction to help normalize some small plaque samples. The extreme sensitivity of the determination has allowed us to analyse protein in some samples for Drs. Cole and Thomson. The amounts of material available for analysis of the plaque fluid samples and the immunofluorescent conjugate was very small and would have required a large part of the sample if done by other methods. We were able to perform analyses for these people while using only a negligible amount of these very limited samples.

In the last report I described some of the limitations to the use of protein for the normalization of dental plaque samples and proposed that the analysis of flavin mononucleotide (FMN) might be used for the same purpose. In the past year we have developed the assay for FMN using bacterial luciferase. The assay is dependent upon bacterial luciferase, the preparation of a long chain aldehyde in a soluble form, and the ability to reduce the substrate to $FMNH_2$. In the presence of these constituents, light can be quantitated using the same instrumentation we have previously described for the analysis of ATP. The assay is very sensitive being able to quantitate FMN in the lower picomole range. The use of hot Tris buffer has proved effective to extract the FMN from bacterial cells. We have demonstrated that the amount of the substrate extracted was proportional to the dry weight of the sample. This relationship has been determined for several organisms commonly found in dental plaque and the specific FMN content was calculated for these pure cultures. When dental plaque samples obtained from monkeys were extracted and analysed in the same manner, we found a specific FMN content which was similar to that obtained from the pure cultures of plaque microorganisms. We have further demonstrated the direct proportionality of the FMN contents of dental plaque samples obtained from monkeys with the dry weights of the same samples. Most importantly, the FMN content of

dental plaque could be quantitated in the presence of amine containing reagents or in the presence of intact proteins. This has allowed us to overcome some of the major limitations imposed by the use of the analysis of protein. We have further demonstrated that the ratio of extractable FMN to protein (dry weight), was constant for 28 samples with widely varying size and consisting of samples which were pooled as well as those obtained from individual teeth. The analysis is not restricted to samples of pooled dental plaque and that the minimum reliable sample size we have established is 30 micrograms dry weight of dental plaque. Samples of this size are of the range of the smallest which one would wish to take from a specific site on an individual tooth.

One of the most interesting reactions we have used this year in our efforts towards the microanalysis of dental plaque is the use of luminol (5-amino-2,3 dihydro-1,4 phthalazinedione). When luminol is mixed with an iron porphyrin, light is given off in direct proportion to the amount of the iron compound. Because we suspect that the major iron containing compound should be catalase, we have used this heme protein as our standard. The reaction is very easy to perform and requires no extraction of the reactive compound as in the case of the analysis for ATP or FMN. The reaction is very sensitive being able to quantitate catalase in the lower nanogram level. Studies are proceeding with an analysis of dental plaque in addition to a variety of pure cultures. When the luminol reaction and dry weight data for dental plaque samples were analysed by linear regression, a lower but definite relationship was shown. Studies have continued by the analysis of pure cultures to determine what fraction is responsible for the data obtained from the plaque samples. We have now shown that there is a direct relationship between the luminol reacting fraction of the sample and the dry weight of A. viscosus. Experiments are in progress to obtain these data with a wide variety of other pure cultures of oral bacteria.

We have made attempts to use other bacterial constituents in order to normalize dental plaque samples. The use of protein or FMN to normalize samples is limited to plaque from smooth surfaces because of the interference of mammalian cells, such as epithelial or white blood cells. In order to circumvent these problems and to have some unequivocal measure of plaque, we have been trying to develop a measure of constituents which are unique to bacteria, such as D-alanine and muramic acid. The determination must be very sensitive in order to be able to quantitate the very small amounts of material obtained in dental plaque sampling. D-alanine can be released after drastic acid hydrolysis of the sample and the hope was to quantitate this material using a coupled enzyme system of D-amino oxidase and L-lactic dehydrogenase. We were able to analyse D-alanine standards but when this was tried with hydrolysates of pure cultures of bacteria, there were too many interfering materials. In the coming year, we hope to couple the reaction of D-amino oxidase to one of several dyes in an attempt to assay the constituent with sufficient specificity and sensitivity. Muramic acid can also be released during acid hydrolysis of bacterial cells. Subsequent alkaline hydrolysis of this will release lactic acid in the D form which can be quantitated with the aid of D-lactic dehydrogenase. Unfortunately, the enzyme is very expensive and the reaction does not have the sensitivity to be able to determine D-lactate in small samples of dental plaque. We are now looking into a variety of other methods to determine this unique constituent of bacteria as a measure of dental plaque.

Attempts to quantitate viable cells in plaque:

In our previous report and in the work which was published this year, we demonstrated that the analysis of ATP could be used to determine the viable portion of dental plaque. A major limitation in the use of this analysis to estimate the number of viable cells in plaque is the fact that the specific ATP content of the cells is quite variable with respect to the growth phase of the organism; e.g., cells in a stationary phase versus those in exponential growth. We have proposed using the function of "adenylate energy charge" (AEC) as described by Atkinson to overcome this problem. This function is the fraction of ATP with respect to the other adenine nucleotides and, in the experience of other workers, AEC is a much more constant value than the specific ATP content. We have succeeded in obtaining these values for both pure cultures and for a limited number of dental plaque samples. We believe that we can define the AEC value in terms of the fraction of viable cells of a population by determining this value using a population of cells with known viable fractions. We have performed several experiments this year in which we establish killing curves of bacteria using physical methods such as heat or a sonic treatment.

Fermentation products from plaque and pure cultures:

We have been continuing our analysis of the fatty acid end products of pure cultures and dental plaque toward an understanding of the energetic balance of dental plaque. During the year we have analysed a variety of standards in order to establish the qualitative analysis of these compounds. In addition to standards, we have made and analysed some of the common derivatives of the fatty acids in order to determine the basic retention functions for the homologous series of both the acids and derivatives. All of the analyses this year, have been made using some outdated equipment obtained from surplus. We have been able to order a new gas chromatograph which should make these analyses considerably easier. We plan to continue these analyses in the coming year and hope to be able to analyse the metabolic end products found in dental plaque samples as well as the fatty acid end products found in dental plaque fluid. The latter analysis is particularly important because we will be able to compare the presence of these products in samples obtained from high and low caries areas.

Robrish, Stanley A., Kemp, Christopher W. and Bowen, William H.: Use of Extractable Adenosine Triphosphate to Estimate the Viable Cell Mass in Dental Plaque Samples Obtained from Monkeys. Appl. Enviorn. Microbial. 35: 743-749, 1978.

Robrish, Stanley A., Kemp, Christopher and Bowen, William H.: The Use of the o-Phthalaldehyde Reaction as a Sensitive Assay for Protein and to Determine Protein in Bacterial Cells and Dental Plaque. Anal. Biochem. 84: 196-204, 1978.

Rölla, Gunnar, Robrish, Stanley A. and Bowen, William H.: Interaction of Hydroxyapatite and Protein-Coated Hydroxyapatite With Streptococcus Mutans and Streptococcus Sanguis. Acta path. microbiol. scand. Sect. B, 85: 341-346, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00164-03-CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978 CT 0600115

TITLE OF PROJECT (60 characters or less)

Effect of School Water Fluoridation and Fluoride Mouthrinsing on Dental Caries

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Meyers	Clinical Investigator	NCP, NIDR
Other:	W. S. Driscoll	Clinical Investigator	NCP, NIDR
	J. A. Brunelle	Chief, Biometry Section	NCP, NIDR
	H. S. Horowitz	Chief, Community Programs Sect.	NCP, NIDR

COOPERATING UNITS (if any)

North Carolina State Board of Health and Duplin county, North Carolina, Public School System

LAB/BRANCH
Caries Prevention and Research

SECTION
Community Programs

INSTITUTE AND LOCATION
National Institute of Dental Research, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.27	0.13	0.14

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

About 1500 children attending grades K through 12 at five schools in Duplin County, North Carolina, are exposed to fluoridation of the school water supply at four and one-half times the level estimated to be optimum for community fluoridation in the same geographic area. In addition, weekly mouthrinsing with a 0.2% NaF solution is done by children in kindergarten through the eighth grade. Teachers dispense 10 millimeters of solution to each participant-child and supervise the one-minute rinsing procedure which is carried out in the classroom. To evaluate the long-term benefits of the combined treatments on the prevalence of dental caries, pre-treatment clinical data will be compared with the data obtained every two years until the completion of the program in 1987. Baseline examinations using the DMF tooth and surface index were conducted in October/November 1975. The project is in its third year. The first follow-up examination was conducted in September 1977.

NIDR Classification: 10600

Objective

The purpose of the study is to evaluate a combined program of school water fluoridation and mouthrinsing with sodium fluoride for the prevention of dental caries.

Methods

The study, a cross-sectional clinical trial, was initiated in October 1975, on approximately 1500 children attending grades K-12 in five public schools located in Duplin County, North Carolina. The County has negligible amounts of fluoride in its sources of drinking water.

All study schools, including high schools, were fluoridated at 4-1/2 times the level estimated to be optimum for community fluoridation in the same geographic area. Surveillance of the fluoride levels is provided by school personnel under the supervision of the North Carolina State Board of Health. In addition, children in grades K-8 rinse weekly, under the supervision of the classroom teacher, with a 0.2 percent sodium fluoride mouthrinse. Children swish 10 milliliters of the solution between their teeth for 60 seconds and then empty the contents of their mouths into a paper cup. Baseline dental examinations, using the DMF tooth and surface index were conducted prior to the installation of fluoridation equipment and the start of the rinse procedures. Follow-up examinations will be compared with baseline findings.

Findings

At the baseline examination, children age 6-15 had a mean DMFS score of 6.74. In 1977, after two years of the preventive program, the DMFS score for children of the same ages was 5.41, or 19 percent less decay. Children, in 1977, had an average of 1-1/4 fewer decayed teeth after two years than did their cohorts at the baseline.

Significance

Populations in the United States residing in areas that have no central water supply are deprived of the benefits afforded by community water fluoridation. For children in these areas a combined program of school water fluoridation and weekly mouthrinsing with sodium fluoride should produce a marked cariostatic effect. These procedures combine the systemic effects of fluoride provided by the ingestion of school water and the topical effects of fluoride provided by the weekly mouthrinses.

Both preventive methods offer a number of advantages for school health programming: simplicity, economy, feasibility, safety, acceptability and minimal requirements for professional personnel.

Proposed Course

Follow-up examinations will be performed every two years, for the first six years of the study, and every three years during the final six years, ending in 1987.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00185-03-CPR

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Antimicrobial Susceptibility of Streptococcus mutans

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Little, Wayne A.	Microbiologist	CPR, NIDR
Other:	Thomson, Lynn A.	Senior Dental Surgeon	CPR, NIDR
	Bowen, William H.	Acting Chief	CPR, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Etiology

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.27	.02	.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study was to examine the antibiotic susceptibility of Strep. mutans. From the results of the screening experiments with Oxoid multidiscs, twelve antibiotics were selected for MIC determinations using a broth microtiter technique.

NIDR Classification: 10510; 10250

Objectives

The involvement of Streptococcus mutans in dental caries and bacterial endocariditis has prompted interest in the susceptibility of this organism to antimicrobial agents. Furthermore, the inclusion of certain agents into selective media has produced preliminary evidence that there may be differences in antimicrobial susceptibility among the different serotypes of S. mutans. The purpose of the present study was to examine the antibiotic susceptibility profiles of S. mutans strains representing serotypes a through f.

Methods

Stock solutions of antibiotics were made with recommended diluents and stored at - 70°C. Further dilutions of the antibiotics were made with trypticase soy broth (TSB). Serial dilutions were carried out manually in microtiter plates using a handlebar that accommodated twelve 50 µl capacity diluters. The microtiter plates were innoculated with 50 µl of diluted culture containing approximately 10^5 colony forming units per ml, covered with clear plastic and incubated aerobically at 35°C for 48 hours. The minimal inhibitory concentration or MIC was the lowest concentration of antibiotic that caused complete inhibition of growth.

Major Findings

Erthromycin, penicillin, lincomycin, methicillin, vancomycin, and tetracycline were the most active antibiotics, having MIC values ranging from .006 to 3.125 µg/ml. Gentamicin, streptomycin, bacitracin, neomycin, kanamycin and polymyxin B had MICs ranging from 1.56 to 400 µg/ml. Bacitracin was most inhibitory for serotype a strains, having an MIC of less than 0.78 U/ml. Strains representing serotypes b through f were inhibited by bacitracin at concentrations ranging from 0.78 to 50 U/ml. Serotype a and b strains were sensitive to polymyxin B at concentrations of 50 U/ml or less. Four of the 10 serotype b strains had MICs of less than 6.25 U/ml. The majority of strains representing the other serotypes were inhibited by polymyxin B at levels between 25 and 400 U/ml Strains of serotypes a and d were generally less sensitive to methicillen, with 60% of the a strains and 75% of the d strains having an MIC greater than 0.39 µg/ml. On the other hand, nearly 100% of the other serotypes were susceptible to methicillin at this concentration.

Significance to Biomedical Research

The differences in the antibiotic susceptibility profiles among the serotypes, although not sufficient to be a practical means of identification, are nevertheless significant when applied to laboratory methodology. Antibiotics such as bacitracin, polymyxin B and neomycin have been incorporated into media to increase selectivity without first thoroughly testing the agent against representative strains of S. mutans. The results of this study demonstrate that such testing is essential in order to avoid possible selection against one or more of the serotypes.

Although the actual mechanisms responsible for differences in antibiotic susceptibility observed among the S. mutans serotypes are not understood, it is notable that bacitracin, polymyxin B, and methicillin utilize the cell wall as the site of action. These findings indicate that certain antibiotics, and perhaps other agents which act on the bacterial cell surface, may not be equally effective against representative strains of S. mutans.

It has been noted by other investigators that there has been a worldwide increase in the number of Lancefield group A streptococci resistant to tetracycline and that the incidence of resistance among S. mutans may also be increasing. In the present study, only animal isolates were resistant at clinical levels to some of the more active antibiotics, including tetracycline and erythromycin. Animal diets are often supplemented with tetracyclines and macrolides, suggesting that prolonged exposure to these antibiotics leads to the appearance of resistant strains of S. mutans in dental plaque. Although a human population study failed to demonstrate resistant S. mutans strains following long term penicillin therapy, similar results might not be observed with tetracycline or macrolide antibiotics.

Proposed Course

The results of this study were presented at the Annual General Session of the AADR, held in June 1977, in Las Vegas, Nevada. Submitted for publication to the Journal of Antimicrobial Agents and Chemotherapy.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00186-03 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Implantation of Streptococcus mutans in germfree animals and antibody production

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Thomson, L. Ariel	Senior Dental Surgeon	CPR, NIDR
COPI:	Bowen, William H.	Acting Chief	CPR, NIDR
	Little, Wayne A.	Microbiologist	CPR, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Etiology

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.36	.26	.10

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project is intended to study the implantation of Strep. mutans in the oral cavity of animals and man. Because implantation studies in humans present several difficulties at present, initial investigation has been limited to implantation of various serotypes of Strep. mutans in gnotobiotic rats. Factors under investigation include the effect of simultaneous introduction of multiple serotypes, the effect of different sugar diets, the effect of prior induction of antibodies to Strep. mutans, and the effect of sequential introduction of each serotype on the implantation of Strep. mutans in gnotobiotic rats.

NIDR Classification: 10570; 10120; 10230; 10110

Project Description

Objectives:

1. To determine whether serum or salivary antibodies develop during Strep. mutans implantation in gnotobiotic rats and whether antibody titers correlate with either disease induction or the prevalence of any serotype.

2. To determine whether implantation of serotypes a, c, d, and e (Strep. mutans) one week prior to the implantation of serotype b (Strain FA-1, of rat origin), will alter the previously observed ability of serotype b to become the most prevalent serotype in simultaneous implantation experiments.

3. To determine if the observed predominance of serotype b organisms in gnotobiotic implantation studies, as determined by serotype specific FA reagent stained fresh smears, is an accurate indication of serotype prevalence.

4. To determine if prior oral administration of nonviable Strep. mutans serotype b organisms to gnotobiotic rats, will through antibody stimulation or other mechanisms alter the ability of serotype b to become the dominant serotype when serotypes a-e are simultaneously implanted later.

5. To determine what other major factors might influence implantation of Strep. mutans in gnotobiotic caries test system.

Methods:

Gnotobiotic Sprague - Dawley rats were used for all experiments; each group consisted of 12 weanling rats housed in an individual isolator containing cages holding 3 animals each. All diets were sterilized by irradition with 6.0 Mrads. The experimental period included from day 21 through day 56, at which time the remaining rats were sacrificed. Although carious lesions were observed in the rats, the small number of animals remaining at the end of the experiment precluded accurate assessment of the caries scores.

The availability of serotype specific Strep. mutans FA reagents as prepared by Dr. Roger McKinney, CDC, Atlanta, Georgia (Interagency Agreement No. Y01-DE-3001-1), has permitted detailed serotype monitoring of Strep. mutans organisms implanted in test animals. Methods and procedures developed under NIDR projects DE-00122-02 and DE-0064-04 specifically permits the monitoring of direct plaque samples for Strep. mutans serotypes. These methods have been described in an FA Workshop held in conjunction with the 1975 IADR meeting and were published in the Journal of Dental Research 55: Special Issue A, 1976. The proportion of each serotype was determined from the number of fluorescent cells and the total cells visible with alternate FA and phase contrast examination of each field.

Efforts to determine the presence of antibodies to S. mutans in the saliva and serum of the gnotobiotic rats after S. mutans had been implanted have met with only partial success. All available anti-rat and anti-mouse immunoglobulin reagents were purchased from commercial sources and also obtained from other research facilities. Characterization of these antisera revealed that contrary to published reports, mouse and rat immunoglubulin do not cross-react sufficiently to permit the anti-mouse antisera to be used in titering rat serum and saliva. Furthermore, with the exception of anti-rat-IgG antisera (Hyland-anti-7-S globulin), satisfactory reagents are not available to determine rat antibody titers.

Results:

Earlier results suggest S. mutans organisms were able to colonize all sites sampled; the serotype prevalence differing considerably as the experiments progressed and according to the carbohydrate ingested by the animals. Serotype b (rat strain FA 1), predominated in specimens from all sites. Serotype d organisms (strain 6715), frequently employed in caries studies involving sucrose diets, required sucrose to colonize and remain on teeth in the gnotobiotic animals studied. In addition, colonization of the animals by S. mutans was observed to stimulate detectable levels of 7-S antibody to S. mutans in serum, but similar titers were not found in the saliva.

Introduction of four S. mutans serotypes (a, c, d, & e) one week prior to infection with serotype b resulted in the dominance of serotype b in fecal samples by day 56 of the experiments. Serotype b represented 76% of the fecal flora and 95% of that found on the rat's tongue. However, serotype b was observed to only account for 32% of the S. mutans detected in tooth plaque. In tooth samples, serotype e accounted for 49% of the S. mutans in the experiment when serotype b was inoculated one week after the other serotypes.

Administration of non-viable serotype b S. mutans cells in the water of the gnotobiotic rats for ten days prior to implantation with equivalent number of serotype a - e, resulted in serotype b dominance at each location studied with the exception of the teeth. Serotype b comprised an average of 78% of the fecal flora, as contrasted with 31% of the serotypes on the teeth. Serotype e was the second most prevalent serotype, accounting for about 8% at sites other than the teeth. For the teeth, serotype e comprised just over 50% of the organisms present.

Animals with glucose and starch diets had serotype b as the dominant serotype in fecal specimens. However, unlike sucrose fed animals, in only one cage of animals did serotype b comprise more than 50% of the flora. In contrast, with sucrose animals serotype b comprised an average of 95% of fecal flora.

Significance to Biomedical Research

Data from gnotobiotic studies conducted to date suggest that although serotype b (at least strain FA-1) colonize the rat more effectively than the other serotypes of Strep. mutans, the actual dominance can be influenced by the type of sugar available in the diet, the sequence of implantation and prior exposure to serotype b organisms. The data support the concept that oral administration can stimulate considerable serum antigenic response. Furthermore the observation that strains not detected in specimens from the tongue or feces, may be present on teeth and detected in plaque samples, supports the view that the teeth are the major reservoir for Strep. mutans. Another finding which has significance in several research areas was the observation that serotype d Strep. mutans, so frequently employed as the etiologic agent in caries studies implicating dietary sucrose as an active factor, apparently requires sucrose to colonize and remain on teeth in the strain of animals studied.

The prevalence of the serotypes of Strep. mutans in both animals and human populations appears to be related to both the frequency of exposure and the greater capacity of some serotypes to colonize certain animals. The apparent unique dependence of serotype d organisms on the presence of dietary sucrose to colonize teeth prompts further interest in the wide use of serotype d in the animal caries studies. Because serotype d has been reported to occur in about 10% of populations studied to date and appears to have unusual sucrose related colonization capacity, the conclusions drawn may be influenced by both specific serotype d-sucrose interaction and serotype d-animal species factors. It is further anticipated that as the project progresses that specific animal findings will suggest the factors and mechanisms which affect Strep. mutans colonization and permit greater understanding of the implantation in humans. Furthermore, an understanding of the implantation process in humans is likely to suggest several disease control measures which would be effective.

Publications

Thomson, L.A., Bowen, W.H., Little, W.A., Kuzmiak-Jones, H.M. and Gomez, I.M. Simultaneous Implantation of Five Serotypes of Streptococcus mutans in Gnotobiotic Rats. Caries Research: In Press

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00189-03 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cohort Sizes in Dental Caries Clinical Trials

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Albert Kingman Statistician-Health CPR, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research Branch

SECTION
Biometry Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.01	.01	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An investigation was undertaken regarding the determination of desirable levels of statistical power for implementation in dental caries clinical trials. The chief concern was to determine sufficiently large cohort sizes so as to be able to reliably detect specified differences in therapeutic efficacy levels among prophylactic agents for dental caries. Another concern was to investigate the consequences of utilizing an insufficient number of subjects in such trials. Statistical tables were also derived for use in the determination of such cohort sizes in terms of the error probability magnitudes and the anticipated efficacy levels.

NIDR Classification: 10500

Background

An investigation was undertaken regarding the determination of desirable levels of statistical power for implementation in dental caries clinical trials. This study was motivated in part by observing that in several clinical trials statistical significance regarding the efficacy of proposed prophylactic agents could not be shown, in some instances even where a 20% to 30% reduction in increment was obtained.

Purpose

The chief concern in this study was the determination of sufficiently large cohort sizes so as to be able to reliably detect specified differences in efficacy levels among prophylactic agents for dental caries. Another concern was to discuss some of the consequences of utilizing insufficient numbers of subjects in such trials as it relates to clinical interpretation of study results.

Results

Statistical Tables were derived for clinical trials in which one agent is tested against a control as well as the multi-group case. These tables can be used by clinical investigators to determine how large a study needs to be conducted for specified clinical differences anticipated and sensitivity levels.

Monte Carlo methods were also employed to derive clinical trials data sets whose relevant parameter values were known to illustrate the practical value of these tables and also document how well one is able to correctly detect known differences in efficacy levels in a practical setting.

Presentation of Findings

The results of this study were presented at the 1976 annual meeting of the AADR held in Las Vegas, Nevada, in June 1976.

Publication

Kingman, A.: Adequate Cohort Sizes for Caries Clinical Trials, Community Dentistry and Oral Epidemiology, 6:30-35, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00190-03 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Extracellular Macromolecules and Virulence of Cariogenic Streptococci

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ciardi, Joseph, E.	Research Chemist	CPRB, NIDR
OTHER:	Bowen, William, H.	Acting Chief, CPRB	CPRB, NIDR
	Reilly, J. Allen	Biologist	CPRB, NIDR
	Hsu, S-Cheng Dana	Biological Aide	CPRB, NIDR
	Cole, Michael	Visiting Scientist	CPRB, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research Branch

SECTION
Etiology

INSTITUTE AND LOCATION
National Institute of Dental Research, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.77	0.67	1.10

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Extracellular molecules produced by S. mutans and implicated in the formation of cariogenic dental plaque are glucosyltransferase, dextranase, lipoteichoic acid, and fructosyltransferase. Investigations of the interactions of these molecules with serum and saliva, antibodies, dental plaque fluid, hydroxyapatite, cariostatic agents, and fluoride have partially elucidated their roles in plaque. Immunization of rodents and monkeys with purifie preparations of these molecules has led to antibody formation. Protection against infection by S. mutans is being evaluated. Preliminary experiments which measured these S. mutans molecules in dental plaque fluid from monkeys suggest a correlation between GTF activity and the presence of S. mutans in plaque. These studies are designed to gain insight into the biochemical and immunological aspects of dental caries which will lead to a rational approach for the control of this disease.

NIDR Classification: 10280; 10240; 10570

Objectives

Several extracellular macromolecules synthesized by Streptococcus mutans have been implicated in cariogenic dental plaque formation. Among these are the glucosyltransferases (GTF), fructosyltransferases (FTF), dextranases and lipoteichoic acids (LTA). The present study proposes: 1) To determine the mechanisms by which these macromolecules are involved in the formation of cariogenic dental plaque. 2) To characterize them both biochemically and immunologically. 3) To test their effectiveness as vaccines for protection against infection by Streptococcus mutans.

Methods Employed

Standard and newly developed methods for quantification of enzyme activities, polysaccharides, and lipoteichoic acids are utilized. Standard immunological methods are employed. Vaccine studies are being carried out in rats and monkeys.

Major Findings

Antibody response in animals immunized through a variety of routes, with cells of S. mutans or cell-free preparations containing GTF, FTF, LTA and/or dextranase synthesized by S. mutans was determined. The results show that the type of immunogenic preparation and the route of its administration can elicit different antibody response and may in part explain the disparity of results achieved by different investigators. The results further emphasize the need to use standardized preparations and carefully prescribed protocols for vaccination. (Ciardi, J.E., Reilly, J.A., Hsu, S.D., and Bowen, W.H. J. Dent. Res. 57: IADR Abs. 114, 1978).

Gnotobiotic studies with rats show that infection with S. mutans alone causes antibody formation to GTF and LTA. Immunization with preparations of GTF or GTF-LTA enhance antibody titers only to GTF; protection against infection with S. mutans is not seen. Immunization with S. mutans cells or with a soluble cell surface fraction increases anti-LTA titer but not anti-GTF; significant protection against infection with S. mutans is observed.

Antiplaque compounds (e.g. bis-biguanides and quaternary ammonium salts) were found to be potent inhibitors of glucan synthesis by GTF. Biological metabolites (organic amine derivatives) present in dental plaque caused a significant potentiation of this inhibition. The effect of these potentiators on inhibition of streptococcal growth and glucan production by antiplaque agents is being studied. The potentiation of GTF inhibition by amine derivatives offers promise toward caries prevention in terms of mouth rinses (Hsu, S.D., Ciardi, J.E., and Bowen, W.H., J. Dent. Res 57: IADR Abs. 972, 1978).

Earlier studies demonstrated that LTA extracted from cells and from culture fluids of S. mutans exhibited a high affinity for hydroxyapatite and that sodium fluoride (NaF) or saliva could inhibit this adsorption. Recent experiments demonstrate that hydroxyapatite beads pretreated with saliva adsorb a decreased amount of LTA or of S. mutans, strain BHT cells; that pretreatment of hydroxyapatite with LTA reduces binding of S. sanguis cells; and that pretreatment of hydroxyapatite with calcium ion caused enhanced adsorption of LTA. These results strengthen the earlier suggestion of a role for LTA in the colonization of teeth by oral streptococci.

Other investigators have shown that LTA can protect streptococci from lysis and that low levels of NaF can enhance lysis. We therefore initiated a study to determine the effect of fluoride on LTA synthesis. The results show that whereas uptake of ^{3}H-glycerol into radioactive LTA was decreased by 50% in the presence of 1 to 2 nM NaF added to the growth medium the total LTA recovered was not different from that in the controls without fluoride. However, the results do sustain a fluoride effect at the cell membrane resulting in a decreased incorporation of exogenous ^{3}H-glycerol into tritiated LTA. (Reilly, J.A., Ciardi, J.E., and Bowen, W.H. J. Dent. Res. 57: IADR Abs. 799, 1978).

Significance to Dental Research:

A more thorough understanding of the mechanisms by which GTF, FTF, LTA and dextranase are involved in the formation of cariogenic dental plaque will lead to the discovery of more effective means of prevention. The development of a safe and effective vaccine against dental caries, comprised of well-defined purified molecules would be well accepted by the population in general and would be instrumental in the control and prevention of this disease.

Proposed Course of Project

Further studies on immunization of monkeys and gnotobiotic or conventional rats with purified macromolecules tested individually and in concert will be carried out. Various routes of administration of immunogens will be evaluated.

Experiments to determine interactions of S. mutans extracellular macromolecules and plaque or saliva components will be continued. The effects of antiplaque agents on these interactions will be studied in order to better understand their mechanisms of action and their structure activity relationships.

The presence and amounts of specific S. mutans macromolecules products in human dental plaque samples from carious and noncarious sites will be determined by very sensitive methods that have been developed in our laboratory.

Publications

1. Ciardi, J.E., Rölla, G., Bowen, W.H., and Reilly, J.A. Adsorption of Streptococcus mutans lipoteichoic acid to hydroxyapatite. Scand. J. Dent. Res. 85: 387-391, 1977.

2. Ciardi, J.E., Bowen, W.H., Reilly, J.A., Hsu, S.D., Gomez, I., Kuzmiak-Jones, H., and Cole, M.F. Antigens of Streptococcus mutans implicated in virulence - production of antibodies. p. 281-292. In J. McGhee, J. Mestecky, and J. Babb (ed). Secretory Immunity and Infection. Plenum Publishing Corp., New York, 1978.

3. Cole, M.F., Bowen, W.H., Sierra, L., Espinal, F., Aquirra, M., Kingman, A., Kemp, L.J., Gomez, I., Reilly, J.A., Hsu, D., Ciardi, J.E. and Gillespie, G. Immunoglobulins and Antibodies in Plaque Fluid and Saliva in Two Populations with Contrasting Levels of Caries. In J. McGhee, J. Mestecky and J. Babb (ed). Secretory Immunity and Infection. Plenum Publishing Corp., New York, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00195-03 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Adherence and Coherence of Cariogenic Streptococci in Dental Plaque.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ciardi, Joseph E.	Research Chemist	CPRB, NIDR
OTHER:	Bowen, William H.	Acting Chief, CPRB	CPRB, NIDR
	Reilly, J. Allen	Biologist	CPRB, NIDR
	Hsu, S. Dana	Biological Aide	CPRB, NIDR
	Cole, Michael	Visiting Scientist	CPRB, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research Branch

SECTION
Etiology

INSTITUTE AND LOCATION
National Institute of Dental Research, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.60	0.40	0.20

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanisms by which oral streptococci interact with teeth, saliva, and with each other to form cariogenic dental plaque are under investigation. Adsorption of S. mutans and/or S. sanguis to hydroxyapatite surfaces does not require sucrose whereas plaque formation by S. mutans on smooth surfaces such as glass appears to involve the formation of adherent glucans from sucrose. Treatment of hydroxyapatite with calcium ions, human saliva, cariostatic agents, or lipoteichoic acid modulates the adsorption of streptococcal cells. Results imply a role of lipoteichoic acid in adsorption of streptococci to hydroxyapatite. Cariostatic agents (e.g. bis-biguanides) which inhibit microbial growth are also potent inhibitors of cell-associated glucan synthesis and adherence to glass. Antibodies to glucosyltransferases and dextranases also prevent S. mutans adherence to glass, but do not effect growth or acid production.

NIDR Classification: 10230; 10240; 10220

Objectives

To elucidate the mechanisms by which oral streptococci adhere to teeth and to each other in the formation of cariogenic dental plaque in order to find effective means to control dental caries. The information obtained from *in vitro* studies on adherence of bacteria to various smooth surfaces and the effect of cariostatic agents on adherence should eventually lead to the formulation of relevant *in vivo* experiments.

Methods Employed

Existing and newly developed methods for measuring the adherence of bacteria to solid surfaces and to each other are utilized. Radioisotope methods are employed to produce radioactive bacterial cells for adherence studies.

Major Findings

The effects of plaque inhibitory agents, sera and antisera on sucrose mediated adherence of cells to glass were determined in growing cultures of *S. mutans*. Chlorhexidine (0.01 mM) and fluoride (50 mM) caused a decreased adherence of cells that was related to inhibition of growth. Low concentrations of these agents did not inhibit cell yield or adherence, although similar concentrations of chlorhexidine but not fluoride inhibited adherence of washed cells under non-growing conditions. Fungal dextranase (0.3 mg/ml) and dextran T500 (0.15%), agents that prevent normal glucan synthesis by *S. mutans*, inhibited adherence 40-60% without effecting growth as determined by terminal pH of the culture. Normal rabbit serum and antiserum with potent activity against glucosyltransferase did not appreciably influence cell yields or acid production when tested at a 1:2 dilution. However antiserum at the 1:2 dilution completely inhibited sucrose mediated adherence and coherence of cells.

S. mutans and *S. sanguis* cells adhere to hydroxyapatite beads in the absence of sucrose. Hydroxyapatite beads pretreated with saliva adsorb a decreased amount of *S. mutans* cells and a decreased amount of lipoteichoic acid. Pretreatment of beads with calcium ion increases adsorption of *S. mutans*, *S. sanguis*, and lipoteichoic acid. Pretreatment with lipoteichoic acid reduces binding of *S. sanguis* cells. These results strengthen the earlier suggestion of a role of lipoteichoic acid in the colonization of teeth by oral streptococci.

Gnotobiotic studies show that glucosyltransferase added to the drinking water supplied to rats did not influence the colonization on teeth of a mutant of *S. mutans* deficient in glucosyltransferases; the parent strain colonizes well. Other gnotobiotic studies with rats show that infection with *S. mutans* alone causes antibody formation to GTF and LTA. Immunization with preparations of GTF or GTF-LTA which enhance antibody titers only to GTF does not effect the colonization of *S. mutans* on teeth. Immunization with *S. mutans* cells or with a soluble cell surface fraction increases anti-LTA titer but not anti-GTF; colonization of *S. mutans* on teeth was significantly decreased.

Significance to Dental Research

A more thorough understanding of the mechanism by which oral streptococci adhere to solid surfaces, to each other and to other oral bacteria will lead to the discovery and use of more effective inhibitors of dental plaque formation. Accurate and reproducible methods for assaying adherence and coherence of cariogenic streptococci will prove useful in testing potential inhibitors of plaque formation.

Proposed Course of Project

Further studies will be carried out with various antimicrobials, glucans inhibitors, antibodies to purified bacterial products, specific hydrolytic enzymes, and other known or potential plaque inhibitors in order to define the mechanism by which S. mutans adheres via glucans to glass surfaces. Similar studies will also be carried out using hydroxyapatite and enamel surfaces. The mechanisms by which other oral bacteria interact with S. mutans in the formation of "plaque" will also be studied in these in vitro systems.

The information obtained from such studies should eventually lead to the formulation of relevant in vivo experiments.

Publications

1. Ciardi, J.E., Rölla, G., Bowen, W.H., and Reilly, J.A. Adsorption of Streptococcus mutans lipoteichoic acid to hydroxyapatite. Scand. J. Dent. Res. 85: 387-391, 1977.

2. Ciardi, J.E., Bowen, W.H., Reilly, J.A., Hsu, S.D., Gomez, I., Kuzmiak-Jones, H., and Cole, M.F. Antigens of Streptococcus mutans implicated in virulence - production of antibodies. p. 281-292. In J. McGhee, J. Mestecky, and J. Babb (ed). Secretory Immunity and Infection. Plenum Publishing Corp., New York, 1978.

3. Cole, M.F., Bowen, W.H., Sierra, L., Espinal, F., Aquirra, M., Kingman, A., Kemp, L.J., Gomez, I., Reilly, J.A., Hsu, D., Ciardi, J.E. and Gillespie, G. Immunoglobulins and Antibodies in Plaque Fluid and Saliva in Two Populations with Contrasting Levels of Caries. In J. McGhee, J. Mestecky and J. Babb (ed). Secretory Immunity and Infection. Plenum Publishing Corp., New York, 1978.

ITHSONIAN SCIENCE INFORMATION EXCHANGE OJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00206-02

RIOD COVERED
October 1, 1977 to September 30, 1978 CT 0600118

TLE OF PROJECT (80 characters or less)
Effect of daily and weekly rinsing with sodium fluoride solutions in a non-fluoride area (C)

MES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER OFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	S. B. Heifetz	Clinical Investigator	NCP, NIDR
ther:	R. Meyers	Public Health Analyst	NCP, NIDR
	H. S. Horowitz	Chief, CPS	NCP, NIDR
	A. Kingman	Statistician	NCP, NIDR

OPERATING UNITS (if any)
Biddeford School Department, Biddeford, Maine

B/BRANCH
Caries Prevention and Research

CTION
Community Programs Section

STITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TAL MANYEARS:	PROFESSIONAL:	OTHER:
0.55	0.35	0.20

ECK APPROPRIATE BOX(ES)
(a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

(a1) MINORS ☐ (a2) INTERVIEWS

MMARY OF WORK (200 words or less - underline keywords)
n October 1976, a sodium fluoride (NaF) mouthrinse study was started in iddeford, Maine, a non-fluoride area. Baseline central examinations (DMFS Index) ere made of 825 children in grades 5-7 attending seven schools in the community. articipants were randomly divided into three groups. Under teacher supervision, hey rinse either weekly with a 0.2% NaF solution or a 0.1% sodium chloride olution (Placebo) or daily with a 0.05% NaF solution. Treatments are to be arried out for three school years. Follow-up dental examinations are scheduled nnually to determine the anti-caries effectiveness of the two fluoride mouth-inse procedures. First year follow-up examinations were made in the fall of 977. The findings are currently being analyzed.

IDR Classification: 10540

IS-6040
lev. 10-76)

Objective

To determine the comparative effectiveness of weekly rinsing with a 0.2% sodium Fluoride (NaF) solution and daily rinsing with a 0.05% NaF solution in a non-fluoride area.

Methods

In October 1976, parental consent to participate in the F mouthrinse study was obtained for 825 students in grades 5-7 attending seven schools in Biddeford, Maine, a non-fluoride area. Baseline caries prevalence was registered and participants were randomly divided into three comparable study groups: Group 1 rinses weekly with a placebo solution containing 0.1% sodium chloride; Group II rinses weekly with a 0.2% NaF solution; and, Group III rinses daily with a 0.05% NaF solution. The mouthrinse procedures are carried out in school under teachers' supervision. Treatments will extend over a period of three school years. Follow-up dental examinations are conducted annually for a period of three years.

Findings

Second-year treatments were completed in June 1978. Interim findings after one year of study are currently being analyzed.

Significance

Although studies have shown that both fluoride mouthrinse procedures can reduce the incidence of dental decay, there is insufficient evidence to determine if one regimen of fluoride mouthrinsing is clearly more effective than the other. Information on the comparative benefits of the two procedures will be helpful to school and health officials interested in implementing a school mouthrinse program.

Proposed Course

Two-and three-year follow-up examinations and third year treatments are scheduled to be carried out as planned. The number of treatments that each child receives during each year of study will be tallied and recorded. Children judged to have "inadequate exposure" will be excluded from interim and final analyses. Interim and final reports will be prepared.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00220-02-CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978 CT No. 0600121

TITLE OF PROJECT (80 characters or less)
Comparison of Daily and Weekly Rinsing with Sodium Fluoride in a Fluoridated Community

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. S. Driscoll	Clinical Investigator	NCP, NIDR
Other:	P. A. Swango	Clinical Investigator	NCP, NIDR
	H. S. Horowitz	Chief, Community Programs	NCP, NIDR
	A. Kingman	Statistician	

COOPERATING UNITS (if any)
Des Moines Independent Community School District

LAB/BRANCH
Caries Research and Prevention

SECTION
Community Programs

INSTITUTE AND LOCATION
National Institute of Dental Research, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.36	0.24	0.12

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The study was initiated in September 1977 with 1000 children in the seventh grade of nine junior high schools located in Des Moines, Iowa, a community that has optimal amounts of fluoride in its supply of drinking water. The children were randomly assigned to one of the following three study groups: Group I (controls) rinse their mouths once every week in school for 60 seconds with a placebo solution; Group II follows an identical procedure using a 0.2% neutral sodium fluoride solution (0.09%F); Group III rinse their mouths once every day in school for 60 seconds using a 0.05% neutral sodium fluoride solution (0.023%F). The procedures are carried out under the classroom teacher's supervision for a period of three years. Baseline dental examinations, using the DMF surface index, were conducted in November 1977. Follow-up examinations will be conducted in May 1979 and May 1980.

NIDR Classification: 10540

Objective

To compare the caries-inhibiting effect of weekly rinsing with a 0.2 percent sodium fluoride solution (0.09%F) and daily rinsing with a 0.05 percent sodium fluoride solution (0.023%F) in children who have been reared during the years of school attendance in an optimally fluoridated community.

Methods Employed

The study, a longitudinal double-blind clinical trial, was initiated in September 1977, with 1000 children in the seventh grade of nine junior high schools located in Des Moines, Iowa, a community that has optimal amounts of fluoride in its supply of drinking water. The children were randomly assigned to one of the following three study groups: Group I (controls) rinse their mouths once every week in school for 60 seconds with a placebo solution; Group II follows an identical procedure using a 0.2% neutral sodium fluoride solution (0.09%F); Group III rinse their mouths once every day in school for 60 seconds using a 0.05% neutral sodium fluoride solution (0.023%F). Under the supervision of the classroom teachers the procedure will be carried out for a period of three years. Baseline dental examinations, using the DMF surface index, were conducted in November 1977. Follow-up examinations will be conducted in May 1979 and May 1980.

Significance

Although the practice of water fluoridation makes a sizable inroad into the widespread problem of dental decay that exists in most communities, complementary public health measures must be developed to prevent the dental decay that still persists despite fluoridation. There is good reason to believe from recently reported data that fluoride mouthrinsing in school may confer significant additional decay preventive benefits when practiced by children who consume optimally fluoridated drinking water. It is the intent of the proposed study to determine the extent of benefit derived from the procedure and to compare the efficacy and feasibility of the weekly procedure with that of the daily procedure. The information that can be gained from this study is needed before fluoride mouthrinsing programs can be recommended and promoted for wide scale use in optimally fluoridated communities.

Proposed Course

The first year of fluoride mouthrinsing has been successfully completed. The mouthrinsing will be resumed as soon as possible after school starts in September 1978. The first follow-up dental examinations are scheduled for May 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00222-02 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Specific and non-specific immune factors in plaque fluid and saliva

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael F. Cole	Visiting Scientist	CP PR NIDR
OTHER:	William H. Bowen	Acting Chief	CP PR NIDR
	Lynn J. Kemp	Biologist	CP PR NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Preventive Methods Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.71	0.31	0.40

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The free aqueous phase was obtained from individual samples of dental plaque and the plaque matrix was then eluted at low pH in an attempt to remove bound protein. The fluid and the low pH phases were assayed for secretory immunoglobulin A (SIgA), IgG, IgM, the third component of complement (C'3), lysozyme, lactoperoxidase and lactoferrin. The presence of these specific and non-specific immune factors in the free and bound phases suggest they are important in host defense at the plaque-enamel interface.

NIDR Classification: 20513; 10260; 20311; 20314

PHS-6040
(Rev. 10-76)

1. Project Description

Objective

The purposes of this study are to measure specific and non-specific defense mechanisms in dental plaque and saliva and to determine their role in host defense at the enamel plaque interface.

Methods

Supragingival plaque free of visible blood was centrifuged at high speed to obtain the free fluid phase. The plaque was then washed with neutral pH buffer until no protein remained. The plaque was then treated with low pH buffer in order to elute proteins bound to the matrix. The proteins were measured by single radial diffusion, rocket immunoelectrophoresis, solid phase fluoroimmunoassay and spectrophotometry.

Major Findings

The levels of the specific and non-specific immune factors found in plaque fluid suggest that predominantly saliva and, to a lesser extent, gingival exudate contribute to the protein pool. Significant levels of these immune factors were bound to the plaque matrix suggesting that they participate in host defense at the enamel plaque interface.

Significance

The study of the qualitative and quantitative composition of plaque fluid in populations with high and low caries experience could help in understanding the mechanisms by which the host protects the tooth surface.

Propose Course

1. Design *in vitro* systems to study the interactions of these proteins.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00223-02 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

An Improved Method for Analyzing Caries Clinical Trials Data

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Albert Kingman Statistician-Health CPR, NIDR

COOPERATING UNITS (if any)

AB/BRANCH
Caries Prevention and Research Branch

ECTION
Biometry Section

NSTITUTE AND LOCATION
NIH, NIDR, Bethesda, Maryland

OTAL MANYEARS:	PROFESSIONAL:	OTHER:
.15	.15	

HECK APPROPRIATE BOX(ES)

(a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

] (a1) MINORS ☐ (a2) INTERVIEWS

UMMARY OF WORK (200 words or less - underline keywords)

In many clinical trials the groups being compared at the end of the trial are not balanced as to initial DMFS scores, even though subjects were randomized to treatment groups initially. Two analytical methods have been used to adjust for these imbalances; the blocking analysis and the covariance analysis. The blocking analysis is the preferred method in that it requires fewer assumptions made of the data.

In this study an adaptation of Grainger's severity index, one method of summarizing the initial DMFS scores, was used as a blocking factor in the analysis of caries clinical trial data. This blocking factor has the advantage of being independent of the data set on which it is used. Further, its high correlation with initial DMFS scores insures that it is utilizing the existent information within subjects at the beginning of the trial. This analytical method was compared with 3 other methods, including the covariance analysis and other blocking techniques to see what increase in efficiency would be realized by using this technique.

NIDR Classification: 10500

Background

A common problem occurring in caries clinical trials is the existence of an imbalance in the initial DMFS scores among the study subjects remaining at the end of the study. Two analytical procedures have been used to adjust the increment scores for this risk imbalance; the blocking or stratification analysis and covariance analysis. The blocking method is the preferred method in that it requires fewer assumptions from the trial data.

Purpose

The purpose of this study was to identify a blocking or stratification factor which could be used in caries clinical trials which had the following properties: (1) able to adjust the group increment scores for initial DMFS imbalances, (2) capable of being defined a priori, (3) is based on objective criteria that can be used by any clinical investigator conducting such studies.

Methods

In this study, an adaptation of Grainger's severity index (one way of summarizing a subject's initial DMFS experience) was compared to three other methods of summarizing a subject's initial DMFS experience. The properties of this index were investigated to see how it would compare with other methods in terms of correcting for differences in risk among subjects who participate in these trials. Six clinical trials data sets were examined by each method and the results compared.

Results

The blocking or stratification method which utilized the adaptation of Grainger's severity index resulted in the greatest efficiency among the methods tested for these six trials data. This would suggest that more information exists in knowing the types of decay present in the subject's dentition than in knowing the subject's total DMFS score.

Presentation of Findings

The results of this investigation were presented at the 1977 annual meeting of the IADR held in Washington, DC, in March, 1977.

Publication

A manuscript containing the results of this study has been completed and is being submitted to an internationally circulated dental journal.

ITHSONIAN SCIENCE INFORMATION EXCHANGE ROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00224-02 CPR

ERIOD COVERED
October 1, 1977 to September 30, 1978

ITLE OF PROJECT (80 characters or less)
Study of the Effect on Dental Caries of 2.5 ppm F in the Drinking Water of Rats

AMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER ROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. H. Bowen	Acting Chief, CPR	CPR NIDR
OTHER:	S. M. Amsbaugh	Biologist	CPR NIDR
	A. Kingman	Statistician	CPR NIDR

OPERATING UNITS (if any)

B/BRANCH
Caries Prevention and Research Branch

CTION
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STITUTE AND LOCATION
NIH, NIDR, Bethesda, Maryland

TAL MANYEARS:	PROFESSIONAL:	OTHER:
.33	.03	.3

ECK APPROPRIATE BOX(ES)

(a) * HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

(a1) MINORS ☐ (a2) INTERVIEWS

MMARY OF WORK (200 words or less - underline keywords)

The purpose of the present study was to determine whether the administration of 2.5 ppm F in the drinking water of rats would confer lifelong protection against dental caries. It was observed that no differences occurred in the caries scores of animals fed fluoride and controls after either 2 weeks or 4 weeks. Animals usually develop pit and fissure caries only during this time. However, significant differences were observed in animals fed fluoride for 8, 12 and 16 weeks.

NIDR Classification: 10550

Objectives

The purpose of this study was to determine whether fluoride would confer a lifetime protection against caries in rats; to observe the pattern of caries development in rats fed fluoride and to identify the optimum duration to run caries investigations in rats.

Methods

Twelve groups of 12 OM rats were used in this investigation. All the animals were infected with Strep. mutans 6715 serotype g and fed diet 2000 ad libitum. Groups of 12 animals, with controls, were sacrificed, having been given 2.5 ppm fluoride in their drinking water for 14, 28, 56, 84, 112 and 140 days. Caries were scored in the usual manner.

Major Results

Little or no protection was observed in animals after 2 or 4 weeks, largely because sulcal caries only develop during this time. Fluoride usually exerts only minimal protection against this type of lesion. However, highly significant differences were observed in animals after 8, 12 and 16 weeks' exposure to fluoride. No significant differences were observed between experimental and control animals after 20 weeks. This apparent anomaly may be explained by the observation that there is no significant difference between the level of smooth surfaces after 8 weeks and 16 weeks in control animals; therefore, the animals fed fluoride for 8 weeks had more surfaces at risk than the corresponding control animals.

Significance

The results show that fluoride exerts its maximum protective effect on smooth surfaces, and therefore experiments must be continued for sufficient time to allow the development of smooth surface lesions if benefits are to be observed. It is also important to observe that the benefits of any agent which does not eliminate caries may disappear if the cariogenic challenge is great enough.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00225-02 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cost Analysis of Implementing School-based Community Mouthrinse Programs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Brunelle, J.A.	Chief, Biometry Section	CPR, NIDR
OTHER:	Miller, Ann	Health Science Administrator	NCP, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research Branch

SECTION
Biometry Section

INSTITUTE AND LOCATION
NIH, NIDR, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.35	.25	.10

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Data on costs of program, student participation and caries experience are being collected from seventeen communities throughout the U.S. and Guam which are conducting three-year demonstrations of school-based mouthrinse programs. Analysis of implementation, acceptance of community and cost-benefit will be performed during and after the three years.

NIDR Classification: 10600

PHS-6040
(Rev. 10-76)

Objective:

To do a detailed analysis of the cost of implementing a school-based mouthrinse program in a variety of community settings.

Methods:

Data on costs of personnel, consumable materials, permanent equipment and building overhead are being collected from 17 sites in the United States and Guam who are participating in a mouthrinse demonstration program. Participation statistics from consent forms, attendance, and drop-out records are also being collected and evaluated.

Findings:

60 to 95% of the total eligible population enrolled in the K-6 or K-8 grade program in the second year. Dental decay was less after two years in more than half of the 17 sites, with 46% the highest reduction. Costs per child per year ranged from $.32 to $8.16. The cost differences relate mainly to the amount and kind of personnel hired to dispense, deliver or supervise the rinse procedure.

Proposed Course:

Data on participation and continuation costs will be collected till the end of study in January 1979. Final analyses of cost-benefit will be made at that time, using the incremental caries scores from the first and final clinical exams as the measure of effectiveness.

MITHSONIAN SCIENCE INFORMATION EXCHANGE ROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00227-02 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Study of the effect of different carbohydrates on dental caries in the rat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William H. Bowen	Acting Chief, CPR	CPR NIDR
OTHER:	Susan Amsbaugh	Biologist	CPR NIDR
	Shou-Hua Li	Visiting Associate	CPR NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.45	.05	.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study is to assess the cariogenicity of various carbohydrates, xylitol, sorbitol, sorbose, and mannitol, when given separately or in combinations as sucrose substitutes in the diet of the rat. The effect on dental caries will be measured as well as the effect on dental plaque. Hopefully, the effective level of one or a combination of carbohydrates can be found that will significantly lower the level of caries, thus becoming a noncariogenic substitute for sucrose.

NIDR Classification: 10270

Objective

This study was carried out to determine the effects of xylitol and sorbose on dental caries in rats.

Methods

Eight groups of 12 rats were infected with Strep. mutans and A. viscosus. The animals were fed diet 2000 or modifications to include the test substances as shown below.

Sugar	56	28	28			14	14	
Starch		28		42	28	28	28	42
Sorbose			28	14	28	14		
Xylitol							14	14

Animals fed a diet containing 28% sorbose gained significantly less weight than other groups of animals. Animals which had all sugar in their diet replaced by starch and sorbose or xylitol developed significantly fewer carious lesions than control. The addition of xylitol or sorbose to diets containing sugar did not prevent caries.

Significance

These observations show that sorbose and xylitol are non-cariogenic and could be considered as substitutes for sugar in part, at least.

Summary

This investigation was carried out to determine the cariogenicity of xylitol and sorbose and to determine whether either substance has cariostatic properties. Neither sorbose nor xylitol promoted dental caries; they did not prevent caries when included in a diet containing sucrose.

SMS. ... ICE INFORMATION EXCHANGE PROJECT ... (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00228-02 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Association of Streptococcus mutans with Dental Caries Reduction in a School Population

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Thomson, L. Ariel	Senior Dental Surgeon	CPR, NIDR
COPI:	Little, Wayne	Microbiologist	CPR, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Etiology

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.55	.25	.30

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS X Children X Clinical Trial

SUMMARY OF WORK (200 words or less - underline keywords)

The project is intended to monitor changes in dental plaque organisms, specifically Strep. mutans, during fluoride preventive procedures provided to a school population over a two year period. Serotypes of Strep. mutans are characterized and enumerated in plaque from individuals using fluoride rinses either daily or weekly, as well as from individuals using a placebo solution. Fluorescent Antibody (FA) reagents specific for the serotypes of Strep. mutans are employed to detect changes in the students' plaque samples. Alterations in the plaque composition during the study period will be related to the changes in the caries incidence.

NIDR Classification: 10110; 10230; 10540; 10120

Project Description:

Objectives

1. To determine the baseline level of the serotypes of Strep. mutans in the dental plaque of a sample of students who will participate in a school fluoride rinse program.

2. To determine any changes in the Strep. mutans plaque organisms which occur during a 12-month period in individuals using fluoride rinses either daily or weekly, as well as from individuals using a placebo solution.

3. Attempt to associate detectable changes in Strep. mutans organisms with any reduction in dental caries. Major plaque changes during the course of fluoride therapy or detection of major differences between the treatment and placebo groups should relate to an interaction with the fluoride.

4. Analyze plaque samples to determine the total fluoride present in children in each group.

Methods

In addition to using routine FA methods, the present investigation currently employs a high intensity xenon arc as the excitation source for all FA procedures. This light source provides a greater amount of primary excitation and less autofluorescence than other currently employed light sources. The use of reagent grade conjugates specific for the serotypes of Strep. mutans has permitted direct FA detection and enumeration of these organisms in plaque specimens. Preparation methods for the conjugates have been published by McKinney and others. The methods for the examination of plaque specimens have been detailed in the proceedings of the FA Workshop, April 1975 (which was published in the Journal of Dental Research 55 Special Issue A, 1976).

The thirty individuals for plaque study were selected randomly by the Biometry Section, CP&RB, NCP. Three dental plaque samples were obtained from each child. The samples consist of a plaque sample for fluoride determination and specific site samples from the upper central incisor-lateral incisor interproximal area (right side if possible) and from the buccal surface of the lower second molar (right side, also). The interproximal samples were obtained with dental floss and the buccal and pooled samples collected using a dental carver number 3S. The pooled samples were placed in plastic 0.5 ml Lancer analyzer cups, capped with polyethylene caps and immediately frozen. The site samples were placed in 3.0 ml Lancer analyzer cups containing 1 ml of RTF transport fluid, sonically dispersed, capped and finally frozen. All specimens were maintained in the frozen state using a dry ice chest.

Major Findings

The results to date suggest that the prevalence of S. mutans in this population as determined using FA procedures is almost identical to that observed with cultural methods. Furthermore, the prevalence of S. mutans is somewhat lower than that observed is several other popoulations. Only 31% of the plaque specimens had detectable levels of S. mutans. This low prevalence may be a direct effect of not pooling plaque from many tooth sites, but of collecting individual site samples. For those who had S. mutans, 92% were observed to have serotype c, 13% had serotype e, and 7% had serotype f. In one student S. mutans accounted for over 90% of the flora cultivatable on MS agar; however, over 70% of the positive samples had less than 20% mutans. Furthermore, when one examines the total number of organisms (phase microscopy, and the proportion which are S. mutans (FA microscopy - polyvalent conjugate) only 6% of the total are S. mutans, even with the sample having 90% on MS agar. The majority of the organisms were Actinomyces viscosus, Actinomyces naeslundii, and Actinomyces israelii. The plaque samples for the plaque samples for the fluoride determinations and the remaining plaque samples are currently being analyzed.

Significance to Biomedical Research

Efforts to date to determine any direct fluoride action on Strep. mutans resident in plaque have been limited by difficult laboratory procedures. Therefore, most studies have provided only qualitative data associating Strep. mutans with caries status. The few studies which have provided quantitative assessment have generally reported some correlation between a fluoride reduction in smooth surface caries and the number of Strep. mutans present. With the development of FA reagents specific for the serotypes of Strep. mutans, studies are now indicated to carefully monitor changes in plaque organisms during fluoride preventive procedures. The present study should provide valuable information on fluoride concentrations in human dental plaque in groups of children exposed to different fluoride therapy and the levels of Strep. mutans resident in their dental plaque.

[illegible] (DO NOT use this space)	DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00229-02 CPR

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Plaque variations in populations ingesting different levels of water fluoride

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Stiles, H. M.	Microbiologist	CPR, NIDR
COPI:	Bowen, W. H.	Acting Chief	CPR, NIDR
Other:	Brunelle, J.	Statistician (Health)	CPR, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH

Caries Prevention and Research Branch

SECTION

Etiology

INSTITUTE AND LOCATION

NIDR

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.29	.49	.80

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Three communities having 0.3, 2.0 and 4.1 ppm fluoride in the drinking water were chosen as study sites. Children 12-18 years of age who had been lifelong residents in the sites comprised the populations. Plaque and saliva samples, collected from each participant, will be analysed for microbial and fluoride content. DMF surfaces were also recorded for each participant. Data will consist of comparisons of various parameters among the three groups.

NIDR Classification: 10550; 10110; 10120

1. Project Description

Objectives:

To examine the differences in plaque fluoride, microbiota, and dental caries among three populations consuming different levels of water fluoride.

Methods:

a. Saliva samples and pooled plaque specimens were collected from each participant, immediately frozen and stored for examination later.

b. Dental caries were assessed according to standard NIDR criteria and scores recorded on NIH Form 1311.

c. Plaque fluoride will be determined by the use of a fluoride electrode; the microbiology will encompass fluorescent antisera and cultural methods.

Major Findings

Plaque and salivary samples are presently being collected so that a number sufficient for statistical analysis is available.

Significance to Program:

This investigation will provide possible mechanisms by which fluoride is effective in preventing dental caries.

Proposed Course:

Because of insufficient individual plaque sample amounts, additional participants will need to be assessed, i.e., other population sites will have to be found. Work will continue in the laboratory on the analysis of the specimens for fluoride and microbiology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-231-02 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Radiation Caries in Primates

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. H. Bowen	Acting Chief, CPRB	CPR, NIDR
OTHER:	M. Cole	Visiting Scientist	CPR, NIDR
	L. Thomson	Sr. Dental Surgeon	CPR, NIDR
	I. Gomez	Microbiologist	CPR, NIDR
	H. Kuzmiak-Jones	Biologist	CPR, NIDR
	J. Swain	Physicist	CPR, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
--

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.06	.03	.03

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two groups of 4 monkeys (Macaca mulatta) were irradiated. The first group received 4 doses of 500 rads over 2 weeks. Considerable desquamation of the oral mucosa occurred. Rampant caries developed but was soon followed by osteonecrosis of the lower jaw. The second group was given 250 rads twice weekly for 2 weeks using Cobalt 60 as a radiation source. The flow of saliva rapidly diminished within 3 weeks of treatment. The animals were fed a diet rich in sugar. In a matter of weeks caries developed which clinically can not be distinguished from that which occurs in irradiated humans. Large numbers of Strep. mutans have been isolated from the irradiated animals. Plaque fluid is now being examined.

NIDR Classification: 10290

Although the monkey is an ideal model on which to study dental caries and means for its prevention, it frequently takes an inordinately long time for caries to develop. By irradiating the animals, it is hoped to use old animals and to reduce the time required for caries development, thusly enhancing the value of the animal and also enabling the screening of cariostatic compounds to be carried out expeditiously.

PROJECT NUMBER (DO NOT USE THIS SPACE)

HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

Z01 DE 00234-01 CP

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Develop Method of Intraoral Telemetry of Various Ions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Roald J. Shern	Clinical Investigator	CP PR NIDR
OTHER:	William H. Bowen	Acting Chief	CP PR NIDR
	Stanley Moss	Research Scientist	Univ. of Utah
	Jiri Janata	Research Scientist	Univ. of Utah

COOPERATING UNITS (if any)
Bioengineering, University of Utah, Salt Lake City, Utah
Microelectrodes, Londonderry, NH

LAB/BRANCH
Caries Prevention and Research

SECTION
Preventive Methods Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.15	.15	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Oral biotelemetry is being developed which enables direct and continuous measurements of intraoral responses such as H^+ and F^- levels following ingestion of various foods and therapeutics. Measurements of ionic levels may be conveyed to the recording equipment by radio or wire transmission. Clinical studies will be initiated soon.

NIDR Classification: 10230; 10540; 10280

PHS-6040
(Rev. 10 /6)

1. Project Description

Objectives

The goal of this work is to develop a method which will be useful in predicting the cariogenicity of foods in measuring the bioavailability of fluoride, and in measuring other biological phenomena.

Methods

Equipment needed for making the various intraoral measurements is not available and must be fabricated. This project which was initiated several years ago remains in the pretest phase. However, substantial progress has been made in developing a workable wire telemetry apparatus. The ion sensing will be made by using either conventional H^+ & F^- sensors or transistor sensors (CHEMFET).

Major Findings

None

Significance

None

Proposed Course

Clinical testing of equipment. Evaluation of foods and therapeutics using telemetry. Telemetry apparati when fully operational will be useful for predicting the cariogenicity of foods and the protective potential of therapeutics.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00235-01 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Induction of secretory immunity in the gnotobiotic rat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael F. Cole	Visiting Scientist	CP PR NIDR
OTHER:	Harriet Kuzmiak-Jones	Biologist	CP PR NIDR
	Christoper Kemp	Microbiologist	CP PR NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Preventive Methods Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.41	0.20	0.21

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Germ free rats were immunized orally, intragastrically and intramucosally with Streptococcus mutans 6715-15. Unimmunized animals served as controls. All rats were infected with S. mutans 6715-15. After 8 weeks the rats were sacrificed and serum, saliva, gut and pulmonary washes were collected. In addition, splenic, salivary, mesenteric and Peyers Patch lymphocytes were assayed. Serum and salivary antibodies were detected in all groups. Primed lymphocytes were detected in spleen and salivary lymph nodes.

NIDR Classification: 20314; 10570; 20513

1. Project Description

Objective

The purpose of this study was to investigate the relative importance of the oral lymphoid tissue and the gut associated lymphoid tissue in the induction of a secretory immune response.

Method

Weanling Osborne Mendal rats were divided into four groups. One group was immunized orally by including 1×10^8 colony forming units (CFU) per ml S. mutans in the drinking water. A second group received 0.2 ml of 5×10^9 CFU/ml S. mutans by stomach tube. Group three also received 0.2 ml of 5×10^9 CFU/ml, but in the vicinity of the salivary glands. The final group served as unimmunized controls. After 8 weeks the animals were sacrificed and serum, saliva, gut and pulmonary washes were collected and assayed for antibodies to S. mutans by microagglutination. The ability of single cell suspensions of spleen, salivary lymph node, Peyer's Patch and mesenteric lymph to undergo blastogenesis in the presence of S. mutans was also determined.

Major Findings

Serum and salivary antibodies were detected in all groups including the unimmunized controls. Intramucosal immunization elicited the greatest response. Primed lymphocytes were detected in the spleen and salivary lymph nodes. Peyer's Patches and mesenteric lymph nodes gave poor responses.

Significance

The success of a vaccine against dental caries will depend upon the induction of high levels of antibody in the oral cavity. Studies of the type outlined above may help to establish the best route, dose and schedule to achieve this goal.

Proposed Course

1. Specifically label cells to determine where the committed salivary lymphocytes originate.

2. Study the immunogenicity of purified components of S. mutans in an attempt to identify protective antigens.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00236-01 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Purification of rat immunoglobulins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael F. Cole	Visiting Scientist	CP PR NIDR
OTHER:	Lynn J. Kemp	Biologist	CP PR NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Preventive Methods Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Immunoglobulins M and G (IgM and IgG) were purified from rat serum and secretory IgA from rat colostrum by molecular sieve and ion exchange chromatography. Purification of IgM was complicated by contamination with α_2 macroglobulin.

NIDR Classification: 20314; 10570; 20513

1. Project Description

Objective

The purpose of this study was to purify immunoglobulin A, G and M in order to raise monospecific antisera to these proteins.

Methods

Rat serum was collected and the immuglobulins precipiated with 20 volumes of 2.5% boric acid. The precipitate was resuspended in 0.1 M Tris HCl pH 8.0 containing 0.01 M glycine and 0.15 M NaCl and chromatographed on Sephadex G200. The exclusion peak containing IgM was concentrated and chromatographed on Sepharose 6B. Immunoglobulin M was found in the second peak and was free of detectable α_2 macroglobulin.

The supernatant from the precipitated serum was dialyzed and chromatographed on QAE Sephadex A-50 to purify IgG. Colostrum was obtained from rat dams following stimulation with oxytocin and was precipitated with 50% $(NH_4)_2$ SO_4. The precipitate was redissolved, dialyzed and chromatographed on Sephadex G200. The exclusion peak was rechromatographed on Sepharose 6B to yield pure SIgA.

Significance

The production of monospecific antiglobulin reagents will allow the detection of antibody forming cells and the quantitation of antibodies in rats which are widely used as a model for dental caries.

Proposed Course:

1. Immunize rabbits in order to produce antisera.

2. Conjugate antisera with fluorescein and rhodamine isothiocyanate.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00237-01 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Immunoglobulins and antibodies in plaque fluid and saliva in two populations with contrasting levels of caries

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael F. Cole	Visiting Scientist	CP PR NIDR
OTHER:	William H. Bowen	Acting Chief, CP&RB	CP PR NIDR
	Lynn J. Kemp	Biologist	CP PR NIDR
	Irma Gomez	Biologist	CP PR NIDR
	J. Allen Reilly	Biologist	CP PR NIDR
	S. Dana Hsu	Biologist	CP PR NIDR
	Joseph E. Ciardi	Research Biochemist	CP PR NIDR
	Albert Kingman	Statistician	CP PR NIDR
	Janet Brunelle	Chief, Biometry Sec.	CP PR NIDR
	Patricia Rodgers	Statistical Assistant	CP PR NIDR
	L. Sierra)		Univ. of Antiquia
	F. Espinal)		Medellin, Colombia, S.A.
	M. Aguirra)		
	G. Gillespie		PAHO, Wash., D.C.

COOPERATING UNITS (if any)
Univ. of Antioquia, Medellin, Colombia, S.A.
PAHO, Wash., D.C.

LAB/BRANCH
Caries Prevention and Research

SECTION
Preventive Methods Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.74	0.26	0.48

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Plaque fluid and saliva were collected from 25 children aged 7-12 years from two communities with contrasting levels of caries and assayed for secretory immunoglobulin A (SIgA), IgG, IgM, complement (C'3) and lysozyme. Significantly more IgA was found in the saliva and lysozyme in the plaque fluid from subjects with low caries experience. Subjects exhibiting antibody reactive with S. mutans serotypes a and d had a lower prevalence of caries than those who failed to demonstrate antibodies.

NIDR Classification: 20513; 10260; 20311; 20314

1. Project Description

Objective

The purposes of this study are to measure immunoglobulins, antibodies and non-specific immune factors in saliva and plaque fluid and to determine their relationship to the level of caries.

Methods

Supragingival plaque, free from overt blood was collected from each subject and centrifuged at 38,000 .g to obtain the free aqueous phase. Whole saliva was also obtained from each subject. Secretory IgA, IgG, IgM and complement were assayed by rocket immunoelectrophoresis. Lysozyme was quantitated by the lysoplate technique.

Major Findings

Significantly more SIgA was found in the saliva of subjects from the low caries community. Low levels of IgG and trace amounts of IgM and complement C'3 were found in saliva from all subjects. No difference in the concentration of SIgA in plaque fluid between the two populations was detected. However, significantly more lysozyme was found in the plaque fluid from the population with low caries experience. Subjects exhibiting antibody reactive with serotypes a and d had a lower prevalence of caries than those who failed to demonstrate antibody.

Significance

Study of the levels of specific and non-specific immune factors in saliva and plaque fluid from subjects with high and low caries experience could aid in understanding the role of host defense factors in protection against dental caries.

Proposed Course

1. Expand the numbers of subjects in the study populations in order to better understand the role of host defense in the etiology of dental caries.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00240-01 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cariogenicity of Foodstuffs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. H. Bowen	Acting Chief, CPR	CPR, NIDR
OTHER:	S. Monell Torrens	Lab. Technician	CPR, NIDR
	S. Amsbaugh	Biologist	CPR, NIDR
	H. Kuzmiak-jones	Biologist	CPR, NIDR
	M. Cole	Visiting Scientist	CPR, NIDR
	I. Gomez	Microbiologist	CPR, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research Branch

SECTION
--

INSTITUTE AND LOCATION
NIH, NIDR, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.14	.01	.13

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

There is considerable interest in identifying snacks which are potentially cariogenic. The purpose of this investigation is to determine whether animals fed sugar-rich snacks under well-controlled conditions in a programmed feeder would develop high levels of caries, and to determine whether foodstuffs could be ranked in their cariogenic potential.

NIDR Classification: 10270

There is growing public interest in identifying foodstuffs which are potentially cariogenic. Manufacturers are also anxious to prepare and sell non-cariogenic snacks. In the past, it has been the custom to mix test materials in a complete diet or to feed it ad libitum. By using the Konig-Hofer machine, it is possible to feed animals up to 17 times daily at pre-selected intervals, and thus rigorously control the frequency of exposure to the test material. Preliminary results have shown that animals which receive 25% of their meals in the form of a highly-sugared cereal develop more carious lesions than animals which receive a cereal which contains little sugar at the same frequency.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00243-01 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Growth Energetics and Interaction of Plaque Microorganism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Robrish, Stanley A.	Research Microbiologist	CPRB, NIDR
Other :	Kemp, C. W.	Microbiologist	CPRB, NIDR
	Bowen, W. H.	Acting Chief, CPRB	CPRB, NIDR
	Dennis, Donna	Physical Science Asst.	CPRB, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Etiology

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.77	0.37	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have continued the studies of the growth properties of pure cultures of oral bacteria in both batch and continuous modes. We have started the calculations of molar growth yields of Streptococcus sanguis and S. mutans on glucose and other substrates. Experiments have been started to investigate the growth of these two organisms, singly or in combination, on several energy sources. Work has continued on the analysis of metabolic end products of pure cultures and dental plaque samples.

NIDR Classification: 10250; 10260; 10280

This work has previously been reported as part of a larger continuing project: Biochemical Products and Energy Requirements of Plaque (Z01-DE-00154-03 CPR B). The efforts expended this year and the anticipated effort for the coming year justifies the establishment of this as a separate project.

Objectives: The objectives of this project are to study the growth properties of a variety of oral microorganisms with the hope of understanding the means by which the oral microflora is established. This study should provide a model for the growth and interactive properties of oral microorganisms as they occur in dental plaque.

Methods: The methods employed are the standard ones which are used for the growth of bacteria in batch or continuous culture. Most of the equipment we have used has been improvised and we are presently acquiring some commercial equipment to grow the organisms in continuous culture. The apparatus for the control of pH has been constructed from existing laboratory equipment. In the coming year we hope to acquire some facilities for continuously monitoring the turbidity of batch cultures grown on various substrates.

Significance to biomedical research and to the objectives of the NCP:
The participation of bacteria in the process of dental caries has been established beyond any reasonable doubt and some organisms are suspected to be specific odontopaths. These bacteria exert their pathogenic potential as part of a complex mixed microflora which we commonly call dental plaque. Little is known about the growth properties of these organisms in pure culture under controlled conditions in which their energetic economy can be measured. It is hoped that an understanding of the growth properties of these organisms in mixed culture will allow us to control the establishment of the suspected odontopath or to control the pathogenic potential of that segment of the microflora once it has been established in dental plaque.

Analysis of growth of plaque microorganisms: Studies of the growth properties of the organisms which are found in dental plaque have continued this year with growth of these organisms in controlled batch and continuous culture. The controlled batch cultures have included ones which have been grown maintaining constant pH and some with a programed feed in order to grow the organisms on limiting energy sources. We have used an improvised pH stat in conjunction with existing feed device to compute the molar growth yields for S. mutans and S. sanguis. Conditions have been developed with this equipment so that the yield of the organisms is proportional to the amount of substrate fed. The values which we obtain for our calculations are comparable to those obtained for similar organisms by other investigators. We hope to collect data in the coming year on the yield of organisms on glucose, galactose and glucosamine in order to try to interpret the possible contribution of these constituents of salivary glycoprotein to the maintenance of the oral microflora

Other experiments have been performed using mixed substrates and

determining their effect on S. mutans. Because S. mutans is one of the few streptococci to use manitol, it was of interest to determine if the organism used glucose and manitol sequentially or simultaneously. When S. mutans was grown in the pH stat with these two mixed substrates, there was no evidence of diauxie by a change in rate of alkali added. The rate of addition and the total alkali added was the same if the two substrates were added separately or together. We intended to continue investigations into this phenomenon in the coming year.

Growth of the organisms at constant pH has allowed us to supply cells to other members of this program. S. mutans and extracellular products from S. mutans have been supplied to Drs. Cole, Ciardi and Bowen for various experiments which they have in progress. This organism has been used in a variety of immunologic experiments including their use as an antigen to be administered by various routes to animals. In addition, they have been used for a variety of in vitro assays. Because of the use of the controlled growth conditions we have been able to supply these constituents in a higher yield and with a more defined composition than has been possible heretofore. The extracellular products of the growth of S. mutans have been supplied to Dr. Ciardi to assist in his studies on glucosyltransferases and lipoteichoic acid.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE (PROJECT NUMBER (Do NOT use this space))	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01-DE-00244-01 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
The Evaluation of a Medium for the Isolation of Actinomyces from Dental Plaque

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Little, Wayne A.	Microbiologist	CPR, NIDR
Other:	Thomson, Lynn A.	Senior Dental Surgeon	CPR, NIDR
	Bowen, William H.	Acting Chief	CPR, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research

SECTION
Etiology

INSTITUTE AND LOCATION
NIDR, NIH Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.22	.02	20

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

CNAC-20 agar was evaluated to determine its usefulness as a differential medium for strains of Actinomyces viscosus and Actinomyces naeslundii. Human and primate plaque specimens, in addition to stock strains of actinomyces and other oral bacteria were cultured on CNAC-20 agar. Plaque isolates were made from colonies judged to be actinomyces or non-actinomyces on the basis of morphology. Verification of these isolates was made with fluorescein labeled antisera specific for A. viscosus and A. naeslundii.

NIDR Classification: 10110

Objectives

In human populations, little is known about the ecology of A. viscosus and A. naeslundii and their relationship to naturally occurring dental diseases. Part of the problem is that the identification of Actinomyces is very difficult and time consuming and does not lend itself to processing numerous samples from clinical studies. The introduction of CNAC-20 agar could greatly facilitate the identification of A. viscosus and A. naeslundii. The purpose of this study was to evaluate CNAC-20 agar and determine its usefulness as a differential medium for strains of A. viscosus and A. naeslundii.

Methods

Human and primate plaque specimens, in addition to stock strains of Actinomyces and other oral bacteria, were plated on CNAC-20 and incubated 48 hrs anaerobically followed by 48 hrs in 90% air 10% CO_2. Verification of plaque isolates was made with fluorescein labeled antisera specific for A. viscosus and A. naeslundii.

Major Findings

Stock Strains

Strains of Rothia dentocariosus, Bacterionema matruchotii, and Propionibacterium avidum produced colonies similar in morphology to A. viscosus and A. naeslundii colonies. In addition one A. naeslundii strain produced an atypical colony.

Plaque

All of the human plaque isolates which were identified as A. viscosus or A. naeslundii on the basis of colonial morphology were also positive by the fluorescent antibody technique (FA). Twenty percent of atypical (non-Actinomyces) isolates gave positive FA staining for A. viscosus or A. naeslundii.

None of the atypical isolates from primate plaque gave positive FA staining for A. viscosus or A. naeslundii. In addition, only 54% of the typical Actinomyces isolates were positive by FA. It is likely that some of these isolates belong to additional serotypes of A. viscosus and A. naeslundii which are not stained with available antisera.

Significance to Biomedical Research

CNAC-20 agar could prove useful as a differential medium for A. viscosus and A. naeslundii as long as its limitations are recognized. Results of pure culture experiments indicate that some strains of A. naeslundii may not grow on CNAC-20. Perhaps a more serious deficiency is the occurrence of atypical Actinomyces colonies. This could lead to an underestimation of the number of Actinomyces in a sample, and could be overcome only by isolating a wider range of colony types for further

identification. In addition, the occurrence of other genera which give rise to actinomyces-like colonies, necessitates the use of a rapid backup method for positive identification. In the IADR, Abstracts (March, 1978) Dr. Ellen reported the use of 0.14M D-mannose in an agglutination test for identifying fresh isolates of A. viscosus and A. naeslundii. As a single test, mannose agglutination was 90% successful in identifying strains of A. viscosus and A. naeslundii. Since it has been postulated that additional serotypes exist that would not be detected by available antisera, an agglutination test could be helpful as a rapid means of verifying fresh isolates from CNAC-20.

Proposed Course

Additional work must be done with CNAC-20 agar before it can be used with confidence as a differential medium for A. viscosus and A. naeslundii. This would include FA identification of additional plaque isolates so that atypical strains can be recognized by colonial morphology. Also D-mannose agglutination should be examined as a rapid method for identification of A. viscosus and A. naeslundii.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1-DE-00249-01 CPR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Evaluation of the Rat Model for Dental Caries

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gordon Huxley	Visiting Scientist	CPR, NIDR
	Suzanne Amsbaugh	Biologist	CPR, NIDR
OTHER:	Janet A. Brunelle	Chief, Biometry Section	CPR, NIDR
	Albert Kingman	Statistician	CPR, NIDR
	Shou-Hua Li	Visiting Scientist	CPR, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Caries Prevention and Research Branch

SECTION
Biometry Section and Preventive Methods Section

INSTITUTE AND LOCATION
NIH, NIDR, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.26	.11	.15

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A series of experiments was designed and run to evaluate various parameters of the rat model. Some of the factors being considered are type of rat, diet, time on experiment, the dental caries scoring system of Konig and Keyes, and both whole and partial recording techniques.

NIDR Classification: 10290

Objective:

The use of the rat model to study dental caries has produced inconsistent and varying results among investigators. Types and age of rats, diets, time on experiment and the scoring systems of König and Keyes will be studied to try and reduce the variation in rat studies so that results from different studies may be more readily evaluated and compared.

Methods:

Six separate experiments were designed and tested in the rat model: (a) Six treatment groups were fed cariogenic diet 2000 for 1, 2, 3, 4, 5 or 6 weeks. All rats were inoculated with S. mutans 6715; (b) A second set were on the same regimen to score teeth with staining; (c) Eight groups were fed diet 2000 or diet SD containing various percentages of sucrose--0, 15, 30 and 56 for 15 days; (d) Eight groups were fed the same sucrose diets for 8 weeks; (e) Four groups were used to test the effectiveness of two known cariostatic agents--2.5 ppm F in the drinking water and 0.4% inorganic phosphate in the diet; (f) 96 rats were started on diet 2000 at different ages--18, 19, 20....25 days old. In all studies litter mates were assigned to different groups. The rats were weighed at the beginning and end of each study. Caries was assessed by an examiner using Keyes technique and a second examiner using König technique. In some experiments, sets of diagonally opposite quadrants were scored by König method, other set by Keyes method to test comparability of half-mouth recording. Analysis of variance will be used to test differences.

Findings:

All studies were run, heads defleshed for scoring, and scoring finished. (a) Analysis of caries incidence over varying periods of time seems to indicate that differences in caries can be detected at four weeks on the cariogenic diet by both scoring methods. Litter mates were not a factor in the variation. (b) Weight gain was significantly less on diet SD. The varying levels of sucrose in diet SD caused different amounts of caries on all surface areas. Litter mates were significantly different for a few surface areas in parts of the scoring technique.

Significance:

If rats can consistently reach a certain level of caries in a short time (4 weeks) and differences between cariostatic agents can

be detected by a particular scoring technique at that time, then testing in the rat model becomes a reliable method of evaluating various new anti-cariogenic agents.

Proposed Course:

Statistical analysis will continue on the remaining studies to answer the rest of the questions.

PART IV

NATIONAL INSTITUTE OF DENTAL RESEARCH

ANNUAL REPORT

INTRAMURAL RESEARCH

OCTOBER 1, 1977 to SEPTEMBER 30, 1978

Compiled by:
Dental Research Data Officer
National Institute of Dental Research
National Institutes of Health

PART IV

NATIONAL INSTITUTE OF DENTAL RESEARCH

ANNUAL REPORT

INTRAMURAL RESEARCH

October 1, 1977 - September 30, 1978

This document was prepared for administrative use at NIH. The comments and declarations of its contributors are their own and do not necessarily represent an official statement of the Institute.

Compiled By

Dental Research Data Officer

National Institute of Dental Research

National Institutes of Health

Bethesda, Maryland

CONTENTS

REPORT OF THE DIRECTOR OF INTRAMURAL RESEARCH

THE NATIONAL INSTITUTE OF DENTAL RESEARCH

October 1, 1977 - September 30, 1978

The scope of research in the Intramural Program encompasses many different fields of biomedical science as can be perceived from reading the summary statements of the Laboratory and Branch Chiefs as well as the individual project reports. These summary statements show that much of the effort is concerned with seeking answers to fundamental questions in order to design methods and procedures for prevention and treatment of diseases and disorders. These studies contribute knowledge that is of general importance to scientists studying diseases in other parts of the body in addition to the particular applications to oral and dental disease. Animal models are used widely while other experiments are performed in vitro using animal organs, tissue cells and cell components or clinical samples. Experiments are also carried out with synthetic compounds. Investigative disciplines presently represented are those of biochemistry, biology, biophysics, chemistry, immunology, microbiology, neurophysiology, and virology.

The Intramural Program's involvement in clinical research has increased gradually during recent years in parallel with the continuing build-up of the data base needed for such endeavors. This is particularly true with respect to the field of periodontal disease. In order to make full use of this potential for progress, the Institute initiated a search for a new Clinical Director last year. The search has continued this past year, but so far the position remains unfilled. Factors which have made it very difficult to attract qualified candidates are limitations on salary, space, and positions as well as restriction of research to candidates with Board eligibility in Periodontology. The Institute has requested relief from the latter restriction which was stipulated in the original search plan approved by the Civil Service Commission. Active search for a qualified candidate will continue once relief is granted.

The search for a new chief of the Laboratory of Biological Structure was concluded with the appointment of Dr. Arthur R. Hand of that Laboratory to the position. This action followed the recommendation of an internal search committee which considered a number of highly qualified external and internal candidates before reaching their decision.

A new Section of Neurocytology and Experimental Anatomy was created by the Director of the Institute in the Neurobiology and Anesthesiology Branch and Dr. Stephen Gobel of the Branch was appointed Chief of the new Section. These actions which were originally requested by the Chief of the Branch were endorsed by the Board of Scientific Counselors following their review of the Branch last year.

Another administrative move has been the creation of a Clinical Dental Associate category within the NIH Associates Program. The Clinical Dental Associates will be assigned to ongoing clinical dental research programs or to other clinical research programs. They will spend 75% of their time carrying out research and the remainder of their time performing dental services for patients at the Dental Clinic. Two Clinical Dental Associates will be appointed each year and appointments will be for 2 years. The first two candidates will be selected next spring. The purpose in setting up this program is twofold: to strengthen clinical research efforts and to create a pool of potential candidates for long term appointment to the Institute staff.

As far as appointments overall are concerned, the Institute has experienced difficulties so far in attracting qualified minority doctoral candidates to its Intramural Programs. A number of efforts have been initiated including 1) writing of a pamphlet about NIDR and careers at NIDR for distribution to appropriate institutions; 2) scheduling of visits by Staff members to Minority Institutions; and 3) development of procedures to insure that in filling vacancies efforts are made to include minority candidates among those considered for the vacancy.

In March, 1978, the Intramural Research Program in collaboration with the Extramural Programs, NIDR, and the National Caries Program, NIDR sponsored a four-day International Conference on the Development, Properties, and Structure of Tooth Enamel. The conference, the third to be held on this topic (the last one met in 1969) was attended by 133 persons, many of whom were from foreign countries. The meeting provided a form for state-of-the-art presentations, reports on current findings, and wide ranging, in-depth discussions of the various presentations. Both formal presentations and transcripts of the informal discussions will appear in print shortly. This publication, like its 2 predecessors, can be expected to serve as the start-off point and guideline for directions of research involving tooth enamel for years to come. In addition, many new contacts were established and new collaborative efforts were initiated which will enhance the Institute's own efforts in this area.

An open workshop on "Feedback Control of Exposure Geometry in Dental Radiography" was held at the University of Connecticut Medical Center on May 16, 1978. It was jointly sponsored by the American Academy of

Dental Radiology, the University of Connecticut School of Dentistry, the Bureau of Radiological Health, and the Intramural Program of the Institute.

The principal objective of the conference was to discuss and exchange ideas on the feasibility of using an on-line, feedback control system to insure comparability of X rays taken at different points in time. This concept was explored both theoretically and practically using a prototype scanning X-ray system as a basis for discussion. Speakers in the first session described the state-of-the-art which provided a rational basis for open discussion of specific details in a subsequent session. The proceedings are being compiled for publication to provide a tangible basis for future development in this area.

The eight-person Board of Scientific Counselors participated in groups of two in four separate program reviews. As was the case last year, ad hoc members were added to provide greater scientific expertise to each review group. In September, the complete Board met for a day and a half to discuss the individual review reports and make recommendations to the Director of the Institute and the Director of Intramural Research. As in previous years comments on lack of space and personnel were common to most of the reviews. These two areas continue to be real obstacles not only to our recruitment efforts as previously indicated but also to the realization of the full potential of our existing programs.

In Fiscal 1978 the following NIDR staff members received awards in recognition of their contributions to the Institute's programs.

NIH Director's Award - Dr. E. David Eanes, Dr. Elliott Schiffmann; NIH Merit Award - Ms. Betty Ho, Mr. Webster C. Leyshon, Mr. Alan H. McKerrow; Commissioned Corps Meritorious Service Medal - Dr. Joost Oppenheim; Commissioned Corps Commendation Medal - Dr. David L. Rosenstreich; NIDR EEO Award - Ms. Barbara Y. Iba, Mr. Garland N. Martin, Dr. Marie U. Nylen; IADR Oral Science Research Award - Dr. Arthur R. Hand.

Report of the Clinical Dental Services Section
Office of the Director of Intramural Research
National Institute of Dental Research
1978

The Clinical Dental Services Section furnishes dental consultative services and dental therapies for Clinical Center patients of all Institutes, originates and carries out its own research projects and provides support and facilities for clinical programs sponsored by the Dental Institute's laboratories and branches.

Active research projects initiated by Section personnel include:

Comparison of high and intermediate lavage volume in third molar surgery (77-D-122) Z01 DE 00119-05 IR. This project is a continuation of studies into the effect on post surgical complications of different volumes of a sterile saline solution used in the irrigation of sockets immediately following the extraction of third molar teeth. A reduction in adverse sequelae associated with this surgical procedure is the goal of the technique being evaluated.

Effect of various preoperative and postoperative rinses on healing after oral surgery (78-D-89) Z01 DE 00241-01 IR. This study will evaluate the effectiveness of four topical solutions to reduce localized osteitis and infection---two adverse sequelae associated with tooth extraction. The solutions (1) chloramine-T, 1%; (2) sodium bicarbonate, saturated solution; (3) normal saline, 0.9% and (4) povidone-iodine, (1% available iodine) will be used twice daily by the patient as topical antiseptic mouthrinses.

Psychological and radiographic evaluations of the orthognathic surgery patient (76-D-249) Z01 DE 00213-02 IR. This study allows an evaluation of the presurgical and postsurgical psychological and functional aspects of individuals with developmental osseous defects of the face and jaws. The psychological impact of the surgical correction and postoperative alterations in bone and tooth position are being studied.

The potential of a fluoride mouthrinse to alleviate xerostomia symptoms (76-D-278) Z01 DE 00217-02 CI C. Preliminary tests indicate that a sodium fluoride mouthrinse (1.23%F) can transiently increase the volume of whole saliva in normal subjects. Present studies evaluate the potential for saliva flow stimulation in subjects xerostomic due to radiation therapy, chemotherapy and Sjogren's syndrome.

An evaluation of individuals with immune deficiency diseases relative to the incidence of dental caries and periodontal disease (protocol pending). Preliminary observations are being conducted to identify if certain dental pathologies are more or less prevalent in a unique patient population being medically evaluated by the Metabolism Branch, National Cancer Institute. This study will attempt to correlate the potential for greater or lesser incidence of dental caries, gingivitis and alveolar bone loss in subjects demonstrating Wiskott-Aldrich Syndrome, IgM or selective IgA deficiency, hypogammaglobulinemia, etc.

An evaluation of periodontal pocket reduction surgery and the topical application of antibacterial agents in the control of periodontitis (78-D-78) Z01 DE 00239-01 IR. This study will compare the effectiveness of two home applied oral hygiene regimens in removing subgingival tooth accumulated bacteria. Either conventional mechanical techniques alone or mechanical techniques in conjunction with topically applied sodium bicarbonate-sodium chloride "paste" will be assigned to periodontitis patients who have had pocket reduction surgery performed in one-half of their mouths.

In addition to the above intra-clinic research projects, the influence of written educational and slide material on the teaching and motivation of dental patients is being evaluated. Attractive pamphlets explaining oral disease prevention and oral problems associated with heart disease and radiation-chemotherapy are being prepared for patient distribution within the NIDR Clinic.

The Clinical Dental Services Section's collaboration with NIDR laboratories and branches involve the following projects:

Laboratory of Microbiology and Immunology

Investigation of cellular immunological mechanisms in periodontal disease and the correlation between periodontal disease and histocompatibility antigens (76-D-330) Z01 DE 00205-02 LMI.

Investigation of the etiology and the extra-salivary complications of Sjogren's syndrome (75-D-54) Z01 DE 00085-05.

Observations on the periodontal microbiota of patients with active periodontal lesions and with lesions controlled by personal oral hygiene and tetracycline therapy (78-D-77) Z01 DE 00096-05 LMI C.

Laboratory of Neurobiology and Anesthesiology

A psychophysiological evaluation of the effectiveness of (and recovery from) intravenous sedation for oral surgery procedures in ambulatory patients (76-D-148) Z01 DE 00132-04 NA.

Assessment and measurement of experimental and clinical acute pain in a dental situation including an evaluation of known pharmacological and non-pharmacological agents used in pain control (75-D-59) Z01 DE 00133-04 NA.

Clinical Investigations Branch

Taste and its disorders Z01 DE 00212-02 CI.

Studies of oral sensation and perception in children and adults (77-D-1) Z01 DE 00182-03 CI.

Oral pharyngeal lipase (74-D-13) Z01 DE 00218-02 CI C.

Clinical evaluation of prefogging panoramic dental radiographs to increase contrast (77-D-47) Z01 DE 00211-01 CI.

The development and evaluation of improved dental radiograph systems, with emphasis on factors influencing diagnostic performance Z01 DE 00065-06 CI.

Laboratory of Oral Medicine

The relationship of mechanical trauma to oral aphthous ulcers (77-D-97) Z01 DE 00094-05 LOM C.

The Clinical Dental Services Section continues to offer a unique and essential opportunity to provide a link between basic research laboratory activities, clinical research laboratory activities and the clinical human disease problem.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00119-05 IR

PERIOD COVERED
October 1, 1977 to August 1978 CT 0600079

TITLE OF PROJECT (80 characters or less)
Effect of Lavage Volumes in Third Molar Surgery

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James B. Sweet	Sr. Dental Surgeon	IR	NIDR
OTHER:	Donald P. Butler	Oral Surg-Anesthesiologist	NA	NIDR
	Alice A. Macynski	Clinical Nurse (General)	IR	NIDR

COOPERATING UNITS (if any)
None

LAB/BRANCH
Office of the Director of Intramural Research

SECTION
Clinical Dental Services Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Individuals from ages 15-35 years with bilateral mandibular third molar impactions indicated for extractions serve as patients. The impacted teeth are removed and the alveolus is immediately irrigated with either 60 or 120 milliliters of normal saline lavage. Each patient is examined postoperatively for localized osteitis.

NIDR Classification: 44420 - 100%

Project Description

I. Objectives:

As reported in the literature, third molar surgical extraction has a higher incidence of postoperative infection and localized osteitis than other exodontia procedures. This research is an attempt to prove whether or not there is a significant reduction in infection and localized osteitis through the use of various volumes of lavage solutions in third molar surgery.

It has been found that high volumes (350 ml) of lavage solution applied under pressure at the surgical site significantly reduce the incidence of localized osteitis from that previously reported. It has also been found that moderate volumes (175 ml) of lavage solution applied under pressure at the surgical site significantly reduce the incidence of localized osteitis when compared with that of low volumes (less than 25 ml) of lavage solution.

In a recent study, the effect of high volume lavage (350 ml) was compared to that of intermediate volume lavage (175 ml) in the same manner as the previous studies. The incidence of localized osteitis was very low in both groups without significant differences occuring between them.

Having demonstrated that it is not necessary to use high volume lavage for prevention of localized osteitis, it is desirable to establish a lower volume that is adequate for prevention of adverse sequelae. A lower lavage volume would be preferable to the surgeon and his staff since delivery time would be less. Thus, the objective of the current study is to determine if similar beneficial results can be obtained by using even smaller volumes of saline lavage.

II. Methods Employed:

1. Patients are screened and selected for removal of two impacted mandibular third molar teeth.

2. Each surgical site is irrigated with a measured volume of lavage solution immediately after tooth removal. A random technique is utilized to preselect the volume of irrigation used at the surgical sites. A sterile saline volume of 60 ml is used at one surgical site just prior to closure and the opposite side is lavaged with 120 ml of the same solution.

3. The patient is examined for signs of infection and localized osteitis 5 days postoperatively. The occurrence of either is recorded and appropriate therapy is rendered.

4. At completion of the study, an evaluation and comparison of the results will be performed using conventional bio-statistical methods.

III. Major Findings:

Results indicate that there is no significant difference in the incidence of localized osteitis with the use of either 60 ml or 120 ml of normal saline lavage following extraction of mandibular third molars. Either volume, however, significantly lowers the normally reported incidence of localized osteitis. No significant differences were noted whether mechanical or conventional lavage was used.

The results also indicate that the age of the patient and whether or not the patient smokes tobacco are important predisposing factors.

IV. Significance to Biomedical Research and the Program of the Institute:

A lavage of at least 60 ml of normal saline solution delivered either mechanically or conventionally following mandibular third molar extractions will significantly lower the expected incidence of localized osteitis. Much postoperative pain and extra treatment visits are eliminated by using this procedure, thus saving both the operator and the patient time.

It can also be recommended from the results of this study, that patients have their impacted third molars removed at a young age and that they refrain from smoking during postoperative healing to decrease the chances of the occurrence of localized osteitis.

V. Proposed course.

Project terminated August 1978.

Publications:

1. Increased Incidence of Postoperative Localized Osteitis in Mandibular Third Molar Surgery Associated With Patients Using Oral Contraceptives: Sweet, J.B., Butler, D.P., American Journal of Obstetrics and Gynecology 127:518 March 1, 1977.

2. Effect of Lavage on the Incidence of Localized Osteitis in Mandibular Third Molar Extraction Sites: Butler, D.P. and Sweet, J.B., Oral Surgery, Oral Medicine and Oral Pathology 44:14 July 1977.

3. Effect of Smoking on the Incidence of Localized Osteitis Following Mandibular Third Molar Surgery: Sweet, J.B., Butler, D.P., Quintessence International. Report 1596:9 February 1978.

4. Predisposing and Operative Factors: Effect on the Incidence of Localized Osteitis in Mandibular Third Molar Surgery: Sweet, J.B., Butler, D.P., Oral Surgery, Oral Medicine and Oral Pathology 46:206 Aug. 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00213-02 IR

PERIOD COVERED

October 1, 1977 to September 30, 1978

CT 0600123

TITLE OF PROJECT (80 characters or less)

Psychological and Radiographic Evaluations of the Orthognathic Surgery Patient

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James B. Sweet	Sr. Dental Surgeon	IR	NIDR
OTHER:	Donald P. Butler	Oral Surg-Anesthesiologist	NA	NIDR
	John Folio	Chief Clin Dental Ser Sect	IR	NIDR
	Richard H. Gracely	Research Psychologist	NA	NIDR
	Harold A. Greenberg	Chief Clinical Care	DCBR	NIMH
	James Madero	Clin Care Cons Psychologist		NIMH
	Alice A. Macynski	Clinical Nurse (General)	IR	NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH

Office of the Director of Intramural Research

SECTION

Clinical Dental Services Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.45	.40	.05

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The psychological and anatomical changes that take place in individuals with middle and lower facial bony defects and malocclusions of the teeth after orthognathic surgery are measured and evaluated. Pre-and post surgery data obtained by tests, interviews, radiographs, photographs and orthodontic study casts taken at specific time intervals for a period of two years postoperatively are compared.

NIDR Classification: 44410 - 50%
44420 - 50%

PHS-6040
(Rev. 10-76)

Project Description

I. Objectives:

Many individuals with middle and lower facial bony deformities or severe malocclusion require surgical correction in addition to routine orthodontic treatment. This includes patients with Class II or Class III malocclusions, patients with apertognathia, and patients with severe facial asymmetry.

The preoperative psychological state of these patients is influenced by their facial appearance, functional disability, and by expected postsurgical changes.

Regression and relapse are common postoperative complications associated with orthognathic surgery.

The specific aims of this study are to establish the psychological impact of the deformities prior to surgery, to determine the expectations from surgery and to correlate the findings after a 2 year follow-up period. Postoperation changes in facial bone and tooth positions which may occur are also being examined.

II. Methods Employed:

1. Patients between 15 and 45 years of age who have malocclusions that can be corrected by surgery, or a combination of surgery and orthodontics are examined and selected for study.

2. Preoperative photographs, study models and panoramic and cephalometric radiographs are taken on each patient.

3. Preoperatively, each patient and a family member is interviewed by a psychiatrist. Personality variables are assessed by standard psychological tests, and preoperative expectations are assessed by a questionnaire.

4. Surgical procedures are performed in the operating room under general anesthesia using commonly accepted techniques.

5. At three months, twelve months, and 24 months following surgery the battery of psychological tests are readministered and the patient is reinterviewed by the psychiatrist. At this time, resolution of presurgical expectations and overall satisfaction of the procedure will be determined.

6. Panoramic and cephalometric radiographs, study models, and facial photographs will be taken at the three, six, twelve and 24 month intervals to assess postoperative stability.

7. At completion of the study a final evaluation and comparison of the results will be done using conventional biostatistical methods.

III. Major Findings:

To date 12 subjects are in various stages of therapy. No findings are available for reporting since this project entails long term post-surgical evaluations.

IV. Significance to Biomedical Research and the Program of the Institute:

The investigation may increase our understanding of personality and psychological profiles in orthognathic surgery patients. This will allow a more thorough pretreatment evaluation and a more realistic definition of the patients expectations, which will result in our providing a better service to the patient.

V. Proposed Course:

Continue until approximately fifty patients have been completed.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00239-01 IR

PERIOD COVERED
June 5, 1978 to September 30, 1978 CT 0600122

TITLE OF PROJECT (80 characters or less)
Evaluation of Surgery and Antibacterial Agents in Control of Periodontitis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	William E. Wright	Sr. Staff Dentist	IR	NIDR
Other:	Paul H. Keyes	Dental Director	LMI	NIDR
	Surya A. Howard	Health Technician (Dental)	LMI	NIDR
	Marjorie L. Meehan	Health Technician (Dental)	IR	NIDR

COOPERATING UNITS (if any)
None

LAB/BRANCH
Office of the Director of Intramural Research

SECTION
Clinical Dental Services Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.25	.20	.05

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The effectiveness of conventional mechanical hygiene alone and in combination with topical applications of sodium bicarbonate-sodium chloride "paste" to control accumulations of oral bacteria will be compared in subjects with moderate periodontitis. Each subject will also undergo periodontal pocket reduction surgery in two of four posterior quadrants to allow assessment of this commonly used mode of periodontal disease therapy. Evaluation criteria will be (1) motile microscopic forms and white blood cells obtained from periodontal pockets will be viewed and numerically estimated by phase microscopy, (2) gingival sulci bleeding index and (3) measurements of gingival attachments relative to the cemento-enamel junction and gingival margin.

NIDR Classification: 20400 - 50%
20511 - 50%

Project Description

I. Objectives:

The purpose of this clinical research project is to compare the effectiveness of two home applied oral hygiene regimens in removing subgingival tooth accumulated bacteria...(1) conventional mechanical techniques-brushing, flossing and water irigation versus (2) the same conventional mechanical techniques in conjunction with topical applications of a sodium bicarbonate-sodium chloride "paste". Periodontal pocket reduction surgery will be performed in two of four posterior quadrants of all subjects selected for this study with the diagnosis of moderate generalized periodontitis.

Combinations of surgical areas versus nonsurgical areas in conjunction with one of the two home therapy regimens will be evaluated as to their role in effectively assisting the patient to prevent or control progressive destruction of the periodontal attachment apparatus.

II. Methods Employed:

1. Male or female patients ranging in age from 20-49 years and demonstrating moderate generalized periodontitis will be selected for study.

2. Pre-study baseline periodontal parameters will be recorded. Evaluation criteria will include (1) motile microscopic forms and white blood cells obtained from subgingival plaque scrapings will be numerically estimated by phase microscopy. (2) gingival sulci bleeding index and (3) measurements of gingival attachments relative to the cemento-enamel junction and gingival margin.

3. Subjects will be randomly assigned to perform one of two oral bacterial control regimens (conventional mechanical only or conventional mechanical plus daily topical applications of an "antimicrobial salt paste"). Random selection of two posterior quadrants to receive periodontal pocket reduction surgery will be used for all subjects.

4. Criteria evaluations will be made before treatment, after surgery one month and at three month intervals throughout the three year study.

5. Patient achievement goals will be an appreciable reduction in the numbers of motile bacterial forms and white blood cells in their subgingival plaque scrapings as determined by phase microscopy. Also a reduction in gingival bleeding index to 10% of total sites tested will be a performance goal.

6. Prevention or control of progressive loss of gingival attachment as indicated by serial pocket measurements over a three year period will be evaluated.

III. Major Findings:

Protocol approved June 1978. No. reportable findings.

IV. Significance to Biomedical Research and the Program of the Institute.

This investigation will give insight into the types of therapy that can be most effective in controlling the common disease, chronic periodontitis. Information should be provided on the potential for treating certain periodontal lesions without surgical intervention. A conservative approach would be of value in treating the great numbers of individuals afflicted with chronic periodontitis for whom surgical therapy is not feasible due to lack of finances, facilities and trained clinicians.

V. Proposed Course:

To evaluate a minimum of 12 subjects as outlined to see if statistical significance can be attributed to control regimens.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00241-01 IR

PERIOD COVERED
June 29, 1978 to September 30, 1978

CT 0600124

TITLE OF PROJECT (80 characters or less)
Effect of Various Preoperative and Postoperative Rinses on Healing After Oral Surgery

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James B. Sweet	Sr. Dental Surgeon	IR	NIDR
CO-PI:	Donald P. Butler	Oral Surg-Anesthesiologist	NA	NIDR
OTHER:	Alice A. Macynski	Clinical Nurse (General)	IR	NIDR

COOPERATING UNITS (if any)
None

LAB/BRANCH
Office of the Director of Intramural Research

SECTION
Clinical Dental Services Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	0.8	0.4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This clinical study is designed to allow the investigators to evaluate the effectiveness of four different solutions used topically before and after oral surgery procedures. Included will be normal saline solution, the conventional rinse, which will serve as a control. Postoperative localized osteitis and infections will be recorded if they occur, as data, to be considered in the overall evaluation of the use of topical solution for possible prevention of these postoperative problems.

NIDR Classification: 44420 - 100%

Project Description

I. Objectives:

The overall incidence of localized osteitis throughout the mouth has been reported from 0.9% to 3.2%. The incidence of localized osteitis in third molar sites has recently been decreased with the use of lavage technique from 20-30% to under 6%, and the infection rate to under 3%. This small percentage of patients, however, still suffer with extreme postoperative pain. Conventionally, patients rinse with a weak saline solution the day following surgery and continue for a few days post-operatively. The saline is used primarily to mechanically clean the extraction wound of any debris present; it will additionally reduce the presence of bacteria. Various other preoperative and postoperative topical solutions have been tried with variable success. Legarth and Munster-Swendsen recently used a chlorhexidine solution for postoperative rinsing of third molar extraction sites and achieved a 45% reduction in the incidence of localized osteitis in a study involving 415 cases of mandibular third molars. Another recent study with a preoperative rinse of chloramine-T reduced the incidence of localized osteitis in mandibular third molar sites from 4% to 1.6%. It is thought that the use of certain antiseptic mouthrinses could achieve similar or possibly better results, especially if used both pre- and postoperatively.

The purpose of this project is to clinically test three different solutions along with the conventional saline used pre- and postoperatively with oral surgery procedures to compare the incidences of localized osteitis and infection rate to determine if there are advantages with the use of other solutions as a rinse.

II. Methods Employed

An informed consent will be signed on GSA Standard Form 522, and a specific informed consent for this study will also be signed.

Immediately before the surgical procedure, the patient will rinse with 10 ml of a solution of either (1) 0.9% sodium chloride (normal saline), (2) 1% chloramine-T, (3) povidone-iodine (1% available iodine), or (4) saturated sodium bicarbonate for a period of one minute. The surgery will then be performed in a normal manner. The patient will receive both oral and written postoperative instructions for use of the mouthrinse for four to six days after surgery. Ten milliliters of the same solution used preoperatively will be used for one minute both morning and evening; 120 ml will be dispensed for the patient's use during this period of time. The patient will begin the rinses the day following surgery and continue their use until his scheduled post-operative observation appointment when sutures will also be removed. The patient will be examined for signs of localized osteitis or infection five days postoperatively, except when the fifth day is on a

Saturday or Sunday. In these cases the patient will be examined either on the fourth or sixth day postoperatively.

Specific criteria for evaluation of a patient with localized osteitis will be: (1) a severe pain in the alveolus of a surgical site two to four days postoperatively, (2) the presence of a foul, grayish exudate from the surgical site, (3) the presence of a necrotic odor from the surgical site, or (4) observation of a denuded, bony alveolus. At least two of these criteria will be present for a diagnosis of localized osteitis to be made.

Specific criteria for evaluation of a patient with an infection will be: (1) a swelling which persists, increases, or appears after four to five days postoperatively, (2) a purulent drainage from the surgical site, (3) a pulse rate significantly increased above the normal for the patient and persistently so elevated, or (4) a fever usually above 38.3°C, which remains elevated even when the patient remains well hydrated. At least one of the above criteria will be present for a diagnosis of postoperative infection to be made.

If an infection or localized osteitis occurs it will be treated and recorded as being present. Systemic antibiotics will not routinely be used postoperatively, except where infection occurs, and in those cases, they will be employed immediately when the diagnosis is made.

After five days (or if the fifth day is on Saturday or Sunday, then either four or six days) the surgical sites will be observed for healing progress. This data will be recorded with the patient's post-operative data sheets. The patient will then be discharged with follow-up appointments if needed. The patient will be instructed to return if he develops any further postoperative problems.

III. Major Findings:

The project was initiated in June 1978, thus insufficient data is available for reporting at this time.

IV. Significance to Biomedical Research and the Program of the Institute:

The results of this study can provide important information relative to a good topical rinse for use after oral surgery procedures. Positive results will allow the recommendation of a topical rinse to oral surgeons and dentists for use in prevention of certain postoperative oral surgery problems.

V. Proposed Course:

Continue project until sufficient data is generated to compare results with previous studies.

Report of the Microbial Systematics Unit

National Institute of Dental Research

The Microbial Systematics Unit is charged with establishing a data bank for information describing diverse strains of microorganisms. Special emphasis is placed on the human oral microbiota. For this purpose, collaborative projects are on-going with microbiologists distributed throughout the world.

At present there are tens of thousands of scientists, physicians, public health personnel, and others involved in some aspect of microbiology. The number of microbial strains isolated, characterized, and (in many cases) preserved, by individuals runs into the millions. Hundreds of millions of bits of information have been developed on these strains. However, these data are not resident in a single, centrally located system, permitting rapid and efficient utilization. Because of the large volume of information involved and because, in several applications such as classification and identification, mathematical manipulations of the data are required, electronic processing of these data is necessary.

In collaboration with personnel of the American Type Culture Collection; the Food and Drug Administration; the Center for Disease Control and numerous academic microbiologists, strain data are being entered into the data bank with the objective of generating a system for providing such services as: data on specific organisms and/or groups of organisms, a locator service for strains with special characteristics, identification of unknown isolates, cluster analysis definition of parameters of taxa, data management and report writing aids for research purposes, aids in quality control of tests, methods, and laboratories, and communication of data via common format.

Data files of primary data on a large number of microorganisms found in the oral cavity and related types are being established. These files provides a resource for asking both ecological and epidemiological questions of interest in dental research.

Programs have been developed and tested to enter, retrieve, and analyze the data in a variety of ways for epidemiological, diagnostic, taxonomic, ecological, etc., uses. The long term goal is to establish a world-wide data bank at a series of cooperating centers. As experience grows, better programs are being designed and implemented.

The system originally developed for bacteria is now being expanded to include the yeasts, molds, algae and protozoa. A series of monographs describing the expanded system is in varying stages of preparation.

An extensive file of descriptions of filamentous organisms is in the final stages of assembly. The file covers all the described types of Mycobacteria, blends into the Nocardia, then through the Actinomycetes (especially a unique set on oral isolates), and finally, Bacterionema. This file is being actively used in collaboration with the submitters of the data as well as numerical taxonomists to revise the badly confused taxonomic relationships of these bacteria. Such revision is necessary to avoid the misidentification (leading to erroneous epidemiological conclusions) which are found in some recent dental research literature.

A second file on non-filamentous oral organisms (streptococci, lactobacilli, veillionella, etc.) is being constructed. It is already being used to study correlations among caries activity, phenetic span of characters, serology, source of isolation, and host descriptions. Preliminary work has shown that Streptococcus mutans is a diffusely heterogeneous group of organisms which does not form internal clusters of relatedness.

One of the long term goals in establishing these particular files is the establishment of probability tables to allow computer-aided probabilistic identification of oral isolates.

Probability matrices, for on-line identification of bacteria (including Gram negative rods, lactobacilli, streptococci, bacilli, etc.) have been constructed. They are available to research workers for field use.

PUBLICATIONS:

Krichevsky, M. I. and Norton, L. M.: The world's culture collections as an information system. Proceedings of the II International Conference on Culture Collections. pp. 41-48, 1976.

Krichevsky, M. I.: Coding and manipulation of strain data. Proceedings of the III International Conference on Culture Collections. pp. 150-156, 1977. University of Bombay Press, Bombay, India.

Krichevsky, M. I.: Coding and Management of Microbiological Data. Developments in Industrial Microbiology 18:309-318, 1977.

Kaneko, T., Atlas, R. M., and Krichevsky, M. I.: Diversity of bacterial populations in the Beaufort Sea. Nature. 270:596-599, 1977.

Kaneko, T., Hauxhurst, J., Krichevsky, M., and Atlas, R. M.: Numerical taxonomic studies on microorganisms isolated from Artic and sub-Arctic marine environments. Proceedings of International Symposium on Microbial Ecology. In press.

Kaneko, T., Krichevsky, M. I., and Atlas, R. M.,: Numerical taxonomy of bacteria from the Beaufort Sea. J. Gen. Microbiol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00044-08 ODIR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Handling of Microbial Strain Information by Computers

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Krichevsky, Micah I.	Chief, Microbial Systematics Unit	ODIR NIDR
OTHER:	Love, Leslie L.	Biological Laboratory Technician	ODIR NIDR

COOPERATING UNITS (if any)

see addendum

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.00	.50	.50

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

Microbial strain data are being entered into a data bank with the objective of generating a system for providing such services as: data on specific organisms and/or groups of organisms, a locator service for strains with special characteristics, identification of unknown isolates, cluster analysis definition of parameters of taxa, data management and report writing aids for research purposes, aids in quality control of tests, methods, and laboratories, and communication of data via common format. Data files of primary data on a large number of microorganisms found in the oral cavity and related types are being established. These files provide a resource for asking both ecological and epidemiological questions of interest in dental research. Programs are being developed and tested to enter, retrieve, and analyze the data in a variety of ways for epidemiological, diagnostic, taxonomic, ecological etc. uses. The long term goal is to establish a world-wide data bank at a series of cooperating centers. The system originally developed for bacteria is now being expanded to include the algae, yeasts, molds and protozoa. A series of monographs describing the expanded system is in varying stages of preparation.

NIDR CLASSIFICATION: 10250, 10110, 20211--100%

COOPERATING UNITS:
R. Gryder, HA, Food and Drug Administration
F. Benedict, EDRO, Food and Drug Administration
R. Gherna, American Type Culture Collection
D. Brenner, Center for Disease Control
V. Dowell, Center for Disease Control
J. Brooks, Center for Disease Control
P. Riley, Center for Disease Control
L. Wayne, Veterans Administration
R. Atlas, University of Louisville

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00250-01 ODIR

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Algorithms for Microbial Systematics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Walczak, Cynthia A.	Computer Programmer	ODIR	NIDR
OTHER:	Krichevsky, Micah I.	Research Chemist	ODIR	NIDR
	Mercer, Paula	Computer Programmer	ODIR	NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH

SECTION
Microbial Systematics Unit

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

A feature frequency analysis program has been written which tabulates features for groups of organisms. The program also tabulates the uniqueness of each feature for each group.

A pilot program to search a master list of codable items was written. The user aassembles and edits a file containing a subset of interest. The results demonstrated feasibility. After optimization of the data base format, the full system will be written.

The traditional algorithms for cluster analysis of microbial data require core storage of two large matrices during program execution. The DEC-10 allows cluster analysis of less than 600 strains at a time. Further, the costs rise exponentially with the number of strains. Modification of the existing program yielded a 90% cost reduction. Pre-sorting strains according to % positive reactions does not change intra-cluster relationships. Mathematical modeling showed that a sorted data set can be partitioned into two overlapping segments, analyzed separately, and the results joined with little loss of meaning. This procedure reduces costs and allows analysis of larger data sets.

NIDR CLASSIFICATION: 10250, 10110, 20211--100%

Annual Report of the Laboratory of Developmental Biology & Anomalies
National Institute of Dental Research

The major research efforts in the Laboratory of Developmental Biology and Anomalies are directed at research on the prevention and treatment of inherited and acquired defects in oral-facial development. Our projects include studies on the formation and development of facial tissues, role of environmental and genetic factors in oral-facial malformations, connective tissue formation and development as well as tumors. These projects are interrelated and information obtained in one area is rapidly applied to another. A particular effort is being used to apply recent research advances to practical applications. Two such applications are discussed below and include the use of cell culture systems for teratogen screening and the use of antibodies against specific collagens in tumor diagnosis.

Some changes have occurred in personnel. Dr. Robert Stern has left to take a position in the Pathology Department at the University of California in San Francisco. Dr. Stern was the principal investigator on Project Z01 DE 00130-03 DB-B, Isolation and Purification of Collagen mRNA, with Drs. M. Zeichner and D. Breitkreutz who have also left. The activities in this area are continuing in Project Z01 DE 00025-12 DB. A new project has been started--Development of Cartilage with Dr. John Pennypacker as Principal Investigator. Initiation of this project acknowledges the increased interest here on the mechanism of chondrogenesis. Many genetic defects and environmental agents alter cartilage development.

In June, 1978, a site visit was held to review the activities of the Connective Tissue Section excluding those individuals involved in Project Z01 DE 00006-18 DB, Studies on Leucocyte Chemotaxis. In September, 1978, the activities of the group studying chemotaxis of cells was presented in a special program for the Scientific Counselors, NIDR.

Connective Tissue Section

Immunological Studies On Connective Tissue - We have found that about one-third of the patients with relapsing polychondritis, a disorder characterized by episodic inflammations of all cartilagenous tissues, have circulating antibody reacting with cartilage. This antibody is specific for type II collagen, the cartilage collagen. The titers of antibody are greatest at the height of the disease and decrease with treatment. No such antibody is found in other chronic inflammatory diseases of cartilage and therefore this represents a unique and perhaps causative association. In future studies the role of cellular immunity to type II collagen in this disorder will be investigated.

Tumors - Antibody to type IV (basement membrane) collagen distinguishes tumors of epithelial origin from undifferentiated metastatic tumors. All 14 breast carcinomas studied (8 primary lesions and 6 metastatic

lesions) showed prominent staining. Individual tumor cells can also be distinguished within lymph nodes while inflammatory cells and macrophages are negative. In addition, lymphomas, osteosarcomas and Ewings' sarcomas fail to stain. This procedure is being tested as a possible diagnostic procedure by the NCI pathologists.

Other - Antibodies against the various collagens have been used to map the locations of types I, III and IV collagen in the rat incisor. Type II collagen was not found. The distribution of type IV collagen throughout other oral tissues is being studied on a contract with the Department of Pathology, University of Innsbrück.

Cell Attachment - Previous studies established that fibroblasts, periosteal cells and hepatocytes attach to collagen via a peripheral membrane protein, fibronectin. Fibronectin is produced by these cells and also found in serum. These cells attach well to types I-V collagen. Chondrocytes use a different protein from serum for attachment and assume a fibroblastic appearance in the presence of fibronectin. Epidermal cells show preferential attachment to type IV collagen and do not use fibronectin. The interactions of cells with specific collagens is mediated by cell specific proteins and is probably of importance in determining tissue architecture in development and repair.

Many tumor cells do not require fibronectin for attachment. Cells from a metastatic mouse tumor were found to attach preferentially to type IV (basement membrane collagen). These cells were found to secrete an enzyme which degraded type IV collagen while other collagenases did not attack this protein. It is possible that these properties allow penetration of tumor cells through capillary basement membranes.

Regulation of Cellular Activities - The synthesis of collagen by fibroblasts is reduced at high cell density. Type I collagen synthesis is inhibited more than type III collagen synthesis. These changes may be brought about by an increase in cyclic AMP levels.

The translation of collagen mRNA has been studied. Two high molecular weight species corresponding to pro α1(I) and pro α2 are produced from calvaria mRNA as well as other proteins. A peptide released from the amino terminal end of the pro α1(I) chain inhibits the translation of mRNA for the pro α1(I) and pro α2 chains but not the translation of other proteins. It is possible that this represents a mechanism for regulating collagen synthesis.

Chondrogenesis - A system has been developed for studying mineralization in tissue culture. Limb mesenchyme cells undergo chondrogenesis in culture. Removal of some of the proteoglycan allows mineralization of the cartilage matrix. Treatment of the mesenchymal cells with vitamin A prevents cartilage formation and induces a highly mineralized matrix. This system should allow the mechanisms underlying mineralization to be studied in an *in vitro* system.

A mouse mutant resembling patients with the Kniest syndrome has been studied. Cartilage from the mutant mice is fragile and disorganized. The proteoglycan is apparently normal while type II collagen synthesis is reduced markedly in comparison with normal cartilage.

Chemotaxis - Leucocytes are attracted to certain n-formylmethioninyl peptides. Now new peptides have been synthesized to establish the relationship between structure and activity. Some of these peptides inhibit chemotaxis. Bacterial products that are chemotactic for leucocytes have been characterized. These factors appear to be methionyl peptides of some 2000 MW.

Craniofacial Development Section

Teratology - Diphenylhydantoin is suspected of causing cleft lip in some children born to women taking this drug. Until recently, the putative teratogenic activity of this compound has been difficult to demonstrate in common laboratory animals. Now it has been found that diphenylhydantoin will produce a high percentage of cleft lip in A strain mice. Our studies indicate that A strain mice metabolize diphenylhydantoin differently than nonsensitive mouse strains such as the C57 strain. It is likely that this difference accounts for the differential susceptibility of the A strain animals to the teratogenic effect of DPH. Similarly, the manner in which the human maternal tissue metabolizes diphenylhydantoin may vary in individuals and determine whether this drug is teratogenic.

Two other teratogens, tunicamycin and vitamin A, may act by altering membrane glycoproteins. Tunicamycin prevents the glycosylation of fibronectin, a membrane glycoprotein produced by fibroblasts and a number of other cells. The nonglycosylated fibronectin is degraded four times more rapidly than normal. As a result fibronectin levels are low on the surface of the cell and the cells are rounded and poorly attached to collagen substrates. Vitamin A impairs chondrogenesis. The fibronectin synthesized by the treated mesenchymal cells contains more mannose than normal and appears to accumulate at the cell surface. As discussed in the Project by Pennypacker, fibronectin causes chondrocytes to convert to a fibroblast-like cell.

Very striking differences have been found in the levels of glucocorticoid receptors present in craniofacial tissues in different strains of mice. Swiss Webster-Frazer mice (highly sensitive) have 10 times the level found in C57 mice (insensitive) while A strain mice (sensitive) have intermediate levels. The susceptibility of the mice to steroid induced anomalies is proportional to the level of receptor in the fetus suggesting that this is the molecular change underlying the genetic difference in susceptibility.

Two in vitro systems, developing neural crest cells and developing mesenchyme cells, are being studied for their ability to respond to teratogens. Addition of known teratogenic agents such as vitamin A

and 5-bromodeoxyuridine blocks the differentiation of these cells, but not their growth in culture. Other compounds such as cyclophosphamide are not active unless a preparation of microsomes with drug metabolizing enzymes is included in the culture. These systems may be used for the in vitro screening of compounds for teratogenic activity.

Whole Embryo Culture and Palate Development - Rat embryos will maintain normal growth and differentiation for at least 36 hours upon transfer to in vitro culture and during this time the primary palate develops. The process of formation of the primary palate has been found by us to be analagous in part to the formation of the secondary palate. That is cells in the zone of fusion of adjacent epithelium cease DNA synthesis while glycoprotein synthesis is increased. The glycoproteins formed are probably used for the adhesion of adjacent tissues while the cessation of DNA synthesis is an early step in the programmed degeneration of epithelial cells on the fusing surfaces. The mechanisms underlying both changes may be mediated by epidermal growth factor and cyclic adenosine monophosphate levels in the cells as shown for similar reactions in the secondary palate.

Experimental Induction of Oral-Facial Anomalies in the Rhesus Monkey - Administration of cyclophosphamide at specific stages of development produces oral-facial malformation in the Rhesus monkey. The abnormalities observed include cleft lip with or without cleft palate, abnormal facies (severe reduction in midfacial growth) and isolated cleft palate. These observations extend those observed previously with the teratogen in rodents. This model should be useful to the oral surgeon in developing improved corrective procedures.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00006-18 DB

PERIOD COVERED
October 1, 1977 to September 30, 1978

NO1 DE 52477

TITLE OF PROJECT (80 characters or less)

Studies on Leucocyte Chemotaxis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Schiffmann, Elliott	Research Chemist	NIDR DB
OTHER:	Corcoran, Barbara A.	Chemist	NIDR DB
	Aswanikumar, S.	Visiting Fellow	NIDR DB
	Venkatasubramanian, K.	Visiting Fellow	NIDR DB
	Muller (Gauss), Verena	Visiting Fellow	NIDR DB

COOPERATING UNITS (if any) LGCB, BP, and LCS of the NIMH; ERRB of the NICHHD; Dept. of Pharmacology, Medical College of Virginia; Dept. of Pathology, University of Connecticut Health Center; Division of Allergy and Infectious Diseases, University of Washington Medical School and Dept. of Medicine, Boston University

LAB/BRANCH
Laboratory of Developmental Biology & Anomalies

SECTION
Connective Tissue Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.00	3.75	.25

CHECK APPROPRIATE BOX(ES)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long term goal of this project is to understand the biochemical processes of cell migration along a chemical gradient. The leucocyte has proven to be a suitable system for this purpose, and studies are proceeding in two directions: on the structural requirements of attractants for recognition by the cells, and on the subsequent molecular events that result in chemotaxis upon the interaction between attractant and cells. It has been found that small formylmethionyl peptides, possibly related to bacterial factors, are potent attractants for leucocytes. These compounds have been used to probe the migratory process in leucocytes and evidence has been adduced to implicate the role of hydrolytic enzymes not only in chemotaxis, but also in the release of histamine and lysosomal enzymes as well. In addition, further information has been obtained on structure-activity relationships of attractants. A specific chemotactic receptor on the neutrophil has also been demonstrated, and a role for methylation has become evident. Therapeutic applications of these peptides are being explored.

I. Introduction - We are attempting to define the biochemical steps in leucocyte chemotaxis. Chemotaxis may be conceived of as occurring in three stages: an interaction between the attractant and a receptor on the cell, transmission of the signal generated by this interaction to the motility apparatus, and activation of the contractile elements to produce directed motion. We are currently investigating these processes as well as studying the biological effects of synthetic attractants. Since synthetic formylmethionyl peptides are chemotactic we are investigating the effect of altered structure on activity. Additionally, we are trying to characterize the highly potent materials produced by bacteria. We are studying the metabolism of synthetic peptides by target cells and their binding to these cells. We have quite recently begun to study the modulation by the cell of its response to these peptides and to investigate certain therapeutic applications of the compounds.

II. Methods - The use of modified Boyden chambers in the in vitro assay for chemotaxis has been described previously (see publications). The assay for peptide binding to the receptor on the neutrophil has been described previously. Neutrophils were obtained from rabbit peritoneal exudates and from the peripheral circulation of human volunteers.

III. Results - Structure activity relationships and chemotaxis - The progress in this area has occurred both in our laboratory and in that of a contractor engaged in work that is a direct extension of our own research. A unique role for methionine has been revealed in a study of the requirements for the N-terminal residue in chemotactic tripeptides. Also the third position for the phenylalanine residue in formylated tetrapeptides is associated with maximal potency: e.g., F Met-Leu-Phe-X is 1000 times more effective than F Met-Leu-X-Phe. Substituting a tBOC group for formyl on the N-terminal of tripeptides yields an inhibitor of chemotaxis that is about 1000 times less effective than the corresponding agonist. Continuing collaboration with E. Gross (NICHHD) has further defined the stereospecificity requirements for chemotaxis. For example, the all L, F Phe-Leu-Phe-Leu-Phe is a more active agonist than the alternating, L. D compound, which in turn is a better agent than the all D compound. Similar results have been found for the antagonist-tBOC pentapeptides. It should be noted also that these reagents contain no methionine. In addition a radiolabelled ligand, (H) F-Phe-Leu-Phe-Leu-Phe, is being prepared which has high affinity for human neutrophils and which is more stable than the methionine-containing tripeptide.

Bacterial factor: This project has been advanced. The material has been separated into two fractions which are homogenous by two successive high pressure liquid chromatography procedures. An amino analysis of part of the purified material showed the presence of methionine, phenylalanine, a preponderance of non-polar amino acids and acidic

residues. The simplest molecular weight is about 2000 daltons, in agreement with earlier estimates from gel filtration results.

Tumor factors that inhibit chemotaxis - Extracts of fibrosarcoma cells from C57 mice in tissue culture have been shown to strongly inhibit neutrophil chemotaxis to a variety of attractants. Control tissue extracts (spleen, liver, kidney) from the same animal do not have this activity. The material has a molecular weight of less than 10,000 daltons, is inactivated by protease and has been isolated, after aqueous ethanol extraction from cells, as a fraction from Sephadex G-50 gel filtration.

Chemotactant receptors - We have extended our studies on the characterization and modulation of the chemotactic receptor. The receptor, as studied in membrane preparations, is inactivated by prior treatment with mercuribenzoate, a sulfhydryl reagent. This can be reversed by treatment with dithiothreitol, indicating a requirement for free sulfhydryl groups. A specific oxidizing agent for thiols, diamide, also inactivates the receptor. The receptor is not inactivated by specific amino group reagent treatments. Under certain conditions low levels (10^{-7} M) of anti inflammatory steroids appear to inhibit binding of peptide to receptor. This may be related to the proteolytic activity of the cell membrane and is being further studied. In addition, we have found that while gold compounds (Na aurothiomalate) do inhibit chemotaxis of neutrophils (10^{-6} M) but they do not appear to act at the receptor. With respect to modulation of the binding of peptide attractant to receptor, it has now been shown in collaboration with J. I. Gallin (NIAID) that this binding is associated with secretory events occurring in the neutrophil. Cells that have been treated with secretagogues such as ionophores show both enhanced binding of peptide to receptor and increased chemotactic rates in response to low levels of these agents (10^{-10} M), at which there is very little secretion of lysosomal enzymes. However, at high levels of secretagogue (10^{-6} M), at which appreciable secretion occurs (30%) there is a marked depression of both peptide binding and chemotaxis. The significance of this may lie in the possibility that phagocytic defense could be affected by an endogenous pyrogen which has been shown to be an exceedingly potent secretagogue for lysosomal enzyme release.

Post-receptor events in chemotaxis - The previous demonstration of chemotactically stimulated methylation of carboxyl groups on protein has been extended to show that particulate preparations of cell homogenates are rich in methylatable substrate, while the supernatant fractions contain most of the methylase enzyme. Also in the whole cell, low levels (10^{-6} M) of an inhibitor of S-adenosylhomocysteine (S-Ado-Hcy) markedly depress chemotaxis and, to some extent, carboxyl methylation. The compound produces a rise in S-Ado-Hcy in cells. This reagent, however, does not affect receptor activity. Inhibitors of adenosine deaminase have less on an effect upon chemotaxis. These

results support a role for carboxyl methylation in chemotaxis. Additionally, there is preliminary evidence that lipid methylation is enhanced in attractant-stimulated cells. The significance of these findings is that methylation may be the chemical signal that couples the attractant-receptor interaction with the motile elements of the neutrophil, resulting in directed movement. These results were obtained through collaborative efforts with Drs. O'Dea and Chiang of the NIMH.

Other collaborative studies - With Drs. Hook and Siraganian (NIDR), it has been shown that histamine release from basophils by peptides appears to require methionine in the peptide, since the corresponding norleucine analogs (either as agonists or antagonists) do not affect this process. Also, there appear to be separate receptors on the basophil for the n-formylmethionine peptides, C5a and antigen-antibody complex. With Drs. Sandberg and Notkins (NIDR) the previously described conjugates of attractant and antibody have been shown to produce a material that retains both biological activities not only as a soluble complex, but also when bound to red cell membrane fragments. This enhances the therapeutic potential of such compounds in target cell directed applications.

Significance to biomedical research - Inflammatory reactions attend bacterial and viral infections as well as the allergic manifestations of immediate and delayed hypersensitivity. Leucocyte migration into affected regions is a major facet of inflammation. Since chemoattractants such as FMet peptides can cause release of histamine as well as lysosomal enzymes from leucocytes, it should be possible to modulate the inflammatory response by appropriate local or systemic administration of these attractants. Thus it may be possible to induce or suppress the accumulation of cells in a particular site. This may have a direct application in the control of periodontal disease. Also, a systematic study of the structure-activity and post-receptor event requirements for chemotaxis could reveal inhibitors not only of chemotaxis but also of the release of histamine and lysosomal enzymes. Finally some basic insights into the nature of cell recognition in general may be gained.

Proposed course of project - In collaboration with a contractor (below) the structure-activity studies will be continued.

In the tripeptide series, in addition to studies on the second residue, the acylating group on the N-terminal position will be varied as follows: Instead of formyl, the thioformyl, glyoxylyl, formamidino and formacetal groups will be placed on the tripeptide and their effects on biological activities determined. Also some typical pentapeptide derivatives of FMet with Phe as the 5th residue will be synthesized and tested. The production of various urethane N-blocked derivatives is continuing in an effort to obtain potent inhibitors.

In this respect the synthesis of t-Boc-Phe-Leu-Met, the 'reverse sequence' tripeptide, is planned. The tumor factor, from a lethal murine fibrosarcoma will be purified by gel filtration and ion exchange techniques. Its effects upon chemotaxis in both neutrophils and macrophages, lysosomal enzyme release from these cells, cell orientation, endocytosis and cell adhesion (with Dr. H. Kleinman, LDBA) will be assessed in attempts to gain insight into its mechanism of action. The effect of this material upon receptor activity and methylation will be studied in this respect.

We plan to pursue studies on the nature of the receptor both in neutrophils and macrophages. Since it appears to be solubilized by Triton extraction, it is feasible to make affinity columns with peptides to purify it. Such material would then be treated with various group reagents to identify the active site as well as to understand how the receptor is coupled to the motility apparatus of the cell. In this respect the roles of methylation and secretory events in leucocyte chemotaxis will be investigated.

The conditions for methylation and demethylation will be studied and some efforts will be made to isolate the protein methylated as well as to identify amino acids that are methylated.

Collaboration with Dr. L. Altman (U. Seattle) has progressed. We have synthesized a conjugate of formylmet-leu and sulfadiazine, which retains in virtually undiminished potencies both chemotactic and antibacterial properties. This has been sent to him and he is in the process of testing this compound as well as various mixtures of attractant and antibiotic for their efficiencies in promoting wound healing in thermally injured animal models. The therapeutic potential of these efforts is considered significant.

Some insight has been gained into the manner in which neutrophils may inactivate peptide chemotactic factors by pathways other than hydrolysis. In initial collaborative studies Dr. R. Clark, Boston University Medical Center, has found that peroxidase enzymes from neutrophils will markedly reduce the activity of FMet-Leu-Phe, but not F Norleu-Leu-Phe, probably a consequence of the oxidation of sulfur in the former compound. This may in part account for the inactivation by phagocytosing cells of C5a which contains one methionine residue near the C terminal end of the molecule. These efforts are continuing with planned studies on peroxidase effects against chemotactic antagonists containing methionine or its isosteric analog norleucine.

At the LDBA in collaboration with Drs. G. R. Martin and H. Kleinman, we plan to study aspects of fibroblast chemotaxis involving requisite collagenous substrata for their movement, the possible involvement of the LETS protein, and a variety of metabolic parameters based upon information gained from the neutrophil. A new visiting fellow will

undertake such studies.

The contract with Drs. R. Freer and E. L. Becker (NO1-DE-52477) is proceeding along the lines previously described. As stated above, the additional plans for modifying the N-terminal blocking groups and enlarging the chain (5 residues) should further define the specificity of the attractant-receptor interaction. In addition the ancillary collaboration with E. Gross (NICHHD), resulting in syntheses of optical antipodes, should markedly contribute to this study.

PUBLICATIONS:

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Aswanikumar, S., Schiffmann, E., Corcoran, B. A., Morell, L., Gross, E. and Pert, C.: Antibiotics and peptides with agonist and antagonist chemotactic activity. Biochem. Biophys. Res. Commun. 80(2): 464-471, 1978.

Isturiz, M. A., Sandberg, A. L., Schiffmann, E., Wahl, S. M. and Notkins, A. L.: Chemotactic antibody. Science 200: 554-556, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00009-17 DB

PERIOD COVERED
October 1, 1977 to September 30, 1978 N01 DE 82412

TITLE OF PROJECT (80 characters or less)
Chemistry and Biosynthesis of Connective Tissue

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Martin, George R.	Chief, LDBA	NIDR DB
OTHER:	Abe, Shigeto	Visiting Associate	NIDR DB
	Foidart, Jean-Michel	International Res. Fellow	NIDR DB
	Rennard, Stephen I.	Research Associate	NIDR DB
	Robey, Pamela G.	Biologist	NIDR DB
	Steinmann, Beat U.	Visiting Associate	NIDR DB
	Tryggvason, Karl	Visiting Associate	NIDR DB

COOPERATING UNITS (if any)
Department of Medicine, Johns Hopkins Hospital, Baltimore, MD; Laboratory of Pathology, NCI; Dermatology Branch, NCI; Department of Anatomy, McGill University of Innsbruck, Austria; Max-Planck-Institute fur Biochemie, Germany

LAB/BRANCH
Laboratory of Developmental Biology & Anomalies

SECTION
Connective Tissue Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7.25	4.50	2.75

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the formation, function and destruction of connective tissue components in normal and diseased states. Particular attention is directed toward collagen and proteoglycan. Current aspects of this project include (1) the collagen in tumors, (2) the role of collagen in diseases and (3) the differentiation of connective tissues.

It is our thesis that the extracellular matrices of connective tissue are used during development to create tissue form. For some years we have studied connective tissue in normal and diseased tissues. Usually we have first defined the structure of matrix molecules since they are major products of the cells and have essential structural roles in the body. The formation and degradation of these matrix components often involves unique enzymes and reactions. We as well as others have found alterations in the formation of connective tissue macromolecules caused by environmental agents as well as defective genes.

We are developing new procedures particularly immunological methods to study the connective tissues in normal and diseased states. Tumors are being studied, since many tumors produce an extracellular matrix. The type of matrix molecule can be used to define the cells in the tumor and the interaction of tumor cells with matrix may clarify the spread of tumors.

Antibodies to Type II Collagen in Polychondritis - Polychondritis is a rare acquired condition with episodic degenerative inflammations of a variety of cartilagenous tissues including the auricles, esophagus, nose, eyes as well as joints. We have found recently that 6 of 16 patients with polychondritis have antibody reacting with cartilage. The antibody has been shown to be specific for type II collagen, the cartilage specific collagen. Antibody titers are highest at the height of the attack and decrease with steroid treatment. Antibodies against other collagens or other constituents of cartilage are not detectable. No antibodies reacting with type II collagen occur in serum from normal persons or in serum from patients with rheumatoid or psoriatic arthritis. We intend to extend our observations to other patients with this disorder and to investigate whether lymphocytes from these patients are sensitized to these collagens.

Foidart and Abe with Dermatology Branch, NCI

ELISA ASSAY for the collagens - Sensitive immunoassays for types I-IV collagens and fibronectin have been developed. The assay depends on measuring an enzyme linked to antibody. The assay is specific for the particular material analyzed and is comparable in sensitivity to radioimmune assays. A variety of studies are underway. These include assaying hybrid cells for fibronectin production. Here we should be able to identify the chromosome containing the gene for human fibronectin. The types of collagens produced by cultured cells is being studied and the effect of such factors as diabetes.

Rennard

Studies on the components of basement membranes - Basement membranes are widely distributed extracellular structures that are thought to establish tissue architecture and promote cell differentiation. They have not been easy to study, since they are very small and normally occur in tissues with many other connective tissue elements. However, we have found that a mouse tumor, the EHS sarcoma, produces basement membrane in quantity and the macromolecules that form this matrix can be extracted and characterized. We have isolated a collagenous protein from this tumor and a 220,000 dalton glycoprotein. Antibodies prepared to these macromolecules react with all authentic basement membranes indicating that they contain similar proteins. We have had success isolating similar proteins from normal tissues. The structure and interation of these macromolecules with one another and with cells is being studied.

Robey, Martin, Foidart and Tryggvason

Selective digestion of basement membrane collagen by an enzyme from a metastatic murine tumor - The basement membrane is thinned, fragmented or absent within the invasion pathway of malignant tumors. This finding suggested that specific enzymes might be liberated by invasive tumors which degrade basement membrane collagen. We have therefore prepared collagenase from an invasive and metastatic murine tumor and studied the ability of this enzyme to degrade type IV collagen.

Collagenase produced by cultured human skin fibroblasts and the invasive murine tumor were compared in their ability to digest soluble native types I, II, III, IV and V (alpha ab) collagen. The skin fibroblast collagenase degraded type I, II and III collagens producing characteristic A and B fragments but failed to degrade type IV or V collagen. In contrast, collagenase derived from cultures of an invasive murine tumor selectively degraded type IV basement membrane collagen producing specific fragments. An enzyme which selectively degrades type IV collagen may play a significant role in tumor metastases and physiologic basement lamina turnover. Attempts will be made to purify this collagenase and to investigate its occurrence in inflammed tissues, wound healing and other conditions.

Abe, Robey and Martin with L. Liotta, NCI

Antibody to type IV collagen distinguishes tumors of epithelial origin - Antibodies to type IV basement membrane collagen were prepared by immunizing rabbits with type IV collagen from the EHS sarcoma (see above). Tissues fixed in formalin and embedded in paraffin were examined after staining with antisera by the immunoperoxidase method.

All fourteen patients with breast carcinomas were studied (8 primary lesions and 6 metastatic lesions) showed prominent intracellular staining with anti type IV antibody. Individual tumor cells could be clearly distinguished within lymph nodes while inflammatory cells and macrophages were negative. No staining was observed with a variety of other antisera including normal goat or rabbit serum, anti human IgG or IgM, etc. Lymphomas, osteosarcomas and Ewings' sarcomas failed to stain.

These studies suggest that specific anticollagen antibodies can be used for distinguishing the origin and nature of tumor cells, particularly undifferentiated metastatic tumors.

Foidart with L. Liotta, NCI

Modulation of type I and type III collagen synthesis by fibroblasts in culture - Human fibroblasts from fetal as well as adult tissue in culture synthesize type I and type III collagen in rather constant proportions although in situ the fetal cells produce much more type III collagen than in culture. However, we have found that the ratio of type III/I collagen synthesis can be increased in culture at high cell density compared to low and medium density. This change is due to a more pronounced reduction in synthesis of type I collagen than of type III collagen. The same relative increase in the ratio can also be observed when low density cells are treated with prostaglandin (PGE). On the other hand epidermal growth factor (EGF) induces a decrease in the ratio of type III/I collagen in dense cultures. It may be possible to use prostaglandins or hormones in vivo to decrease fibrotic reactions.

Steinmann and Abe

Significance - Many disorders alter normal connective tissue development as well as function. In our studies the function of various types of collagen is being investigated. These studies show that these collagens can be used as molecular markers to identify specific cell types. Specific antibodies have been prepared to these collagens and used in histological and pathological studies. Defects in one or another collagen are associated with certain diseases and developmental disorders.

PUBLICATIONS:

Dessau, W., Adelmann, B., Timpl, R. and Martin, G. R.:
Identification of the sites in collagen α chains that bind serum anti-gelatin factor (cold insoluble globulin). Biochem. J. 169: 55-59, 1978.

Timpl, R. Martin, G. R., Bruckner, P., Wick, G. and Wiedemann, H.: Nature of the collagenous protein in basement membranes. Eur. J. Biochem. 84: 43-52, 1978.

Kram, D., Schneider, E. L., Singer, L. and Martin, G. R.: The effects of high and low fluoride diets on the frequencies of sister chromatid exchanges. Mutation Res. 57: 51-55, 1978.

Liotta, L., Vembu, D., Kleinman, H. K., Martin, G. R. and Boone, D.: Collagen required for proliferation of cultured connective tissue cells but not their transformed counterpart. Nature 272: 622-624, 1978.

Kleinman, H. K., McGoodwin, E. B., Martin, G. R. and Klebe, R. J.: Binding of cell attachment protein to collagen: Effects of chemical modifications. Annals N.Y. Acad. Sci. 312: 436-438, 1978.

Kleinman, H. K., Murray, J. C., McGoodwin, E. B., Martin, G. R. and Binderman, I.: Attachment of bone cells to collagen. Proceedings, Mechanisms of Localized Bone Loss, Eds. Horton, J. E., Tarpley, T. M., and Davis, W. F. Special Supplement to Calcified Tissue Abstracts, Information Retrieval Inc., Washington, D.C., pp. 61-72, 1978.

Abe, S., Kleinman, H. K., Martin, G. R. and Steinmann, B.: Collagen: Function and malfunction. Birth Defects, Eds. Littlefield, J. W. and Grouchy, J. de. Excerpta Medica, Amsterdam, pp. 280-286, 1978.

Kleinman, H. K., McGoodwin, E. B., Martin, G. R., Klebe, R. J., Fietzek, P. P. and Woolley, D. F.: Localization of the bindings site for cell attachment in the 1(I) chain of collagen. J. Biol. Chem., 253: 5642-5646, 1978.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00024-12 DB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Developmental Processes In Genetically Controlled Malformations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Brown, Kenneth S.	Medical Director	NIDR	DB
OTHER:	Harne, Leslie C.	Bio Lab Technician (Animal)	NIDR	DB
	Strong, David M.	Bio Lab Technician (Animal)	NIDR	DB
	Wind, Marilyn	Postdoctoral Fellow	NIDR	DB
	Austin, W. Lena	Postdoctoral Fellow	NIDR	DB

COOPERATING UNITS (if any)
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Laboratory of Developmental Biology & Anomalies

SECTION
Connective Tissue Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.00	3.25	2.75

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are to describe the genetic mechanisms and developmental processes in mutant and highly inbred animals with hereditary predisposition to congenital malformations, particularly involving the face and limbs, and to utilize these animals as experimental systems for the study of the processes of congenital malformation. Mouse strains with hereditary malformations as Mendelian or non-Mendelian traits have been discovered in this laboratory or are obtained from others. Comparison of normal and abnormal development are carried out in embryos of timed gestational age using gross examination of living embryos, whole mount preparations and histological sections as well as biochemical analyses of tissues and or cultured organs and cells. Genetic analysis involves segregation analysis, selection of sublines, cross breeding of abnormal and normal lines and genetic linkage studies. Agents such as drugs, vitamins and hormones are used as probes both for the study of gene action in hereditary malformations and for the study of possible human teratogens.

Objectives

The objectives of this program are to determine the genetic and biochemical patterns associated with malformations. Specific individual malformations in mice are studied as models of human birth defects. The overall pattern of mouse defects, both genetic and in response to teratogens is being studied as an indicator of the pattern of biochemical risk in man.

Methods

We use selected mouse stocks carrying hereditary malformations and highly uniform inbred laboratory mice as our experimental test systems. By using the defined genotypes and uniform behavior of these genetic traits we are able to follow the pathogenesis of malformation at the morphological and biochemical levels. By comparing many of these defective strains we hope to be able to learn not only about the specific processes leading to malformations in mice but also the distribution of the types of these processes among many different mutants. A specific trait in mice may, or may not, correspond to a recognized individual human malformation but the broad pattern of pathogenesis is likely to represent the general pattern for mammalian species including man. Just as hereditary traits are used we also use genetically different but highly uniform inbred strains as test systems for the study of the pathogenesis of teratogen produced malformations and for the interaction of the genotype and specific teratogenic agents in the production of malformation. For the determination of the specific pathogenesis we collaborate with anatomists, embryologists, histopathologists and biochemists to examine the embryos and young of specific genetic types during the periods of pathogenesis for malformations.

Findings

During the past year the brachymorphic (bm/bm) mutant has been studied in several aspects. We have previously described this dwarf mouse as having an abnormality in sulfation of the proteoglycan of the epiphyseal plate of the enchondral bones. In collaboration with Dr. N. Schwartz, it has now been further characterized biochemically as having a defect in the synthesis of the sulfate donor phosphodenyl-sulfate (PAPS) which acts as a carrier to sulfate into proteoglycan. The localized failure of the normal increase of sulfate and glucoaamine incorporation in the proliferative zone of the epiphysis has been demonstrated radioautographically in collaboration with Drs. Greene and Pratt. The undersulfation of proteoglycan has been found by Drs. Pennypacker and Kimata to be present in skin cells as well as cartilage although no histological or morphological defect of skin is found in these animals.

The brachymorphic mouse has also been found to represent an unusual class of susceptibility to steroid teratogenesis. In collaboration with Dr. D. Salomon, we have found that the number of steroid receptors in brachymorphic mice are low, like the background C57BL/6J strains, and thus the susceptibility to steroid teratogens might be expected to be low as has been suggested by Goldman et al. and others. On the contrary we find that brachymorphic mice are very sensitive to this class of teratogen indicating that difference of receptor levels is not the only controlling factor in susceptibility to steroid teratogens. Brachymorphic mice have responses similar to the resistant background of C57BL/6J mice when tested with retinol in teratogenic amounts, which suggests that the sensitivity to steroids may be specific.

The open eyelid with cleft palate (oel) strain which segreates for the recessive oel gene had been found earlier to have an unusual sensitivity to retinol as a teratogen. During this year we have demonstrated that the difference in susceptibility is not related to the genotype of the mother but to the fetus genotype. We have looked for possible retinol and retinoic acid receptor differences between the sensitive oel/oel and resistant +/+ lines of this strain. Dr. Wind has found differences between these genotypes in retinol binding proteins isolated by sucrose density centifugation from maxillary processes of new born animals. This contrasts to their similar levels of retinoic acid binding and cytosol steroid receptors which suggests that this receptor difference may be significant in the different susceptibility of these strains to retinol as a teratogen. Fetus cells from the two strains are being established in culture for further study of these differences.

During the last year we have characterized the previously unnamed mutant strain disproportionate micromelia (Dmm) morphologically by bone measurement and histology of mutant animals, genetically by appropriate mating experiments and biochemically by several techniques. Dmm is an incomplete autosomal dominant. We discovered Dmm/Dmm dies after birth with cleft palate and extreme limb shortening which is most severe in the proximal parts. Dmm/+ has limb shortening of moderate degree but lives and breeds well. Histologically Dmm/Dmm have severe disorganization of the epiphyses and "Swiss Cheese" spaces similar to the Kniest Syndrome, a rare lethal human dwarfism, which also shares an abnormal localization of matrix collagen around chondrocytes. Dmm/Dmm has reduced type II collagen in the epiphyses and cartilage matrix proteoglycans are also generally reduced as shown both histologically and biochemically. There are also proteoglycans abnormalities of tracheal cartilage associated with weakness which may be part of the cause of death of Dmm/Dmm newborns.

The possible role of mycotoxins as teratogens and the role of host genotype in modifying teratogenicity are being studied by Dr. W. L. Austin. She has found that cytochalasin B has very low teratogenicity in relation to toxicity for adult mice while D and E are teratogenic and produce characteristic neural tube defects. The teratogenicity of

D is greater than E and differs between mouse strains. These compounds are produced by fungi growing on grains and vegetables.

Significance to Biomedical Research

We have demonstrated several types of genetic defects that produce malformations or growth defects of severe degree. Defects of collagen synthesis, defects of proteoglycan synthesis and abnormalities of receptors to hormones and vitamin are each associated in specific way with abnormal development. Each mutant has its own specific metabolic block however in the case of bm/bm the metabolic block apparently has multiple effects. At the present time the overall pattern of defects in mice is not apparent. The neural tube defects produced by cytochalasins are similar to those associated with eating "blighted potatoes" in humans.

Future Course of Research

We will continue to study the pathogenesis and cell biology of the bm/bm and oel mice using both biochemical and genetic techniques to identify the specific biochemical steps involved in their sensitivity to different teratogens. The Dmm limb development will be studied further in younger stages. Three new mutants with probable cartilage defects are being introduced into the colony and will be studied by the same approaches. A mutant with abnormal oral epithelium resulting in fusion of all intraoral surfaces is under early stages of study as a possible model of infant respiratory distress syndrome.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00025-12 DB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Regulation of Collagen Biosynthesis in Normal and Diseased Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paglia, Larry M.	Staff Fellow	NIDR DB
OTHER:	Diaz de Leon, Lino	Visiting Fellow	NIDR DB
	Wilczek, Joseph	Biologist	NIDR DB
	Martin, George R.	Chief, LDBA	NIDR DB

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Developmental Biology & Anomalies

SECTION
Connective Tissue Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.75	3.00	.75

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to investigate the regulation of collagen biosynthesis in normal, diseased and malignant cells. Here we are concerned with intracellular steps in collagen formation and the factors that control the amount and kind of collagen synthesized by cells. Collagen synthesis is studied in cell-free systems including polysomes from various connective tissues.

During development, both the type and the amount of collagen produced is carefully regulated. To study these regulatory phenomena at the molecular level, we have optimized the isolation and cell-free translation of polysomes and mRNA from collagenous tissues. This allows us to monitor transcriptional and translational changes in collagen synthesis.

To obtain full sized mRNA, we have had to resort to the proteinase K or guanidine method for extracting RNA from cells. These procedures minimize degradation during extraction. The resulting RNA is efficiently translated into polypeptides similar in size to the chains of procollagen in a rabbit reticulocyte cell-free system. Isolated polysomes can also be translated in this system with even higher efficiency. The cell-free products have been identified by collagenase sensitivity, immunoprecipitation, and gel electrophoresis. Type I procollagen chains are synthesized in a 2:1 ratio which is characteristic of the in vivo synthesis of procollagen type I.

When the mRNA from a rat chondrosarcoma is assayed, a single high molecular weight, collagenase-sensitive band is observed. Part of the protein synthesized is precipitated by antibody against type II collagen. Several other systems under similar investigation include cells from patients exhibiting osteogenesis imperfecta and Ehlers-Danlos syndromes, murine osteosarcomas, chondrosarcomas and fibrosarcomas. Also under current investigation is the role of procollagen amino terminal extension peptides in the regulation of collagen synthesis. Our preliminary data indicate a possible specific feedback inhibition by these peptides on collagen synthesis. We are now proceeding with confirmation of the specificity of their action and attempting to elucidate the mechanism of action.

Significance: Factors regulating the synthesis of collagen are not understood. Very different amounts of collagen are synthesized at different stages and there are abrupt shifts in the molecular species of collagen. Regulation could occur during the transcription of DNA to mRNA or during the translation of the mRNA. We have carried out studies that allow us to measure the amount and kind of mRNA in cells and should allow us later to measure the genes themselves.

PUBLICATIONS:

Diaz de Leon, L., Paglia, L., Breitkreutz, D. and Stern, R.: Evidence that the messenger RNA for collagen is monocistronic. Biochem. Biophys. Res. Commun. 77(1): 11-19, 1977.

Breitkreutz, D., Diaz de Leon, L., Paglia, L., Zeichner, M., Wilczek, J. and Stern, R.: The synthesis of presumptive procollagen messenger ribonucleic acid in the calvaria of the developing chick embryo. Biochim. Biophys. Acta 517: 349-359, 1978.

Breitkreutz, D., Diaz de Leon, L., Paglia, L. Zeichner, M., Wilczek, J. and Stern, R.: Characterization of procollagen messenger ribonucleic acid in a murine chondrosarcoma. Biochim. Biophys. Acta. In press, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00230-02 DB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Attachment of Cells to Collagen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kleinman, Hynda K.	Senior Staff Fellow	NIDR DB
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	Murray, J. Clifford	Visiting Fellow	NIDR DB
	Hewitt, A. Tyl	Postdoctoral Fellow	NIDR DB
	Martin, George R.	Chief, LDBA	NIDR DB

COOPERATING UNITS (if any) Dept. of Human Biological Chemistry & Genetics, The University of Texas Medical Branch, Galveston, TX; National Cancer Institute; National Heart, Lung and Blood Institute; and Dept. of Medicine, University of Wisconsin, Madison, WI

LAB/BRANCH

Laboratory of Developmental Biology & Anomalies

SECTION

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INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.25	4.00	.25

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the mechanism of cell attachment to collagenous matrices. Particular attention is directed towards (1) the requirement of a collagen matrix for cell growth, (2) the attachment properties of various cultured and non cultured cells and whether cells synthesize or utilize attachment proteins different than fibronectin and (3) the nature of the attachment protein(s) specificity for different collagens and reconstituted collagen.

Introductions and Objectives - Many cells exist surrounded by a collagenous matrix. The role of collagen in the tissue is probably more than structural since collagen matrices promote the growth and differentiation of cells. Recently it was discovered by Kiebe that CHO cells do not bind directly to collagen but are linked to the collagen by a large glycoprotein, fibronectin. We are investigating the role of collagen and fibronectin in cell attachment. Our studies indicate that a variety of collagen attaching proteins exist including different ones for fibroblasts, chondrocytes and epidermal cells.

Methods - The interaction of cells with collagen is studied in vitro. Cells are obtained either as cell lines or from a variety of tissues and purified by standard methods. Human skin fibroblasts and CHO cells were obtained from American Type Culture Collection while SV-3T3 and Ki-3T3 were obtained from S. Aronson (NCI). Periosteum cells were obtained from rat embryonic periosteum, connective tissue cells from adult mouse skin, chondrocytes from chick embryonic sternum, hepatocytes from adult rat liver, epidermal cells from guinea pig epidermis and the rat osteosarcoma, monkey hepatoma and mouse fibrosarcoma were obtained as cultured cells from various NIH investigators.

Studies on the attachment protein - When serum is passed through a collagen affinity column, the eluate is unable to promote cell attachment of fibroblasts while the bound material (when eluted) is active. The ability to attach cells to collagen has also been found in LETS-type proteins suggesting that the serum protein is related. Current studies are directed towards determing whether the defactorized serum is active on cells other than fibroblasts and whether a specific protein exists for type IV collagen. In addition, the level and specificity of the attachment protein will be examined in serum from normal individuals and from patients with connective tissue diseases.

Attachment properties of cultured and non-cultured cells - The interaction of cells with the collagen-attachment protein complex is likely to play a major role in determining the distribution of cell types in tissues. A variety of cells are being tested for their attachment properties, i.e. rate, serum dependence and collagen specificity. Cultured cells such as human skin fibroblasts, CHO, 3T3, SV-3T3, Ki-3T3, periosteum and connective tissue cells all show enhanced attachment to collagen and require serum fibronectin for attachment. Cells such as hepatocytes derived directly from tissue also show serum dependence while epidermal cells do not. Epidermal cells probably synthesize their own attachment protein because (1) they attach very slowly, (2) their attachment is not enhanced by fibronectin, (3) they do not make fibronectin, (4) they adhere specifically to type IV collagen, and (5) they differentiate into multilayered squamous epithelial type cells on type IV collagen. Circulating cells such as monocytes and neutrophils attach to all surfaces (plastic or collagen) but differ in their rates and serum requirements. Monocytes attach

within 90 minutes and require serum while neutrophils attach within 5 minutes and are inhibited by both serum and fibronectin. Three tumorigenic cell types (an osteosarcoma, a hepatoma and a fibrosarcoma) grew better on plastic than on collagen, were not dependent on serum for adherence and synthesized little or no collagen. We are currently investigating other normal and transformed cells as well as high and low metastatic tumor cells to determine whether collagen attachment plays a role in the location of the neoplastic metastases.

Identification of chondrocyte attachment requirements - Unlike fibroblasts, chondrocytes do not make fibronectin in vivo and will make it in vitro only after they are cultured for several days. Serum has been found to promote the adhesion of chondrocytes, but purified fibronectin does not. In addition, when fibronectin is removed from serum, chondrocytes (but not fibroblasts) are still capable of adherence to collagen substrates. Preliminary fractionation of the chondrocyte attachment factor by size suggest that it is greater than 300,000 in molecular weight. Further purification and identification of the chondrocyte adherence factor will be made from serum and cartilage extracts.

Role of collagen and fibronectin in cell adhesion and growth - The role of newly synthesized collagen in the growth of normal and transformed cells in culture was investigated using cis-hydroxyproline, a proline analogue which blocks the deposition of a collagen matrix. Addition of the analogue to growing normal cells resulted in a marked reduction in cell number after two days but had no effect upon transformed (tumorigenic) cells with treatment extending up to 21 days. Normal cells cultured with cis-hydroxyproline on collagen substrates grew normaly. These results indicate collagen is required for the growth of normal cells in culture, but is not required by tumorigenic cells.

Studies by Grinnell and Rubin et al. suggest that cells will adhere to native collagen substrates in the absence of fibronectin. However, we find that the attachment of cells to native collagen requires fibronectin in proportion to the amount of collagen on the dish. The other groups used too little fibronectin. Further, when the collagen substrate is prepared with high levels of phosphate as is the normal practice, cells will adhere in the absence of fibronectin. Also addition of phosphate to denatured collagen and to a non-fibronectin binding collagen from Ascaris cuticle promotes cell attachment in the absence of fibronectin. The high levels of phosphate may precipitate calcium and/or magnesium and the cells may be binding to the precipitate. This possibility is supported by the presence of precipitates on the collagen and the failure of trypsin to free the bound cells. This indicates that, with the possible exception of mineralized tissue, this is not a physiological attachment.

Significance - Various genetically distinct collagens exist in association with a specific type of cell including chondrocytes with type II collagen and epidermal cells with type IV collagen. Such cells produce proteins that mediate the attachment. Our studies explain the molecular basis of this sorting out and the manner in which tissues are formed from the interaction of cells with the extracellular macromolecules they produce.

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Kleinman, H. K., Pennypacker, J. P. and Brown, K. S.: Proteoglycans and collagen of "Achondroplastic" (cn/cn) neonatal mouse cartilage. Growth 41: 171-177, 1977.

Klebe, R. J., Rosenberger, P. G., Naylor, S. L., Burns, R. L., Novak, R. and Kleinman, H. K.: Cell attachment to collagen: Isolation of a cell attachment mutant. Exp. Cell Res. 104: 119-125, 1977.

Liotta, L. A., Vembu, D., Kleinman, H. K., Martin, G. R. and Boone, C.: Collagen required for proliferation of cultured connective tissue cells but not their transformed counterpart. Nature 272: 622-624, 1978.

Kleinman, H. K., McGoodwin, E. B., Martin, G. R. and Klebe, R. J.: Binding of cell attachment protein to collagen: Effects of chemical modifications. Annals N.Y. Acad. Sci. 312: 436-438, 1978.

Kleinman, H. K., Murray, J. C., McGoodwin, E. B., Martin, G. R. and Binderman, I.: Attachment of bone cells to collagen. Proceedings, Mechanisms of Localized Bone Loss, Eds. Horton, J. E., Tarpley, T. M., and Davis, W. F. Special Supplement to Calcified Tissue Abstracts, Information Retrieval Inc., Washington, D.C., pp. 61-72, 1978.

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Kleinman, H. K., Murray, J. C., McGoodwin, E. B. and Martin, G. R.: Connective tissue structure: The attachment of cells to collagen. J. Invest. Dermatol. In press, 1978.

Silbert, C. K. and Kleinman, H. K.: Studies of cultured human fibroblasts in diabetes mellitus: Changes in heparan sulfate. Diabetes. In press, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00253-01 DB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Development of Cartilage

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Pennypacker, John P.	Staff Fellow	NIDR DB
OTHER:	Binderman, Itzhak	Visiting Scientist	NIDR DB
	Kimata, Koji	Visiting Associate	NIDR DB
	Lee, Willard A.	Chemist	NIDR DB

COOPERATING UNITS (if any)

None

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SECTION
Connective Tissue Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.25	2.25	2.00

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The factors influencing chondrogenesis are under study. Particular attention is being directed towards the extracellular matrix of developing cartilage and its relationship to normal cellular function. The cartilage system has certain advantages for study in this laboratory since it contains a unique type of collagen and proteoglycan which can be utilized as phenotypic markers, and the expertise and technique are available here to extend these studies to the level of transcriptional or translational control.

In these investigations, use is being made of limb mesenchyme cell cultures, mature chondrocytes in culture, cartilage-producing tumors, and cartilage from various animal mutants which may serve as models for human chondrodystrophies.

Studies on Chondrogenic Expression in vitro

I. Embryonic limb mesenchyme cells plated at high density differentiate into cartilage within four days. With this culture system, one can study normal differentiation, as well as the effects of various teratogenic drugs. However, these experiments are limited by the small amounts of limb mesenchyme initially available and the requirement for a high plating density. When cells are plated at low density, chondrogenesis does not occur. It would be an advantage to be able to initiate cultures at lower cell densities, stimulate their proliferation and still obtain chondrogenesis. Several growth factors have been tested with limited success. In future experiments, culture conditions for chondrogenesis will be optimized by utilizing a collagen substrate, decreasing fibronectin levels in serum, and reducing the oxygen tension. The tumor promoter, phorbol myristate acetate, which acts as a mitogen for these cells, as well as other growth factors will be tested.

II. Recently, we demonstrated that fibronectin added to chondrocyte cultures causes the cells to assume a fibroblastic morphology, decrease proteoglycan synthesis, and increase de novo fibronectin synthesis. Chondrocytes were found to be particularly sensitive to the fibronectin in serum when grown on a collagen substrate. These observations suggest that the fibronectin normally found in serum and used routinely in culture can alter specialized cells. Manipulation of fibronectin serum levels should become an important approach to cell culture. Interestingly, the transition to a fibroblastic phenotype appears to be incomplete as type II collagen is still synthesized by fibronectin-treated chondrocytes. Therefore, the relationship of fibronectin and chick embryo extract is now being investigated. Chick embryo extract completely inhibits chondrogenesis, including type II collagen synthesis. These two systems, one of which dissociates collagen synthesis from other chondrocyte specific synthetic activities and the other which completely inhibits chondrogenic expression may provide important model systems for studies of molecular control.

III. Preliminary studies on a rat osteosarcoma demonstrated that cells from the tumor become chondrocytes and produce cartilage in culture. This suggests that the tumor may arise from a stem cell population. Experiments are now in progress to select for a line of cells in culture which continually gives rise to chondrocytes.

Pennypacker and Kimata

Animal Models for Human Chondrodystrophies

Previous studies from this laboratory have defined the molecular lesion in brachymorphic mice to be a defect in proteoglycan sulfation. Recent work on this mouse has demonstrated that the defect is not

cartilage specific but occurs also in skin proteoglycan, a genetically distinct macromolecule. The generalized defect may present as a cartilage problem due to the importance of proteoglycans to the structure of cartilage.

Work has also progressed on another mouse mutant, disproportionate micromelia, which has a cartilage disorder characterized by a reduction in type II collagen synthesis. This mouse resembles in histological appearance, the Kniest syndrome, one of the human chondrodystrophies. Further investigations are planned to determine whether the lack of type II collagen is due to a translational or transcriptional defect.

Two other mouse mutants will also be studied. Both are characterized by disproportionate micromelia, which include distinctive craniofacial features. These are the stumpy mouse (stm) and cartilage matrix deficiency (cmd). It is expected that such mutants may provide animal models for certain human chondrodystrophies and that their study will contribute to our understanding of normal skeletal development.

Pennypacker, Brown, Kleinman, and Kimata

Calcification in vitro

Recent studies have demonstrated calcification to occur in vitro in cultures of bone cells. However, the process requires some weeks and could be due to the degenation of the cells. Now we have developed a culture system in which to study calcification using the limb mesenchyme cells. In our system, cells are plated at high density in a small area of the dish. Normally, day 4 cultures contain a central zone of cartilage, which coincided with the region of initial mesenchymal attachment, and a peripheral zone of fibroblastic cells. Increasing the phosphate concentration in the medium induces mineral formation only in the peripheral zone. Treatment with streptomyces or testicular hyaluronidase induces mineralization of the central portion of the culture. This suggests that partial removal of proteoglycan is required for cartilage mineralization. Alteration of cellular differentiation with drugs greatly affects mineralization. Treatment with 5-bromo-2'-deoxyuridine (BudR) or vitamin A prevents chondrogenesis. While the vitamin A treated cultures exhibit extensive mineralization, the BudR treated cultures do not mineralize. This, we believe, demonstrates cellular regulation of calcification.

Dr. Binderman who was involved in this project, has returned to Israel and will continue to study this system there. However, we will continue here also as well as collaborate with him.

Pennypacker and Binderman

PUBLICATIONS:

Pennypacker, J. P., Lewis, C. A. and Hassell, J. R.: Altered proteoglycan metabolism in mouse limb mesenchyme cell cultures treated with vitamin A. Arch. Biochem. Biophys. 186: 351-358, 1978.

Hassell, J. R., Pennypacker, J. P. and Lewis, C. A.: Condrogenesis and cell proliferation in limb bud cell cultures treated with cytosine arabinoside and vitamin A. Exp. Cell Res. 112: 409-417, 1978.

Lewis, C. A., Pratt, R. M., Pennypacker, J. P. and Hassell, J. R.: Inhibition of limb chondrogenesis in vitro by vitamin A. Alterations in cell surface characteristics. Develop. Biol. 64: 31-47, 1978.

Hassell, J. R., Pennypacker, J. P., Yamada, K. M. and Pratt, R. M.: Changes in cell surface proteins during normal and vitamin A inhibited chondrogenesis in vitro. Ann. N.Y. Acad. Sci. 312: 406-409, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00135-04 DB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cellular and Biochemical Mechanism of Vitamin A Induced Malformations

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hassell, John Robert	Senior Staff Fellow	NIDR DB
OTHER:	Pennypacker, John P.	Staff Fellow	NIDR DB
	Cannon, Frances B.	Biological Lab Technician	NIDR DB
	Wind, Marilyn	Postdoctoral Fellow	NIDR DB
	Pratt, Robert M. Jr.	Research Chemist	NIDR DB

COOPERATING UNITS (if any)

None

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NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.50	2.50	1.00

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The administration of vitamin A to pregnant mice produces facial and limb malformations which resemble some of those observed in certain human syndromes. The purpose of this project is to determine the cellular and biochemical mechanism by which vitamin A causes malformations. The results indicate that the vitamin A induced malformations are due to an inhibition of neural crest cell migration and the inhibition of chondrogenesis. Studies, using developing limb bud cell cultures, showed that vitamin A causes contact inhibition and increased cell-cell adhesion suggesting cell surface changes. Cell surface labeling studies indicate that vitamin A treatment increases the accumulation of a 220,000 dalton cell surface glycoprotein.

Objectives: The purpose of this study is to determine the mechanism by which vitamin A causes facial malformations in developing embryos. This is determined by mainly using limb mesenchyme cells and chondrocytes in culture and assessing the effects of vitamin A on the synthesis and degradation of extracellular matrix components including proteoglycans and cell surface glycoproteins.

Methods Employed: Vitamin A has been administered to (1) pregnant mice at certain gestational ages, (2) 2-3 day old chick embryos in whole embryo culture, (3) cells in culture derived from pre-chondrogenic mouse (day 10) and chick (stage 24) embryos, and (4) chondrocytes in culture derived from serum of day 10 chick embryos. The morphological effects of vitamin A were monitored by light and electron microscopy. Biochemical studies involved evaluating changes in proteoglycans and glycoproteins.

Major Findings: Our studies show that vitamin A alters the development of facial and limb tissues at very specific stages of development. For example, the administration of vitamin A inhibits the migration of neural crest cells from the neural tube to the facial region but has little effect on these cells after migration. Vitamin A inhibits the differentiation of mesenchymal cells to chondrocytes but has little effect after chondrogenesis is initiated. More extensive studies have been carried out in cultures of mesenchymal cells derived from pre-chondrogenic limb buds and in cultures of mature chondrocytes. Vitamin A inhibits cellular proliferation in dense but not sparse cultures and the number of close cell contacts is increased. Separation of the cells from one another and differentiation of the cells to cartilage do not occur. Such cultures incorporate greater than normal amounts of mannose into glycoproteins. Vitamin A treatment also increases the accumulation of a 220,000 dalton cell surface glycoprotein (fibronectin). This is not due to altered synthesis of fibronectin but increased accumulation of this glycoprotein at the cell surface. This cell surface protein may be responsible for the effect of vitamin A on developing tissues.

Significance: High doses of vitamin A cause developmental malformations in animal models that resemble those of unknown origin in human populations. An understanding of the mechanism by which vitamin A produces malformations could be useful in developing procedures for preventing human disorders.

The principal investigator, Dr. John Hassell, has transferred effective June 1, 1978, to the Clinical Branch of the National Eye Insitute. Dr. John Pennypacker (project #Z01 DE 00253-01 DB) will continue to examine the effect of vitamin A on differentiating limb mesenchyme cells. Drs. Pratt and Greenberg will examine the effect of vitamin A on neural crest cell development (project #Z01 DE 00149-04 DB) and Dr. Wind will examine the biochemical basis for the strain difference in

malformations observed in response to vitamin A (project #Z01 DE 00024-12 DB).

PUBLICATIONS:

Hassell, J. R., Greenberg, J. H. and Johnston, M. C.: Inhibition of cranial neural crest cell development by vitamin A in the cultured chick embryo. J. Embryol. Exper. Morph. 39: 267-273, 1977.

Hassell, J. R., Silverman-Jones, C. S. and DeLuca, L. M.: The stimulation of mannose incorporation into mannosylretinylphosphate, dolichylmannosylphosphate and specific glycopeptides of rat liver by high doses of retinyl-palmitate. J. Biol. Chem. 253: 1627-1631, 1978.

Hassell, J. R., Pennypacker, J. P. and Lewis, C. A.: Chondrogenesis and cell proliferation in limb bud cell cultures treated with cytosine arabinoside and vitamin A. Exp. Cell Res. 112: 409-117, 1978.

Pennypacker, J. P., Lewis, C. A. and Hassell, J. R.: Altered proteoglycan metabolism in mouse limb mesenchyme cell cultures treated with vitamin A. Arch. Biochem. Biophys. 186(2): 351-358, 1978..

Lewis, C. A., Pratt, R. M., Pennypacker, J. P. and Hassell, J. R.: Inhibition of limb chondrogenesis in vitro by vitamin A; alterations in cell surface characteristics. Develop. Biol. 64: 31-47, 1978.

Hassell, S. R., Pennypacker, J. P., Yamada, K. M. and Pratt, R. M.: Changes in cell surface proteins during normal and vitamin A inhibited chondrogenesis in vitro. Ann. N.Y. Acad. Sci. 312: 406-409, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00138-04 DB

PERIOD COVERED
October 1, 1977 to September 30, 1978

NO1 DE 52452

TITLE OF PROJECT (80 characters or less)

Pharmacological Mechanisms of Various Oral-Facial Teratogens

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Horigan, Elizabeth A.	Biologist	NIDR DB
	Mosley, General L. Jr.	Biological Lab Tech-Animal	NIDR DB
	Pratt, Robert M. Jr.	Research Chemist	NIDR DB

COOPERATING UNITS (if any)

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LAB/BRANCH
Laboratory of Developmental Biology & Anomalies

SECTION
Craniofacial Development Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.00	2.25	.75

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The metabolism, placental transfer, and mechanism of action of various teratogens that affect the craniofacial region and skeletal system are under investigation. Studies with two therapeutically dissimilar drugs, norchlorcyclizine and trifluoperazine, have shown that they induce the same syndrome of malformations of the palate, limbs and vertebral column in rat embryos. Biochemical characterization of the cleft palate defect has shown an acceleration of glycosaminoglycan degradation. Animal models, including rodents and subhuman primates, of specific oral-facial malformations have been developed to evaluate craniofacial development. Teratogens used in these studies include cyclophosphamide and diphenylhydantoin. An in vitro test system using differentiating cells has been developed to assess teratogenic compounds.

I. Alteration of Glycosaminoglycan Metabolism and Cleft Palate Induction

Previous studies have indicated that enhanced degradation of glycosaminoglycans (GAG) in palatal shelves of rat embryos was responsible for cleft palate induced by norchlorcyclizine. Current work indicates that this mechanism is not unique for norchlorcyclizine but is probably the method by which various phenothiazine drugs induce cleft palate in rats. Trifluoperazine, a phenothiazine which contains a piperazine side chain, produces exactly the same array of malformations as norchlorcyclizine; the histology of affected palatal shelves is similar; and trifluoperazine causes an enhanced degradation of hyaluronic acid. Other investigators have reported that cortisone or aspirin, both of which produce cleft palate in mice, decrease the amount of GAG in palatal shelves. If altered palatal GAG metabolism proves to be a major mechanism for cleft palate induction then xenobiotic compounds that substantially alter GAG metabolism may present a threat for human malformation if the embryo is exposed to them during critical times of palate formation. This information would be of value in predicting potential cleft palate teratogens in the human.

II. Diphenylhydantoin Teratogenesis and Metabolism

Diphenylhydantoin (DPH) therapy for epilepsy in pregnant women is suspected of causing craniofacial and other congenital malformations in offspring. Administration of DPH to pregnant mice induces a high incidence of cleft lip and palate or isolated cleft palate in fetuses. The expression of experimentally-induced DPH malformations is under genetic control and this may be related to differences we observe in DPH metabolism and distribution in sensitive and non-sensitive mouse strains. Using DPH and several DPH analogs which are metabolized differently, we are attempting to determine drug metabolic pathways in mice which may correlate with the teratogenic response. Such information may be helpful in identifying predisposing factors for human malformations.

III. An In Vitro Test for Teratogens

Several in vitro screening systems are currently available to detect mutagens and possible carcinogens. These tests, however, are not comprehensive enough to detect potential teratogens because they only measure DNA damage, whereas teratogenic compounds most likely act through a variety of mechanisms in addition to causing DNA damage. Since teratogens interfere with normal developmental processes, embryonic cells or tissues that undergo differentiation in culture should constitute appropriate screening systems for teratogenesis. We have developed short term tests employing differentiating cells representative of "early" and "late" developmental events. One cell type used is chick cranial neural crest cells which differentiate into pigment cells in the presence of fetal calf serum or into neuronal-like cells in the presence of horse serum. (see Annual Report Z01 DE 00149-04 DB). Another cell type is chick limb bud mesenchyme

cells which at high density differentiate into chondrocytes.

Several drugs were selected for testing including cyclophosphamide, 5-bromodeoxyuridine, acetylsalicylic acid, and retinoic acid which are proven animal teratogens and glutethimide and isoniazid which are not thought to be teratogenic. Our results demonstrate that teratogenic compounds elicited selective toxicity in one or more of the cell types and that the damage in vitro appeared to reflect and correspond to the damage in vivo. For example, β-aminoproprionitrile, a lathyrogen, whose teratogenic effects are limited to connective and skeletal tissue inhibited chondrogenesis in limb mesenchymal cell cultures but had only transient effects on neural crest cells. Nonteratogenic compounds had no effect on growth or differentiation of cells in either system. It is evident from these studies that in vitro assays for teratogenesis are rapid, sensitive, and capable of distinguishing between teratogens with different actions. Such systems should prove to be powerful adjuncts to animal testing.

IV. Research and Development Studies Relative to the Experimental Induction of Oral-Facial Malformation (Contract NO1-DE-52452)

The purpose of this contract is to produce craniofacial malformations in subhuman primates in order to study abnormal development and to obtain animal models for the oral surgeon.

Two syndromes of malformations have been induced in embryos by treating pregnant monkeys with the antineoplastic drug, cyclophosphamide, at different times in gestation. The first is cleft lip and palate Cl(P) with exopthalmos. The second is a "craniofacial dysmorphia" characterized by marked underdevelopment of midfacial bones, highly arched closed palate and either meningoencephalocele or persistant anterior fontanel. Limb defects are often found in treated animals. Several syndromes of human malformation bear a resemblance to these anomalies including, acrocephalosyndactyly, oral-facial digital syndrome, and Larsen's syndrome. Efforts in the past year have concentrated on development of these animals models to a point where they can be used by oral surgeons and in studies on pathogenesis. Optimal dosing schedules were determined to maximize the yield of specific malformations. The frequency of Cl(P) is 60% while the frequency of craniofacial dysmorphia is 100%. Alpha-fetoprotein levels in maternal serum are being monitored to aid in diagnosis of malformations in utero.

Future Plans

The principal investigator is leaving NIH at the end of the current fiscal year. Studies on diphenylhydantoin teratogenesis will continue with emphasis placed on determining its mechanism for teratogenesis. Research and development studies relative to induction of oral-facial malformations in Rhesus monkeys will continue with Dr. Robert Pratt as project officer.

PUBLICATIONS:

Wilk, A. L., King, C.T.G. and Pratt, R. M.: Enhancement of chlorcyclizine teratogenicity in the rat by coadministration of calcium chelating agents. Teratology. In press, 1978.

Wilk, A. L., King, C.T.G. and Pratt, R. M.: Chlorcyclizine induction of cleft palate in the rat: Degradation of palatal glycosaminoglycans. Teratology. In press, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00149-04 DB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Cellular Proliferation and Differentiation During Craniofacial Development

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Cannon, Frances B.	Biological Lab Technician	NIDR	DB
	Figueroa, Alvaro	Visiting Fellow	NIDR	DB
	Greenberg, Judith H.	Sr. Staff Fellow	NIDR	DB
	Greene, Robert M.	Staff Fellow	NIDR	DB
	Leyshon, Webster C.	Biologist	NIDR	DB
	Mosley, General L. Jr.	Bio Lab Tech (Animal)	NIDR	DB
	Salomon, David S.	Senior Staff Fellow	NIDR	DB
	Verbruggen, Leon A.	International Fellow	NIDR	DB

COOPERATING UNITS (if any)
None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.00	6.00	2.00

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The long range purpose of this project is to understand the regulation of cellular proliferation and differentiation in craniofacial tissues by: (1) defining the factors involved in the in vitro differentiation of cranial neural crest cells; (2) developing procedures to culture the rodent embryo during the time of primary palatal development; (3) determining the biochemical events that regulate palatal epithelial cell adhesion and death; (4) defining the molecular basis for sensitivity to glucocorticoid-induced cleft palate.

The areas of research and the investigators included in this project are divided into the following major subdivisions (I) Cranial Neural Crest Cell Development--Dr. J. H. Greenberg, (II) Primary and Secondary Palatal Development--Drs. A. Figueroa, R. M. Greene, D. S. Salomon, L. A. Verbruggen, Mr. W. C. Leyshon, Mr. G. L. Mosley and Ms. F. B. Cannon provided technical support for these studies.

I. Cranial Neural Crest Cell Development

Cranial neural crest cells migrate from the neural tube at the time of closure and give rise to most of the facial mesenchyme as well as to nervous tissue and pigment cells. Defects in the migration and/or differentiation of these cells are suspected as causes of such anomalies as cleft lip and cleft palate in humans. The differentiation of these cells can be controlled in cell cultures. Differentiation of the neural crest cells into pigmented or neuronal derivatives occurs during culture in fetal calf serum or horse serum, respectively. Specific assays have been developed for macromolecules indicative of each cell type, choline acetyltransferase (neuronal) and melanin for pigmented cell types. The type of collagen synthesized by the crest cells in culture has been examined and it has been found that after 6 days in the presence of horse serum, these cells begin to accumulate type III collagen. The factors in the sera that are responsible for this dramatic difference in differentiation are of high molecular weight, and we are attempting to isolate them. Differentiation into the pigmented cell type is irreversibly inhibited by 5-bromodeoxyuridine (BudR) and reversibly inhibited by dimethyl sulfoxide. A number of chemicals which cause craniofacial anomalies in vivo also affect crest differentiation in vitro including BudR, vitamin A, β-aminoproprionitrile and metabolites of cyclophosphamide. Therefore this system may serve as an in vitro screening test for testing the teratogenic potential of various drugs and environmental agents. Future studies are aimed at testing a variety of suspected agents in this system and also in conjunction with cultures from the limb mesenchyme cells (see Annual Report Z01 DE 00138-04 DB).

II. Primary and Secondary Palatal Development

Clefts of the primary and secondary palate constitute a major birth defect of special interest to the NIDR. Normal palatal development involves the growth, alignment and fusion of the palatal processes. We believe that an alteration in any one of these processes could cause cleft lip or palate.

A method developed for culturing whole rodent embryos during the period when the primary palate is developing has provided new insights into normal development. It has been shown, using this culture system in conjunction with labeled precursors and autoradiography, that cells of the presumptive fusion zone are programmed for terminal cell

differentiation in a manner similar to that occurring in the epithelial cells of the developing secondary palate. Techniques have been developed to inject drugs of interest directly into the primary palate region. Tunicamycin, an inhibitor of glycosylation, injected locally appears to inhibit formation of essential glycoproteins necessary for primary palatal adhesion. We have also observed in the chick embryo fibroblast that tunicamycin causes a reduction in a major cell surface glycoprotein (fibronectin) by stimulating the rate of degradation, presumably due to the lack of glycosylation of fibronectin.

Previous studies have shown that higher levels of steroid receptors are present in the palatal mesenchyme cells of mouse strains which exhibit high sensitivity to steroid-induced cleft palate. Now we have found that cultured palatal mesenchymal cells from the more sensitive strains respond to exogenous glucocorticoids with a greater inhibition of growth than nonsensitive strains (as measured by decreased cell numbers and inhibition of DNA synthesis). We have also found that the endogenous levels of glucocorticoids in maternal as well as fetal plasma during midpregnancy are identical in the sensitive and resistant strains further indicating that the different levels of receptors explain the different sensitivities. Studies are in progress to determine whether there are differential alterations in the synthesis of specific enzymes involved in glycoprotein production or in the synthesis of specific matrix components following steroid administration in vivo or in vitro. Studies are also in progress to examine glucocorticoid receptor levels in fibroblasts from patients with a genetic predisposition to oral clefts, and also the response of those cells to exogenous glucocorticoids and diphenylhydantoin.

We have provided evidence that hormones (glucocorticoids) and growth factors (EGF) play a role in normal craniofacial development. EGF, glucocorticoids and their receptors are present and synthesized by the midgestation mouse embryo suggesting an involvement in normal craniofacial development. EGF can specifically inhibit in vitro the terminal cell differentiation in the presumptive fusion zone of the secondary palatal epithelium. This suggest that lack of EGF by these cells may normally account for their terminal differentiation.

Future plans are aimed at further defining the hormones necessary for palatal development and whether teratogens alter the levels of hormones or their receptors. Most of these studies will be conducted with palatal organ culture or cell cultures with certain lines of mouse teratocarcinoma cells possessing embryonal carcinoma stem cells (ECC) which are pluripotent in vivo and in vitro. These cells undergo developmental changes which are analogous to those observed during normal mouse embryogenesis, they may be useful for studying hormones and other factors involved in normal growth and differentiation or for testing teratogenic agents.

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ANNUAL REPORT OF THE LABORATORY OF BIOCHEMISTRY
NATIONAL INSTITUTE OF DENTAL RESEARCH

The senior personnel and major programs in the Laboratory are unchanged from the past several years. The 27 persons in the Laboratory (as of July, 1978) are approximately evenly divided among senior investigators (seven, two as guests), postdoctoral-level scientists (eleven) and support personnel (nine, office and laboratory). There has been the usual turnover of senior guests and postdoctoral-level scientists, their stay being usually 1-5 years. As usual, laboratory members participated actively in scientific meetings in this country and abroad. Quite a few were also involved in reviewing and editing manuscripts and a variety of other professional activities as well as conducting research.

The major programs of the Laboratory are Enzyme Chemistry, Protein Chemistry and Proteoglycan Chemistry. The last two are administratively in one Section but a new Section has been proposed for the protecglycan chemistry group. Approval is expected early next fiscal year. The Laboratory programs were "site-visited" by a review committee composed of two members of the NIDR Board of Scientific Counselors and two outside experts. All aspects of the Laboratory programs received excellent reviews. These findings of high quality and significance were gratifying to all. A comment of note made by the review committee was that the conduct of research was greatly handicapped by the lack of adequate space. There does not seem to be a way to alleviate this problem in the near future.

Some of the major research findings this past year are summarized below to illustrate the research going on in the Laboratory. Results are described according to research problem, contributions in many cases coming from more than one investigator in the Laboratory and often being collaborative.

I Protein Chemistry

The efforts of the group continue to be directed largely to collagen structure, but elastin, proteoglycans and other macromolecules have been studied as the interest arises either directly from a biological relationship or indirectly when a particular technique can be used profitably on a new problem.

Collagen Structure -- The primary structure of a protein contains the information that determines higher levels of structure. Understanding the relationship in general is a very difficult problem. In the case of collagen, it may be easier because of the several levels of coiling by assumming that the helical parameters are constant. In that case contacts between chains and molecules in the collagen fibril should give rise to repeats in the amino acid sequence of collagen chains. Such repeats have been found by Fourier analysis. The major ones are 43-residue repeats of large hydrophobic residues and a 39-residue repeat of both hydrophobic and charged residues. Based on these results, a model was proposed in which the pitch of the chains in the molecule is 39

residues, molecules are staggered and rotated to form a five-stranded microfibril, molecules are super coiled with a pitch of 426 residues, and microfibrils are aligned and packed on a square lattice to form the fibril. This rope-like structure is also consistent with other evidence.

These model-building studies assume a highly ordered, rigid structure for the collagen fibril. However, nuclear magnetic resonance (nmr) studies of collagen labeled biosynthetically with one of several ^{13}C-amino acids show that the order is more like that of a liquid crystal. Amino acid side chains move very freely and the collagen molecules oscillate about their axes. Therefore, the order deduced from models represents an average structure about which there is considerable mobility. It is evident that although molecular interactions determine this order, it is the covalent crosslinks between molecules that provide the structural stability and strength of the collagen fibril.

Recently developed computer graphic techniques make it possible to visualize molecular structure in great detail. Both wire models and space-filling models in color can be constructed on a CRT display and photographed. Collagen was examined this way and a short, but very dramatic movie was made with the help of DCRT personnel. It is hoped that the movie will both instruct and entertain.

Collagen Fibril Formation -- The collagen fibril must of course be assembled in some manner from collagen molecules. As a beginning to the understanding of the biological process, studies of *in vitro* assembly are being actively pursued. Beginning with collagen monomer in a cold acidic solution, fibril formation can be initiated by raising the pH and the temperature. Kinetic analysis and electron microscopy have shown that there are several steps in assembly. The initial step is temperature dependent and leads to an unknown intermediate. The second step is linear growth of thin filaments which may be the five-fold microfibrils discussed above. The third step is lateral assembly of the thin filaments to form native-banded fibrils. If the collagen molecules can crosslink as they do *in vivo*, assembly is faster and irreversible. This complex process *in vitro* poses a difficult physical chemical problem. *In vivo* assembly promises to be an even more difficult biological problem.

Elastin Structure -- This is a problem in which there is a continuing interest. It was earlier shown by nmr that the polypeptide chains in elastin are highly mobile and elasticity therefore is similar to that of a rubber. Elastin has now been biosynthetically labeled with ^{13}C-amino acids that are restricted to either the crosslink regions or to regions between crosslinks. Consistent with the proposed model, it was found by nmr that mobility is greater in regions between crosslinks than near crosslinks. These details of structure illustrate the power of the nmr techniques.

Hemoglobin S -- Sickle cell anemia is characterized by a sol to gel transition of deoxyhemoglobin S. The rigid gel is responsible for the

sickle formation of red blood cells. Methods to follow the transition are important as a means of studying the mechanism and as assays of gel formation in the presence of agents with a potential therapeutic (inhibiting) effect. The nmr techniques developed for connective tissue studies have been found to be useful in this regard since a quantitative measure of the fraction in the sol or gel phase is provided. The technique is applicable to both hemoglobin S in solution and in whole red blood cells. These studies were collaborative with investigators in NIAMDD.

Gel Structure -- Another collaborative study (with investigators in DCRT) arose from the development of a laser light scattering method for connective tissue studies. It was found that light scattering from gels such as polyacrylamide is affected by mechanical vibrations and that the effect can be used to measure certain properties of the gel such as the elastic modulus. The method should be useful in studying biological gels such as fibrin and collagen.

II Proteoglycan Chemistry

Proteoglycans are a component of all connective tissues and occur in large amounts in cartilage. Although an understanding of their chemistry, structure and biological function is not as well advanced as that of, for example, collagen, recent breakthroughs in methods have led to a model that has proven to be highly useful in designing experiments and interpreting results. Proteoglycan chemistry has become a very active area of research and new results can be integrated with results from related areas such as collagen chemistry to provide an ever-broadening picture of connective tissue structure and function.

Comparative Chemistry -- A rat chondrosarcoma provides large amounts of proteoglycan for chemical and structural studies. It is similar in essential aspects to bovine nasal cartilage proteoglycan but has the advantage that its biosynthesis can be studied in culture and related to its structure. The proteoglycan can be cleaved by several proteolytic enzymes to give two fragments. One contains the glycosaminoglycan (GAG) side chains; the other contains the site which, with another protein (link protein), binds specifically to hyaluronic acid (HA). These fragments are suitable for further chemical studies. In addition, antibodies can be prepared and used for assays of biosynthesis and for localization in whole tissue or in culture systems.

The chondrosarcoma proteoglycan and a similar proteoglycan made by chick limb bud chondrocytes in culture have been found to contain, in addition to GAG side chains, short oligosaccharide side chains containing mannose that are apparently similar to glycoprotein oligosaccharides. Reevaluation of older data suggests that bovine nasal proteoglycan also has this feature. The significance of this new finding is not yet known, but it opens the way to new investigations.

Porcine ovarian follicular fluid contains a proteoglycan that is about the same size as bovine nasal proteoglycan but the GAG side chains are

longer and there are fewer of them. Mannose-containing oligosaccharides are also present. However, the proteoglycan does not form a complex with HA. These results, obtained in collaboration with investigators in NICHHD, illustrate how proteoglycans vary from tissue to tissue to fulfill specific biological functions.

Proteoglycan Biosynthesis -- Cells from either the rat chondrosarcoma or chick limb bud grown in culture provide a convenient system to study proteoglycan biosynthesis. In both cases the cells synthesize proteoglycan with a structure similar to that formed by cartilage in vivo. HA oligomers were used to study proteoglycan complex formation in culture. HA oligomers with 10-20 carbohydrate units compete with proteoglycan-HA complex formation but oligomers with 26-50 units are required to compete with the formation of proteoglycan-HA-link protein complex. These studies help to define the size and nature of the interacting components and show that complex formation occurs extracellularly.

Another way to probe proteoglycan synthesis is to add xylosides to cells in culture. The xylosides compete with the proteoglycan core in the synthesis of GAG chains. Although synthesis of complete proteoglycan molecules is strongly inhibited, and there is much less extracellular matrix, the cells seem to differentiate and mature normally forming characteristic nodules. Apparently in this system there is no direct feedback control of development.

III Enzyme Chemistry

The efforts of the enzyme chemistry program for some years have been directed largely to studies of the mechanism of action of the transglutaminases (TGase). These enzymes catalyze the formation of covalent crosslinks between the ε-amino group of lysine and the γ-glutamyl of glutamine in polypeptide chains. They form a class of crosslinks distinct from the aldehyde-derived crosslinks in collagen and elastin and the disulfide crosslinks common to many proteins. Several TGases are known. There are specific enzymes in liver and other organs, plasma, platelets, hair follicles, seminal plasma, epidermal tissue and possibly cell membranes. They apparently perform a variety of biological functions, many of which have only recently been observed and perhaps some yet to be discovered. With a strong base in the enzyme chemistry of the transglutaminases now available, increasing emphasis has been directed in the last few years to biological functions of these enzymes.

Mechanism of TGase Activity -- A variety of model peptides have been made to allow mapping of the active site. A particularly active peptide is an 11-residue sequence identical to one from β-casein containing an active glutamine. Removal of hydrophobic residues from either end of the peptide reduces activity markedly. Peptides containing an active lysine residue have also been made. A nearby hydrophobic residue enhances binding; differences in the location of the residue and stereospecificity of the region can be related to a presumed structure of the

active site of TGase. Diamines and polyamines are also active substrates for the TGases. Since these compounds have several important biological roles, evidence that they serve as substrates *in vivo* is being sought.

Cold-Insoluble Globulin (CIG) -- This protein has long been known as a plasma protein. It has recently been shown that it is identical or very similar to a cell surface-associated protein called fibronectin (and several other names) which is involved in cell-collagen interactions. There is also recent evidence that interactions involving fibrin, collagen, fibronectin and cells may be stabilized or in some way modulated by TGase. The story is presently not clear, but the chemistry of CIG is being studied as a beginning to an understanding of this potentially important biological system. Physical chemical studies in collaboration with investigators in NIAMDD have shown that this very large protein has several rigid domains joined by flexible regions. This structure is consistent with its polyfunctional interaction properties.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE-00002-27 LB

PERIOD COVERED

October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Chemical and Structural Studies on Collagen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	B. L. Trus	Senior Staff Fellow	NIDR LB
OTHER:	K. A. Piez	Chief	NIDR LB

COOPERATING UNITS (if any)

T. Porter and R. Feldmann, DCRT

LAB/BRANCH

Biochemistry

SECTION

Protein Chemistry

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	1.50	0.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this project is an understanding of collagen structure from the molecular to the fibril level. Emphasis is presently on three aspects of the general problem. 1) Computer analysis of the primary structure of collagen shows distribution or interactional patterns related to molecular packing. 2) Three-dimensional computer models illustrate structural features and can be used to study detailed portions of the molecule or the microfibril. 3) Computer enhancement and analysis of electron micrographs reveals detail not obvious to the eye.

1. Project Description

Objectives:

The structure of the collagen fibril is presently an area of active research in many laboratories. The amino acid sequence of the polypeptide chains is being analyzed for regularities and other structural characteristics which may be related to higher level structure. This is possible because the regular coiled coil structure allows the positions of residues to be predicted from their positions in the sequence and known or calculated helical parameters. Several models have been evaluated and a comparison of the effects of models and different sequences from different type collagens is underway.

The Evans and Sutherland Picture System and Frame Buffer have been used to graphically describe collagen models and detailed structural features both for examination, and as an educational tool. The real time display features of this system allow the scientist to easily change model parameters and ascertain their effects on structure.

Electron micrographs of various molecules and molecular arrays have been enhanced by computer techniques to clarify their structure. This can be applied to collagen fibrils and extract regularities, periodicities, or other structural information which is related to molecular parameters. A semiautomatic system to easily and rapidly scan electron micrographs, perform routine computer enhancement techniques, and display results on the Evans and Sutherland Frame Buffer is being designed.

These approaches are analogous to working from the bottom up with models and the top down with computer enhancement of electron micrographs in order to fully understand collagen structure.

Methods Employed:

Analysis of the primary structure of collagen for relationships to higher level structure is being done by computer modeling methods and Fourier analysis of the primary sequence. The real time graphical display programs have dials representing important variables allowing the researcher to dynamically change models to a number of different published values. Analysis of electron micrographs uses both standard enhancement techniques and procedures developed in this laboratory specially suited to the problem.

Major Findings:

Fourier analysis of type I collagen has led to a model for molecular structure in which the three α-chains are supercoiled about each other

with a pitch of 39 residues. With this pitch the molecule is two-sided. One side has alternating charged and hydrophobic regions with a spacing of 39 residues, while the other side contains an excess of hydrophobics with a spacing of 43 residues. In the model, the molecules are helically arranged in a microfibril with the 39-residue spacing inside which is explained by the 39-residue pitch of the α chains. The molecules are also supercoiled with a pitch of 426 residues which changes the α chain pitch to 43 residues along the microfibril axis and explains the 43-residue spacing as arising from contacts between microfibrils. The molecular supercoil also gives rise to a 4-fold screw axis as suggested by x-ray diffraction. Although this model explains all of the known data, proof of its correctness will depend on more direct evidence.

Line drawings of molecules and microfibrils together with CPK space filling computer drawn color images are being combined into a movie which will demonstrate important features of collagen structure as summarized above. In addition, the modeling programs are a valuable tool allowing the researcher to model collagen in real time. Useful parameters such as pitch, supercoil pitch, radius, orientation, and sequence are variables which are easily changed.

Computer analysis of electron micrographs is in a preliminary stage, but development of some of the computer and photographic methods is well along. The availability of a new high-speed and very accurate microdensitometer to be installed soon, together with the picture processing capability of the Evans and Sutherland Frame Buffer, will offer new capabilities. We are also dependent on high quality electron micrographs (see project No. Z01 DE-00215-02 LB; Drs. R. A. Gelman and B. R. Williams.)

Significance:

Collagen is the major protein of connective tissue and is found in various forms throughout the body. It performs a structural role in a variety of different ways. Through interactions with other macromolecules such as proteoglycans, mineral, and cells, it also plays an important role in many biological processes during development and in pathological states. These studies on collagen structure are important to understanding these functions.

Proposed Course:

The sequence analysis is continuing using additional data from type II and III collagen as it becomes available. When complete type II or III data are known, a detailed comparison of similarities and differences between these and type I collagen will be investigated.

The real time collagen display system will be kept current as additional sequence and structural information becomes available. It will be used to help evaluate more detailed descriptions of collagen.

The electron micrograph enhancement techniques will be converted for use on a new computer and the new microdensitometer will be interfaced so that the reading and enhancement of EM plates can approach real time. This will allow the processing of much more information and searching more samples for particular structural characteristics.

2. Publications

Piez, K. A. and Trus, B. L.: Sequence Regularities and Packing of Collagen Molecules. J. Mol. Biol. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE-00134-04 LB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Structure and Biosynthesis of Proteoglycans

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHERS:	S. M. De Luca	Senior Staff Fellow	NIDR LB
	S. Lohmander	Visiting Associate	NIDR LB
	D. K. MacCallum	Research Scientist	NIDR LB
	J. H. Kimura	NIH Postdoctoral Fellow	NIDR LB
	C. B. Caputo	NIH Postdoctoral Fellow	NIDR LB

COOPERATING UNITS (if any)
Arnold Caplan, Case Western Reserve University, Contract (NO1-DE-72403)
Timothy Hardingham, Kennedy Insitute of Rheumatology, London, England.
Dick Heinegard, University of Lund, Sweden. Masaki Yanagashita, RR, NICHHD.

LAB/BRANCH
Biochemistry

SECTION
Protein Chemistry

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9.00	6.00	3.00

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the chemical and physical properties of proteoglycans, primarily those derived from cartilaginous tissues. Topics of present interest include: 1) chemistry of proteoglycans isolated from bovine hyaline cartilages and from the Swarm rat chondrosarcoma, 2) mechanisms of proteoglycan aggregation and interaction with hyaluronic acid, 3) biosynthesis of the protein core of proteoglycans by chondrocyte cultures, 4) changes in proteoglycan structure during chondrogenesis and maturation of chick limb bud chondrocyte cultures, 5) characteristics of proteoglycans derived from follicular fluid during follicular development.

1. Project Description

Background

Cartilage proteoglycans are large macromolecules (MW 1-4 million) in which large, but variable, numbers of sulfated polysaccharide chains, chondroitin sulfate (CS) and keratan sulfate (KS), are covalently attached to a core protein. Such a molecular architecture yields macromolecules which occupy large hydrodynamic volumes and which exhibit reversible compressibility, characteristics that help provide cartilages with resiliency and resistance to compressive forces. The core protein of cartilage proteoglycans consists of three distinct regions. One end, referred to as the HA-binding region, has a portion of protein (MW about 70-90 thousand) which is devoid of polysaccharide and which interacts in a highly specific way with hyaluronic acid (HA) and with a small molecular weight protein referred to as the "link" protein. These interactions are critical for the organization of proteoglycans into aggregate complexes, the predominant form of the proteoglycans in the tissue matrix. Adjacent to the HA-binding region is a portion of the core protein, referred to as the KS-enriched region (MW about 25-40 thousand), which contains an average of about 65% of the KS chains but less than 10% of the CS chains present in the intact molecules. Distal to the HA-binding region is a portion of the core protein, referred to as the CS-enriched region (MW variable, from a few thousand to 200,000), which contains an average of more than 90% of the CS chains but less than 35% of the KS chains present in the intact molecules. This region has a variable length which appears to be proportional to the number of CS chains present on any individual proteoglycan molecule. The average proteoglycan molecule contains about 100 CS chains (average MW about 20,000 per chain) and 50 KS chains (assuming an average MW of about 5000 per chain).

This program continues to collaborate with: 1) Dr. Dick Heinegard in studies he initiated while a Visiting Associate in our laboratory two years ago concerning the structure of skeletal keratan sulfate. 2) Dr. Timothy Hardingham in studies he initiated while a Guest Worker in our laboratory last year concerning the biosynthesis of proteoglycans in chondrocyte cultures isolated from the Swarm rat chondrosarcoma. 3) Dr. Dennis Torchia of this laboratory in studies relating to ^{13}C-NMR of ^{13}C -serine and ^{13}C-glycine labeled proteoglycans prepared from cultures of chick limb bud chondrocytes (Project # Z01 DE-00157-04.) 4) Dr. Robert A. Gelman of this laboratory in studies relating to the interaction of proteoglycans with collagen during fibrillogenesis (Project # Z01 DE-00215-02.) 5). Dr. Gretchen Hascall, Laboratory of Biological Structure, in studies of the morphology and radioautography of the Swarm rat chondrosarcoma cultures (Project # Z01 DE 00163-02 LBS.) 6) Dr. Arnold Caplan in studies relating to the characteristics of proteoglycans isolated from the chick limb bud chondrocyte cultures. (This

work, detailed in section 3 is being supported in part by a contract, NIDR #NO1 DE-37042.) The projects listed in last years report as "4) Characteristics of enzymes which depolymerize hyaluronic acid, 5) Interaction of hyaluronic acid oligomers with HA-binding protein and link protein, and 7) Development of CsCl gradient stabilized sedimentation technique for investigating transport properties of proteoglycans;" have been completed.

The following sections describe our ongoing projects.

1. Characteristics of proteoglycans isolated from the Swarm rat chondrosarcoma.

Sufficient quantities of proteoglycan aggregates can be purified from this transplantable rat chondrosarcoma for extensive studies of the chemical structure of these complex macromolecules. Further, the tumor provides a convenient source for isolating large numbers of chondrocytes for studying proteoglycan biosynthesis in culture (see 2 below). The effects of the proteolyic enzymes, trypsin, chymotrypsin and clostripain, on the aggregate are being studied using Sepharose chromatography and SDS-polyacrylamide gel electrophoresis. Partial digests with each enzyme yield several distinct fragments from the core protein with molecular weights around 120,000. More extensive digests yield limit fragments of about 70,000 (trypsin and clostripain) and 55,000 (chymotrypsin) for the HA-binding region. Procedures are being developed for isolating various of these fragments for further characterization. Antibodies have been raised in rabbits against the purified HA-binding fragment from trypsin digests and against purified link protein. These antibodies are being characterized for subsequent studies in the chondrocyte cultures.

2. Characteristics of proteoglycans synthesized in culture by chondrocytes prepared from the Swarm rat chondrosarcoma.

Chondrocytes isolated from the tumor and grown in vitro synthesize proteoglycans characteristic of cartilage. ^{35}S-Sulfate and ^{3}H-serine have been used to study the kinetics of synthesis and secretion of proteoglycans. The newly synthesized monomer molecules are secreted from the chondrocytes about 10 min after sulfation and about 15-20 minutes after the core protein is synthesized. The monomers subsequently form link protein-stabilized aggregates in the extracellular space. Oligomers of hyaluronic acid (HA) between HA_{10} and HA_{20} retard link-stabilized aggregation because these oligomers can reversibly interact with the active site in the HA-binding region of monomers thereby inhibiting interaction with macromolecular HA. Oligomers between HA ∿26 and HA∿50 are large enough to accomodate a link protein in addition to a monomer thereby forming a stable ternary complex and blocking subsequent interaction with macromolecular HA. About 5.5%

of the ^{3}H-serine in purified ^{3}H-serine-labeled aggregates was found to be present in link protein suggesting that aggregates contain one link for each bound proteoglycan molecule. ^{3}H-glucosamine was used to label the hyaluronic acid and chondroitin sulfate in aggregate preparations. About 2% of the glycosaminoglycan was found to be HA; no keratan sulfate was found. Additionaly the monomer proteoglycan was found to contain an alkaline-labile oligosaccharide approximately six monosaccharides long which contained ^{3}H-glucosamine. This oligosaccharide was then recovered from purified proteoglycans isolated directly from the tumor. It is linked to the core protein by a galactosamine glycosidic linkage to serine (threonine) and contains sialic acid as well as glucosamine. This oligosaccharide has not been previously identified in proteoglycans but it appears to be present in proteoglycans isolated from bovine nasal cartilage and chick limb bud chondrocyte cultures and may therefore be common to most cartilage proteoglycans. Its structure is being determined. Experiments are underway to determine the kinetics of entry of different amino acid precursors into different parts of the core structure of the proteoglycan molecule to define better the size and characteristics of the core structure. The effect of puromycin and cycloheximide on the synthesis of the core protein is being investigated in similar experiments.

3. Proteoglycans from chick limb bud chondrocyte cultures.

a. Effects of β-xylosides on proteoglycans.

When chick limb bud mesenchyme cells from stage 23-24 embryos are plated at high density, they rapidly divide and differentiate to chondrocytes during the first 2-3 days in culture. Between days 4 and 8, the chondrocytes mature and elaborate an extracellular matrix. The proteoglycans synthesized by newly emergent chondrocytes differ from those synthesized by either the prechondrogenic mesenchyme cells or the mature day 8 cells. For example the proteoglycans synthesized by the prechondrogenic cells are smaller, contain larger chondroitin sulfate chains, contain no keratan sulfate and do not interact with HA, while the proteoglycans synthesized by mature day 8 chondrocytes have much longer keratan sulfate chains and do interact with HA. The effect of adding β-xyloside to these cultures was investigated. This compound acts as an exogenous acceptor for chondroitin sulfate synthesis and therefore competes with the normal proteoglycan core protein acceptor. Cultures were grown from day 0 or day 2 in the continuous presence of 1 mM β-xyloside, a concentration which gives maximal stimulation of chondroitin sulfate synthesis. The proteoglycans synthesized by these cultures on day 8 were essentially identical to those synthesized by day 8 cultures to which β-xyloside was added only one hour before labeling. The molecules were able to bind to hyaluronic acid, contained about 1/3 as many chondroitin sulfate chains but the same number of keratan sulfate chains as for normal day 8 proteoglycans. Additionally, both the chondroitin sulfate

and keratan sulfate chains were about 20% shorter than for the normal day 8 proteoglycans. The proteoglycans synthesized by cultures maintained on β-xyloside until day 8 and then switched to medium with no β-xyloside one hour before labeling were essentially identical to those of normal day 8 cultures although the amounts synthesized were much greater for the released cultures. These data suggest that stage 23-24 mesenchyme cells undergo chondrogenic differentiation and maturation in culture in the presence of β-xylosides even though (i) most of the glycosaminoglycans are synthesized on the exogenous substrate, (ii) the proteoglycans that are synthesized contain fewer and shorter chondroitin sulfate chains, and (iii) the extracellular matrix is greatly depleted in proteoglycan content.

b. Structure of keratan sulfate and of an oligosaccharide which contains mannose.

Mannose is considered to be a constituent of keratan sulfate isolated from mammalian cartilage proteoglycans. For this reason 2-^{3}H-mannose was used in combination with ^{35}S-sulfate to label proteoglycans in day 8 cultures of chick limb bud chondrocytes. Keratan sulfate was isolated from the purified proteoglycan monomers after chondroitinase digestion and alkaline-borohydride treatment. The keratan sulfate chains contained about 9% of the total ^{35}S-sulfate activity in the proteoglycan but none of the ^{3}H-mannose. The keratan sulfate chains had an average size of about 26 monosaccharides which is about 40% larger than keratan sulfate isolated from proteoglycans of bovine nasal cartilage. On the other hand, the ^{3}H-mannose was found in two oligosaccharides with average sizes of about 13 and 6 monosaccharides. The smaller appears to be similar to the oligosaccharide isolated from the rat chondrosarcoma proteoglycans (see 2 above). The larger oligosaccharide is being characterized further. Preliminary evidence obtained with proteoglycans from bovine nasal cartilage suggests that a similar oligosaccharide is present in mammalian cartilage proteoglycans as well and that the apparent content of mannose in the keratan sulfate may be due to the similar average sizes of keratan sulfate and the oligosaccharide which allow them to be co-purified. The mannose-rich oligosaccharides, then, constitute newly discovered constituents of the proteoglycans and they will be characterized further.

4. Characteristics of proteoglycans isolated from porcine follicular fluid and synthesized by cultures of rat granulosa cells.

Monomer proteoglycans isolated from porcine ovarian follicular fluid are similar in size to those from bovine nasal cartilage. They contain about 20% protein, 50% co-polymeric chondroitin-dermatan sulfate and 20% oligosaccharides rich in sialic acid, galactose, mannose, glucosamine and galactosamine. The amino acid composition is significantly different from that of cartilage proteoglycans. The alkali released

chondroitin-dermatan sulfate chains are larger (average MW = 56,000) than chondroitin sulfate chains from cartilage proteoglycans (average MW = 25,000). Two major sialic acid-containing oligosaccharides, which constitute about 15% of the proteoglycan and correspond to penta and heptasaccharide size, were isolated. The pentasaccharide, accounting for approximately 77% of all the sialic acid, contained sialic acid, galactose, glucosamine and galactosamine with the latter linked to the protein core by an O-glycosidic bond. The follicular fluid proteoglycans, unlike those from cartilage, do not interact with hyaluronic acid. Digestion with plasmin released chondroitin-dermatan sulfate-peptides nearly as small as those released by papain. In contrast, cartilage proteoglycans are resistant to plasmin. The molecular structure of follicular proteoglycan molecules is probably responsible for the viscosity and water retention by the follicular fluid. Since granulosa cells produce plasminogen activator at the time of ovulation, plasmin mediated breakdown of proteoglycans would decrease the viscosity of follicular fluid and facilitate release of the ovum.

Granulosa cells have been isolated from rat follicles and grown in vitro for up to 48 hours. The kinetics of the synthesis of proteoglycans by these cells has been monitored with ^{35}S-sulfate. Two ^{35}S-labeled proteoglycans have been recovered from the cultures. One of these is recovered from the bottom of CsCl density gradients and has molecular properties similar to those of the proteoglycan isolated from porcine follicular fluid and described above. The other is recovered from the upper fractions of the density gradient. It appears to contain only a single polysaccharide chain bound to a protein which gives the macromolecule a low buoyant density. The low buoyant density proteoglycan also appears to be in the porcine follicular fluid but has not yet been characterized since it is not recovered in the purified proteoglycan fraction. The rat granulosa cells, then, synthesize proteoglycans characteristic of follicular fluid. The cells will now be studied in media with different hormones such as FSH and gonadotropins to determine more about the regulation of proteoglycan synthesis and degradation in this system.

Significance

Proteoglycans are major structural components of connective tissue. This is most obvious in cartilage where they in part determine physical properties and form, and are critical for normal skeletal function and development. Proteoglycans are also found in all other connective tissues, although sometimes only in small amounts. Their role in these tissues is in general not well understood, but is undoubtedly critical to function.

Proposed Course

The project will continue to investigate such parameters as the physical and chemical properties of proteoglycans, their interactions with other matrix molecules in the organization of an extracellular matrix, the mechanisms involved in their biosynthesis and catabolism, and the changes they undergo during tissue development and transitions. A broad approach will be continued emphasizing not only basic physical and chemical studies but also the role of proteoglycans in biological systems.

2. Publications

Hascall, V. C.: Interactions of Cartilage Proteoglycans with Hyaluronic Acid. J. Supramol. Structure 7, 101-120, 1977. (also published in Cell Surface Carbohydrates and Biological Recognition 181-200.)

Pita, J. C., Muller, F. J., Oegema, T. R. and Hascall, V. C.: Determination of Sedimentation Coefficient Distributions for Cartilage Proteoglycans. Arch. Biochem. Biophys 186, 66-76, 1978.

De Luca, S., Heinegard, D. K., Hascall, V. C., Kimura, J. H. and Caplan, A. I.: Chemical and Physical Changes in Proteoglycans During Development of Chick Limb Bud Chondrocytes Grown In Vitro: J. Biol. Chem. 252, 6600-6608, 1977.

Hascall, V. C. and Heinegard, D. K.: Structure of Cartilage Proteoglycans. Proceedings of the 4th International Meeting on Complex Carbohydrates (Symposium) in press.

Kimura, J. H., Osdoby, P., Caplan, A. I. and Hascall, V. C.: Electron Microscopic Examination of Isolated Proteoglycan Aggregates. Proceedings of the 4th International Meeting on Complex Carbohydrates (Communication). in press.

Kimura, J. H., Osdoby, P., Caplan, A. I. and Hascall, V. C.: Electron Microscopic and Biochemical Studies of Proteoglycan Polydispersity in Chick Limb Bud Chondrocyte Cultures. J. Biol. Chem. 253. 4721-4729, 1978.

De Luca, S., Caplan, A. I. and Hascall, V. C.: Biosynthesis of Proteoglycans by Chick Limb Bud Chondrocytes. J. Biol. Chem. 253, 4713-4720, 1978.

Faltz, L. L., Reddi, A. H., Hascall, G. K., Martin, D., Pita, J. C. and Hascall, V. C.: Characteristics of Proteoglycans Extracted from the Swarm Rat Chondrosarcoma with Associative Solvents. J. Biol. Chem. in press.

Faltz, L. L., Caputo, C. B., Kimura, J. H., Schrode, J., and Hascall, V. C.: Structure of the Complex Between Hyaluronic Acid, the Hyaluronic Acid-Binding Region, and the Link Protein of Proteoglycan Aggregates from the Swarm Rat Chondrosarcoma. J. Biol. Chem. in press.

Oegema, T. R., Hascall, V. C. and Eisenstein, R.: Characterization of Bovine Aorta Proteoglycan Extracted with Guanidine Hydrochloride in the Presence of Protease Inhibitors. J. Biol. Chem. in press.

Yanagashita, M., Rodbard, D. and Hascall, V. C.: Isolation and Characterization of Proteoglycans from Porcine Ovarian Follicular Fluid. J. Biol. Chem. in press.

Heinegard, D. K. and Hascall, V. C.: The Effects of Dansylation and Acetylation on the Interaction Between Hyaluronic Acid and the Hyaluronic Acid-Binding Region of Cartilage Proteoglycans. J. Biol. Chem. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE-00157-04 LB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Biophysical Studies on the Structure of Connective Tissue

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	D. A. Torchia	Biophysicist	NIDR LB
OTHERS:	W. W. Fleming	NIH Postdoctoral Fellow	NIDR LB
	L. W. Jelinski	NIH Postdoctoral Fellow	NIDR LB

COOPERATING UNITS (if any)
Dr. A. N. Schechter, NIAMDD

LAB/BRANCH
Biochemistry

SECTION
Protein Chemistry

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.25	3.00	2.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to investigate the molecular structure of fibrous proteins, proteoglycans and the mineral in connective tissues. The structural information obtained will be correlated with function. Areas of present interest are 1) Structure of collagen and elastin. ^{13}C magnetic resonance techniques are being used to study the structure and interactions in collagen and elastin fibers. 2) Proteoglycan structure. ^{13}C magnetic resonance is also being used to study the molecular mobility of the polysaccharide and protein chains in the chick limb bud proteoglycan monomer. 3) Mineral structure in connective tissue. ^{31}P magnetic resonance is being used to probe the structure of the phosphate moieties in mineralized tissue. ^{13}C magnetic resonance is being used to study the extent and mechanism of hemoglobin S gelation in cell-free preparations and in erythrocytes. A magnetic resonance spectrometer has been assembled which obtains high resolution spectra of solids using high-power decoupling in either the cross-polarization or standard Fourier transform modes.

Introduction

The goal of this work is to determine the molecular structure and interactions of macromolecules in connective tissue and to elucidate structure-function relationships. The primary research tool employed is nuclear magnetic resonance (nmr). Until recently, high resolution nmr studies had been limited to flexible macromolecules since linewidths of high molecular weight rigid structures were too broad to detect. However, structured molecules can now be studied in the solid state by decoupling the dipolar interactions between proton and ^{13}C or ^{31}P nuclei. Cross polarization can be used to enhance ^{13}C or ^{31}P signals in rigid molecular lattices where long spin-lattice relaxation times make normal Fourier transform techniques impractical.

Since high resolution solid state nmr spectrometers are not commercially available we have converted our instrument into a spectrometer suitable for work with solids. High resolution cross polarization spectra and normal Fourier transform spectra (of ^{13}C and ^{31}P) can be routinely obtained for samples ranging from inorganic crystals to whole tissues. Relaxation times in the laboratory and rotating frames, cross polarization times and chemical shift anisotropies can all be measured and provide information about molecular orientation and molecular motions covering the frequency range 10^3 to 10^{10} Hz.

Unlike ^{31}P, the natural abundance of ^{13}C is low (1%). Hence, if labeled amino acids are incorporated into the protein the time required to obtain the ^{13}C data is significantly reduced. Furthermore, the presence of the label greatly simplifies interpretation of the spectra since the ^{13}C resonance can readily be assigned to the labeled site. For these reasons we have used biosynthetic techniques to incorporate ^{13}C labeled amino acids into collagen, elastin and proteoglycans.

Progress

1. Collagen and Elastin Structure. Chick calvaria collagen samples containing ^{13}C labeled Gly, Ala, Lys, Glu and Met have been prepared in tissue culture. Line shapes and relaxation times obtained from the spectra of the labeled reconstituted fibrils provide strong evidence that rapid, anisotropic molecular motion occurs in the helix backbone and in the labeled sidechains. These results indicate that the interactions within fibrils produce liquid crystalline-like order, with fluid boundaries between molecules. Cross-linked elastin samples, containing Val, Ala and Lys residues labeled at backbone carbonyl carbons, have been prepared from chick aorta tissue cultures.

Spectra of the Val labeled samples confirm the conclusion of earlier studies that the bulk of the elastin chains undergo rapid, essentially isotropic motion. The data further indicate more restricted motion for Ala and Lys, presumably since these residues are found in regions where cross-linking occurs.

2. Proteoglycan structure. Although polysaccharide signals are readily observed in natural abundance in the spectra of proteoglycans, protein resonances are not, because protein constitutes less than 10% of the proteoglycan. For this reason we have prepared samples of the chick limb bud proteoglycan monomer containing serine ^{13}C labeled at C^{β}. Spectra of these samples clearly show two separate Ser resonances, respectively corresponding to free Ser C^{β} and Ser C^{β} covalently attached to polysaccharide. A narrow line is observed for the free Ser, consistent with the expectation of rapid internal motion. Of greatest interest is the result that line width of the polysacchride linked C^{β} suggests that rapid, segmental chain motion occurs at the point of attachment of polysaccharide to the core protein. (N. B. This is a collaboration with Dr. V. C. Hascall, see project (#Z01 DE-00134-04 LB.)

3. Hemoglobin-S. ^{13}C nmr spectroscopy has been used to determine the fraction of HbS which polymerizes (fp) when protein solutions or erythrocytes are deoxygenated. The nmr spectra support the idea that the hemoglobin S gel behaves qualitatively like a two phase syatem, consisting of monomer in equilibrium with paracrystalline polymer. However, nmr and centrifugation studies yield significantly different values for f_p as a function of concentration, suggesting that hemoglobin solubility near the surface of the polymer may be markedly different than in free solution (N. B. This is a collaboration with Dr. A. N. Schechter, NIAMDD.)

Significance

Interactions involving specifically labeled sites have been investigated using the new experimental technique of high resolution nmr in solids in conjunction with ^{13}C labeled tissues. New information about the molecular dynamics and interactions at specific sites in intact connective tissue has been obtained. This information has provided a basis for understanding some of the structural features of these tissues at the molecular level.

Future Plans

The general strategy of using solid state nmr to study labeled macromolecules will be followed. However, we plan to feed ^{2}H labeled amino acids to weanling rats in order to obtain specifically deuterated samples of collagen and elastin in intact

tissues. A ^{2}H probe and electronics are currently being built to carry out the deuterium nmr studies. A magic angle spinning probe is being built to remove homonuclear dipolar broadening from ^{31}P spectra of mineralized tissue. We expect this probe to provide high resolution spectra which will be particularly useful for studying phosphorous chemistry in mineralized tissue. The hemoglobin work will concentrate on determining f_p in erythrocytes as conditions are varied.

Publications

Torchia, D. A.: The Measurement of Proton Enhanced Carbon-13 T_1 Values by a Method which Suppresses Artifacts. J. Magn. Res. 1978, in press.

Torchia, D. A. and VanderHart, D. L.: High Power Double Resonance Studies of Fibrous Proteins, Proteoglycans and Model Membranes, in Topics in Carbon-13 NMR Spectroscopy, Vol. 3, G. G. Levy, Ed. Wiley, New York, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 DE-00215-02 LB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Connective Tissue: Formation and Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI. :	R. A. Gelman	Staff Fellow	NIDR LB
	B. R. Williams	Senior Staff Fellow	NIDR LB
Other :	K. A. Piez	Chief, Laboratory of Biochemistry	NIDR LB

COOPERATING UNITS (if any)

Dr. S. E. Lamberg; Johns Hopkins University; Dr. Bruce Wetzel; NCI; Dr. Beat Steinman; LDB&A, NIDR; Drs. R. J. Nossal and S. L. Brenner; DCRT

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Protein Chemistry

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.00	2.50	1.50

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the long range purpose of this project to study interactions and relationships between connective tissue macromolecules as a way to understand connective tissue formation and structure. The topics of present interest are: 1) High resolution electron microscopy of fibrils and other aggregates of collagen. 2) The mechanism of collagen fibril formation. 3) Quasielastic light scattering. 4) Abnormal forms of collagen and other connective tissue molecules.

Project Description

Objectives

1. High resolution electron microscopy of fibrils and other collagen aggregates.

The properties of connective tissues are determined in part by the packing of collagen molecules into functional units, the native fibril. The precise relationship between monomers in a highly ordered fibril - for example, a tendon - is not known. Electron microscopy has provided structural information regarding longitudinal placement of molecules from knowledge of the primary structure and certain residues which have affinity for heavy metal stains, thereby producing characteristic band patterns. Although several models have been proposed, information regarding lateral placement, supercoiling and possible subfibrillar units has been limited by the methods available. Electron microscopy has the potential of providing information down to 0.5 to 1 nm if certain instrument modifications are utilized and preparation of the specimen both conserves native structure and optimizes electron density differences. The major purpose of this aspect of the project is to obtain improved electron microscopic information through improved techniques and the use of in vitro aggregates of collagen in which the structures are altered by varying the conditions of aggregation.

2. The mechanism of collagen fibril formation.

The development of a well-characterized, reproducible system for the study in vitro of collagen fibril formation and of models of fibril structure has made it possible to investigate, in greater detail than previously possible, the mechanism of collagen assembly. It has been established that the rate of assembly is sensitive to, among other conditions, the temperature at which the reaction occurs. This parameter can be used to manipulate the assembly process allowing the study of early stages. It is known that collagen undergoes cross-linkages via aldehyde groups; chemical reduction will prevent these linkages from being formed. It is therefore possible to study the effect of this variable on the assembly mechanism and to study the effect of other variables in a system which is now reversible.

3. Quasielastic light scattering.

Quasielastic light scattering affords a technique for the study of the size and shape of macromolecules in solution. This technique should prove to be well suited for studies of the early stages of collagen assembly where small intermediates are believed to form. Development of instrumentation has been stimulated by this problem. The application

of the technique in investigations into the structure of other biological macromolecules is also of interest.

4. Abnormal forms of collagen and other connective tissue macromolecules.

Abnormalities of the extracellular matrix as expressed in human tissue and in cultured fibroblasts are being compared with normal matrix. In particular, the morphology of macromolecular aggregates in normally functioning tissue are compared with aggregation states occurring when these molecules either exhibit biochemical defects which have already been established or are present in a system in which some other structural macromolecule is defective or deficient. Examples potentially include collagen, elastin and proteoglycans.

Methods Employed

Collagen is prepared from rat skin or rat tail tendon, and purified and characterized by standard biochemical and biophysical methods. Fibril structure and kinetics of formation are being studied by electron microscopy, turbidimetry, and quasielastic light scattering. Increased resolution of electron micrographs is being sought by systematically investigating various preparative methods for specimens and by utilizing techniques to minimize beam damage. A quasielastic light scattering instrument has been built using plans developed at the National Bureau of Standards. It is fully operational.

Progress

1. High resolution electron microscopy of the fibril and other collagen aggregates.

Native collagen fibrils and reconstituted collagen fibrils have been examined by electron microscopy using standard methods. Observation of collagen in various stages of assembly and under various conditions has shown that intermediate aggregates are formed early in assembly and that the formation of long thin filaments precedes banded fibril formation. If the collagen cannot crosslink, cooling of banded fibrils gives thin filaments again. The filaments are loosely associated and entangled. The lower limiting width is 2-4 nm which suggests that they may be five-fold helical microfibrils as proposed by x-ray diffraction and model building studies. Preliminary studies, in collaboration with Dr. B. Whetzel, NCI, using scanning electron microscopy have been started.

2. The mechanism of collagen fibril formation.

A set of optimal conditions for the self-assembly process has been selected and used in an investigation of the mechanism of collagen

assembly. It has been found that collagen assembly occurs via a mechanism of at least three steps. The first step, initiation, involves a temperature-dependent change which leads to an unidentified intermediate that in the second step spontaneously grows. The second step is linear growth of filaments by a temperature-independent process. The third step is lateral association of filaments by a temperature-dependent process. The rates of the second and third steps, and the lack of a measurable critical concentration, are suggestive of a mechanism which is unlike a classical nucleation-polymerization process. Assembly of collagen which cannot crosslink (reduced collagen) occurs by the same mechanism as nonreduced protein, but the rate is slower. This can be explained by the reversibility of the third step (lateral association) which is prevented by crosslinking.

3. Quasielastic light scattering.

Computer programs designed to analyze the data obtained from the light scattering instrument have been written; analysis involves use of a non-linear regression routine which utilizes an on-line computer. The instrument has been calibrated using polystyrene latex particle and testing of the program continues. Preliminary results on collagen in solution have confirmed earlier observations of a slight concentration dependence in the diffusion coefficient of the molecule.

The instrument has been used in collaboration with Drs. R. J. Nossal and S. L. Bremner, (DCRT) to determine the possible uses of quasielastic light scattering in investigations into the mechanical properties of gels. It has been demonstrated that quasielastic light scattering can be used to determine elastic moduli of gels since discrete resonant peaks occur at frequencies that are related to the mechanical rigidity of the gel. Measurements of polyacrylamide gels have shown that the shear modulus can be determined directly. This measurement is of the bulk material, not just the polymer lattice. Preliminary studies have recently been started on fibrin gels, following changes in the gel structure during the course of fibrin polymerization and/or the crosslinking of the fibrin network.

4. Abnormal forms of collagen and the structure of extracellular matrix of fibroblasts in culture.

The Ehlers Danlos Type VII condition in humans and related hereditary diseases in animal models result in the formation of collagen fibrils which contain pN-collagen (incompetely processed collagen containing N-terminal precursor portions) apparently incorporated in the fibrils. In animal models, such fibrils are bizarre and sometimes exhibit no band patterns. In the human condition, the fibrils show lateral packing errors and disorientation at higher orders of structure. However, the

banding pattern and cross-sectional order are normal. These studies are collaborative with Dr. Steinman, LDB&A. Cells from cultured calf ligamentum nuchae produce the microfibrillar component of elastic tissue in culture in a manner similar to that observed in vivo. The ultrastructure and chemical characteristics of this component, not contaminated by amorphous elastin, have been studied in collaboration with Dr. Lamberg, Johns Hopkins University.

Significance

Macromolecules provide the basic properties of connective tissues. Of these, collagen is the major structural protein. These molecules are present as native fibrils, which vary in diameter and in higher levels of organization as a function of species, tissue and developmental stage. Information regarding the mechanism of assembly and interactions with other macromolecules are the basis for continued research on development in tissues and on the relationship of structure to disease.

Proposed Course

1. Increasing emphasis will be placed on high resolution electron microscopy to resolve structural questions related to fibril formation. Of particular interest is the structure of early intermediates and of the thin filaments that we observe. In addition to standard transmission electron microscopy, scanning transmission will be investigated as a tool for these studies.

2. Our studies of the mechanism of the self-assembly of collagen will be continued with primary emphasis on the early stages of assembly. Modified collagens and manipulation of assembly conditions will be used. The effect of other macromolecules on the assembly process will be investigated.

3. Laser light scattering will be used to study the hydrodynamic properties of monomeric collagen in acidic solution. This will be followed by an investigation into the nature of the species present during the early stages of assembly of collagen. It is also anticipated that the laser light scattering instrument will be used in other studies, yet to be planned, since it provides fundamental physical chemical data useful in a wide variety of problems.

4. Our interest in abnormal forms of collagen will continue, largely in collaboration with other investigators when ultrastructural questions arise.

Publications

Brenner, S. L., Gelman, R. A. and Nossal, R.: Laser Light Scattering from Soft Gels. Macromolecules 11 202 (1978).

Williams, B. R., Gelman, R. A., Poppke, D. C. and Piez, K. A.: Collagen Fibril Formation. Optimal in Vitro Conditions and Preliminary Kinetic Results. J. Biol. Chem., in press.

Gelman, R. A., Williams, B. R. and Piez, K. A.: Collagen Fibril Formation. Evidence for a Multistep Process. J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
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NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE-00001-27 LB

PERIOD COVERED

October 1, 1977 to September 1978

TITLE OF PROJECT (80 characters or less)

Specificity and Catalytic Site Studies on Transglutaminases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI :	J. E. Folk	Chief, Enzyme Chemistry Section	NIDR LB
	J. Schrode	Staff Fellow	NIDR LB
Others:	J. J. Gorman	Visiting Fellow	NIDR LB
	G. Seelig	NIH Postdoctoral Fellow	NIDR LB

COOPERATING UNITS (if any)

None

LAB/BRANCH

Biochemistry

SECTION

Enzyme Chemistry

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.50	4.00	1.50

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Active site mapping studies and specificity studies on transglutaminases using synthetic model substrates and macromolecular substrates, respectively, are underway. Special emphasis is being placed on the blood coagulation enzymes (activated plasma and platelet factor XIII) with intent to correlate their specificities to their probable roles in ε(δ-glutamyl)lysine crosslink formation in the physiological processes of wound healing and cell adhesion.

1. Project Description

Objectives

Studies carried out over the past several years have been directed toward characterization of enzymes termed transglutaminases that are responsible for the formation of γ-glutamyl amide bonds in proteins and polypeptides. These enzymes catalyze transfer reactions at the carboxamide group of peptide-bound L-glutamine with a high degree of stereospecificity. In the presence of acceptor amines, the transfer reaction results in the formation of substituted amides ($-CONH_2 + RNH_2 \rightarrow -COHNR + NH_3$).

One of the well-characterized covalent crosslinks between and within protein molecules is the ε(γ-glutamyl)lysine bond. There has been increasing evidence for the presence of this crosslink in fibrin clots, cell membrane, glycerinated myofibrils of muscle, proteins of seminal plasma, native wool keratin, and the citrulline-containing protein fraction of hair. We have recently observed that the transglutaminases can catalyze formation of a hitherto undescribed crosslink. This crosslink between peptide chains is formed by means of a transfer reaction between the carboxamide group of a glutamine residue in each chain and both primary amino groups of a diamine or polyamine. The wide occurrence and obvious importance of γ-glutamyl amide bonds have led us to focus attention on the enzymes responsible for their formation, as well as on the occurrence and functions of these bonds in various proteins.

This project is in part collaborative with S. I. Chung project number Z01 DE-00049-07 LB.

Major Findings

The specificity of activated human blood coagulation factor XIII is being systematically investigated. Since, it was known that mixed casein is an excellent substrate for this enzyme, purified αls -, β-and κ-caseins isolated from the milk of cows homozygous for single variants of these caseins were examined as substrates. β-casein, A variant was found to be by far the best substrate.

β-casein was labeled with a fluorescent amine substrate by use of factor XIIIa and the labeled casein, containing a single derivatized glutamine, was subjected to chemical and enzymic degradation. Peptide mapping and sequence studies identified the glutamine residues as Gln-167. Solid phase synthesis of an 11 member peptide containing the sequence of β-casein surrounding this glutamine

residues was carried out. This peptide was found to be an excellent substrate for factor XIIIa as compared to other synthetic peptides. Products of enzymic digestion of this peptide in which hydrophobic residues were removed from either end of the peptide were found to be poor substrates.

As a continuation of active site mapping studies on transglutaminases, the primary amine binding site of acyl intermediates of both liver transglutaminase and factor XIIIa are under investigation. Most recent findings using a series of heptapeptides of the general structure, X-X-X-L-Lys-X-X-X, where X is glycine or L-leucine and L-Lys is L-lysine, show that the binding of lysine substrates is influenced only by hydrophobic residues contiguous to the lysine residue. When L-leucine was on the amino side of L-lysine, the peptide showed the highest substrate activity. When L-leucine was on the carboxyl side of L-lysine the opposite effect was noted. Thus, it was concluded that the peptide chain itself has little or no effect on the transfer of the acyl portion of the acyl enzyme to amine. If the conclusion that the peptide chain does not bind to the acyl enzyme is correct, then the orientation of the peptide must be determined by the position of the hydrophobic residue adjacent to lysine. That is, the peptide Gly_3-L-Lys-L-Leu-Gly_2 binds to the acyl enzyme surface in the opposite direction (i.e. carboxyl to amino direction) to Gly_2-L-Leu-L-Lys-Gly_3. The fit of Gly_3-L-Lys-L-Leu-Gly_2 to the acyl enzyme surface in this reverse manner would be analogous to the fit of Gly_2-D-Leu-D-Lys-Gly_3 However, Gly_2-D-Leu-D-Lys-Gly_3 would presumably bind less tightly than Gly_2-L-Leu-L-Lys-Gly_3 since the hydrogen atom on the optically active α-carbons of the D-Leu and D-Lys would preclude the close fit of the L-forms where these hydrogens point in the other direction, i.e. away from the acyl enzyme surface. In order to test this theory, a series of peptides of the forms Gly_2-Leu-Lys-Gly_3, Gly_3-Lys-Leu-Gly_2 and Gly_3-Lys-Gly_3 were prepared which contained all combinations of D and L residues (10 peptides). Presuming that the peptide backbone has little or no influence on the binding of substrate peptides, the following pairs of peptides should show equivalent substrate specificity:

Gly_2-L-Leu-L-Lys-Gly_3	vs	Gly_3-D-Lys-D-Leu-Gly_2
Gly_2-L-Leu-D-Lys-Gly_3	vs	Gly_3-L-Lys-D-Leu-Gly_2
Gly_2-D-Leu-L-Lys-Gly_3	vs	Gly_3-D-Lys-L-Leu-Gly_2
Gly_2-D-Leu-D-Lys-Gly_3	vs	Gly_3-L-Lys-L-Leu-Gly_2
Gly_3-L-Lys-Gly_3	vs	Gly_3-D-Lys-Gly_3

This proved not to be the case. The peptides containing L-lysine were better substrates than their D-counterparts. This indicates that the peptides are bound to the acyl enzyme in the same direction as a result of peptide backbone binding and that this specific alignment dicates L-specificity. The subtle differences in the position and stereospecifity of the leucine in the peptides can be rationalized by the strain on the normal binding of the peptide backbone induced by 1) the attempt of leucine to fit into a hydrophobic pocket and 2) the spacial exclusion of this bulky group from the enzyme surface. These conclusions were substantiated by preparation and testing of a number of analogs of lysine peptides in which there are no assymetric carbon atoms. Results with these derivatives also support a notion that a number of other amino acids with hydrophobic side chains can serve in place of leucine to enhance specificity toward L-lysine residues.

It was found that both liver transglutaminase and activated blood coagulation factor XIII are capable of forming crosslinks through diamines and polyamines. This was observed with low molecular weight glutamine substrates, as well as with casein substrate. Methods were developed for identifying this type of crosslink in tissues, fluids and cells.

Significance

Understanding of the molecular characteristics of the transglutaminases is vital to determination of the function of these enzymes in normal and diseased tissues, pharmacological control of activity, and hormonal regulation.

The minimal substrate structural requirements for transglutaminases have been defined over the past several years through studies such as those described above. We now have a better understanding of the mechanism of enzyme-substrate interactions in transglutaminase-catalyzed reactions. The techniques used for these studies, as well as ones used in determining specificity toward glutamine and lysine residues in macromolecular substrates, most certainly will show similarities, as well as differences, in the members of this important group of enzymes.

Both polyamines and transglutaminases are widely distributed and both may play important roles in control of growth and other biological processes. The possibility that polyamines may be natural substrates for transglutaminases, that enzyme-catalyzed incorporation of these amines can occur through either one or both primary amino groups, and that the degree to which crosslinked and non-crosslinked products are formed can be controlled by the level of amine seems solid enough grounds on which to base further investigation.

Proposed Course

Active site mapping studies will be continued with special emphasis placed on the transglutaminases involved in hemostasis and wound healing. Studies on the substrate specificity of factor XIIIa will be continued with the principal objective of determining the importance of amino acid side chains in close vicinity to substrate glutamine and lysine residues. Special attention will be given to fragments of fibrin, the normal substrate for factor XIIIa. Preparation and testing of these fragments as substrates and preparation of other synthetic peptide substrates is underway.

A thorough knowledge of the substrate properties of glutamine and lysine derivatives should furnish the necessary information for design of reagents for localizing and determining the orientation of membrane proteins and other structural proteins.

A survey is underway on tissues, blood, urine, and cultured cells to determine if polyamines exist in nature covalently linked to proteins, and in particular if they serve as portions of crosslinks between or within protein molecules. If, indeed, they do attempts will be made to determine if transglutamines are responsible for formation of the covalent linkages and in what way this relates to growth and development.

2. Publications

Gross, M., Whetzel, N.K. and Folk, J.E.: Amine Binding Sites Acyl Intermediates of Transglutaminases: Human Blood Plasma Enzyme (activated Coagulation Factor XIII) and Guinea Pig Liver Enzyme. J. Biol. Chem. 252: 3752-3759, 1977.

Folk, J.E. and Finlayson, J.S.: The ε(δ-Glutamyl)lysine Crosslink and the Catalytic Role of Transglutaminases. In Anfinsen, C.B., Edsall, J.T. and Richards, F.M. (Eds.): Advances in Protein Chemistry Academic Press, 31: 1-133, 1977.

Abe, T., Chung, S.I., DiAugustine, R.P. and Folk, J.E.: Rabbit Liver Transglutaminase: Physical, Chemical and Catalytic Properties. Biochemistry 16: 5495-5501, 1977.

Schrode, J., and Folk, J.E.: Transglutaminase-catalyzed Crosslinking through Diamines and Polyamines. J. Biol. Chem. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 DE-00049-07 LB

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Physiological Role and Metabolism of Transglutaminases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: S. I. Chung Research Chemist NIDR, LB

COOPERATING UNITS (if any)

Dr. S. S. Alexander, Jr.: NIAMDD Dr. H. Edelhoch: NIAMDD
Dr. J. Finlason : FDA

LAB/BRANCH
Biochemistry

SECTION
Enzyme Chemistry

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.00	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The physiological function and the mode of regulation of the transglutaminases which catalyze the formation of covalent cross-links between protein molecules are being studied. Three distinct classes of these enzymes participate in stabilization of the fibrin clot and in cross-linking of other proteins. The roles of individual transglutaminases in formation of cellular membranes and in the membrane-mediated stimulation of specific cellular function, and the regulation of tissue matrix stabilization following injury by the release and generation of active transglutaminase from the cells involved in wound healing are under investigation. The ameobocytes of Limulus Polyphemus show cellular properties similar to human plateletes in their ability to adhere, to aggregate, and to release cellular transglutaminase for formation of a matrix of protein polymers at the site of injury. Thus this system was chosen to serve as a model to study the complex wound healing process. Since cold-insoluble globulin is known to play a role in cell attachment, this protein is being characterized.

1. Project Description

Objectives

The transglutaminases catalyze formation of covalent ε(γ-glutamyl)-lysine cross-links within and between protein molecules. These bonds are of vital importance in proper blood coagulation, in seminal clot formation and in maintaining the structural integrity in certain hair, wool, and skin proteins. Furthermore, they may play an important role in wound healing and in cell membrane formation. Limulus Polyphemus, one of the most ancient animal species, uses the haemostatic processes as an efficient defense mechanism. That is, picogram quantities of bacterial endotoxins induce the aggregation of blood cells and the release of coagulating components in this crab. A transglutaminase is also released from the blood cells. This enzyme appears to participate in the wound-closure processes.

Cold-insoluble globulin (CIG), a large glycoprotein of blood plasma, is also found associated with the surfaces of a number of types of cells including fibroblasts and chondrocytes. In its cell-associated form it has been termed LETS, CPS or fibronectin and has been shown to be responsible for certain adhesive and morphological properties of cells. Transglutaminases catalyze the covalent polymerization CIG and the attachment of CIG to other protein molecules through ε(γ-glutanyl)lysine cross-links. Such covalent interactions may play an important role in adhesive of proliferating cells to matrices.

Thorough characterization of the various transglutaminases is underway with special emphasis toward determining their physiological functions and modes of regulation. Efforts are directed toward characterizing the protein substrates for these enzymes and defining the manner in which their covalent interactions relate to wound healing and cell adhesion.

Major Findings

In continuation of studies carried out in the previous years, studies on the biosynthesis of protransglutaminases (various forms of factor XIII) were extended to other types of cell systems in which the zymogen and binding globulin are rapidly released into plasma. For this study, guinea pig plasma protransglutaminase was isolated in homogeneous form. This zymogen was shown to be composed of catalytic subunits and binding globulins. Microheterogeneity in molecular weight was observed only in the binding globulin. Utilizing fluorescent antibody techniques, megakaryocytes were identified as the cells where protransglutaminase is synthesized. This occurs prior to the transformation of these cells into blood platelets. Most of the other blast cells in bone marrow on

the other hand, appear to react positively with anti-tissue transglutaminase IgG.

The trace amounts of transglutaminase present in lymphocytes has definitively been identified as tissue transglutaminase. Both cytosol and membrane-bound enzyme, the activity of which is enhanced by Con A or PHA treatment of the intact cells, appears to be identical to the enzyme from liver and other tissues.

Cold-insoluble globulin mammalian species and the primary, secondary and tertiary structure have been examined in an effort to understand the relationship between molecular properties and various cellular functions. Studies of the secondary and tertiary structure of CIG were carried out in collaboration with Drs. S. Alexander and H. Edelhoch of NIAMDD. The various spectroscopic parameters (CD measurement, fluorescent polarization) are unaffected by pH between 7.0 and 9.0. In contrast, there is a small but definitive increase in frictional ratio. Since the spectroscopic properties depend on organizational constraints, i.e., secondary or tertiary structure, and the frictional properties depend on the dimensions of the protein, the change in frictional ratio should represent an expansion of the protein without loss in structure. The stability of CIG has been evaluated by observing the modifications in several structural parameters produced by urea and guanidine solutions. A single fairly broad transition curve is observed with each denaturant. All of the organized structure of CIG appears to be completely eliminated in the transitions since the final parameters are typical random coil polypeptides. However, this was not the case in the thermal denaturation of CIG where elements of structure persisted after the thermal transition. This suggests that the organized domains that are unfolded by urea or guanidine may be of similar size to those of lower molecular weight proteins, i.e. ribonuclease, lysozyme etc. The protein does not have a rod-like rigid structure since the high helical content of other fibrous proteins (tropomyosin, collagen) is not present in CIG. Thus it would suggest that CIG has highly organized domains which are linked by random polypeptide segments affording the molecule considerable flexibility and high frictional ratio. Such a structure may be important to CIG in its function as an adhesive protein.

Tissue repair following injury involves rapid generation of specific enzymes and timed clearance of these enzymes. The generation of transglutaminase activity from its zymogen, factor XIII, is catalyzed by thrombin. However, in the environment of the dynamic circulation, the transglutaminase must be confined to its activation site and it's catalytic activity must be limited to specific substrates. We have examined the affinity of factor XIII and the active enzyme for plasma proteins and for collagen. This work was carried out in collaboration with Dr. J. Finlayson of BOB, FDA. Gel-filtration, sedimentation analysis, and fibrinogen-agarose were used in the binding analyses

with I^{125}-labeled plasma factor XIII (A_2 B_2) platelet zymogen (A_2), and enzyme (A'_2) prepared from each zymogen by thrombin activation. Fibrinogen showed high affinity for A'_2, but weak association with A_2. Fibrin on the other hand binds both A_2 and A'_2 tightly. These results suggest that zymogen reactivation occurs at, and enzyme activity is confined to the site of fibrin formation. A clearance study showed that fibrinogen is the only plasma protein which binds both A'_2 and tissue transglutaminase, and clears both enzymes rapidly from the circulation.

Significance

A comprehensive study of various transglutaminases has shown that the enzymes are widely distributed in various cells (in cytosol and membranes) and suggests that all may be involved in the formation of ε(γ-glutamyl)-lysine cross-links. These linkages are essential in maintaining the permanent rigid structure of many protein molecules. The finding that the activity of a transglutaminase, tightly bound to lymphocyte membrane and human fibroblast, is enhanced by membrane stimulators, (Lectins; Con A or PHA) suggests the additional role of transglutaminase in cell membrane protein interaction.

Cold insoluble globulin (CIG) was shown to contain highly organized structural domains which are linked by random polypeptide segments. This affords the molecule considerably flexibility which may account for its adhesive properties.

The localization of transglutaminase activity during hemostasis probably results from the high affinity of the zymogen and enzyme for the fibrin polymer (substrate). Transglutaminases,released into the circulation are adsorbed by fibrinogen molecules and subsequently cleared from the blood circulation.

Proposed Course

1. Preliminary studies suggest a primary role for transglutaminases in wound-healing. Attention will be focused on the possible interaction between fibrinogen and collagen as catalyzed by transglutaminases and the possible relationships of these interactions to wound-healing.

2. Efforts will be made to determine the mechanism of seminal plasma clot formation, and especially to determine which transglutaminase is responsible for cross-link formation in the coagulable protein.

3. Studies on the biosynthesis, release, and catabolism of platelet factor XIII will be studied in the megakaryocyte of the guinea pig.

4. Efforts will be made to relate the possible involvement of cold insoluble globulin in cross-linking with fibrin, collagen, and other epidermal proteins involved in wound-healing, and also studies on the biosynthesis of CIG, and its interaction with cell membrane and contractile proteins of cell will be investigated.

2. Publications

None

ANNUAL REPORT
LABORATORY OF BIOLOGICAL STRUCTURE
NATIONAL INSTITUTE OF DENTAL RESEARCH

The research program of the Laboratory of Biological Structure is directed toward advancing the scientific knowledge of the structure and function of the hard and soft tissues of the oral cavity. The individual research projects of the Laboratory staff encompass five broad areas concerned with biological structure from the molecular to the tissue level: (1) the structure, composition, formation and maturation of the mineral phase of calcified tissues and of synthetically prepared calcium phosphate salts; (2) the identification, isolation and characterization of the non-collagenous proteins of fetal and adult enamel and dentin matrices and their role in mineralization; (3) the factors regulating endochondral bone differentiation and bone cell-matrix interactions; (4) the structure of cartilage cells, matrix and isolated matrix proteoglycans; and (5) the structure and function of exocrine secretory tissues *in vivo* and *in vitro*. The results of these investigations are essential for a thorough understanding of the factors involved in the maintenance of oral tissue organization and integrity, and their destruction by disease processes.

Because of the diverse and complex nature of these tissues, the Laboratory has developed a broad-based and multi-faceted approach in its investigative methodology. Instruments ranging from nuclear magnetic resonance, infrared and laser Raman spectrometers, x-ray diffractometers, electron and light microscopes are employed to analyze the structural characteristics of molecules, crystals, cells and tissues. Biochemical procedures ranging from routine chemical analyses to complex chromatographic and electrophoretic separation techniques are utilized to identify, quantitate, isolate, purify and characterize the various organic and inorganic components of oral tissues. Cytochemical, radioautographic and immunohistochemical microscopic techniques allow correlation of the chemical and structural properties of the tissues. Finally, the respective advantages of *in vivo*, *in vitro* and synthetic experimental systems are utilized to their full potential in pursuing the goals of the Laboratory's program.

The deposition of mineral in biological systems is a complex process influenced by many variables. One approach to the systematic investigation of the chemical and physical factors involved in biological calcification is the use of synthetic calcium phosphate precipitation systems. Over the last few years thermodynamic, chemical, x-ray diffraction and electron microscopic analyses of these synthetic systems have provided considerable insight into the mineralization process. During the past year, solubility and solution chemistry studies have confirmed that the crystalline phase first formed from the initial amorphous calcium phosphate (ACP) precipitate is octacalcium phosphate (OCP). The lifetime of this OCP crystalline phase is dependent upon the pH of the solution, and it undergoes a hydrolytic transition to form a more basic apatitic phase with a tricalcium phosphate stoichiometry. In addition to the pH, small amounts of various inorganic ions have been found to affect the growth and stability of the various

solid phases. Magnesium, in concentrations as low as 0.3 mM, extends the solution lifetimes of both the ACP and OCP phases, delaying the appearance of the final apatitic phase by a factor of two or more. Previous work had shown that fluoride also affected the initial solid phases formed in spontaneously precipitated systems, stabilizing ACP and, depending upon the fluoride concentration, reducing the lifetime or completely suppressing the formation of OCP. These observations were extended to the precipitates formed by the addition of apatite crystals to supersaturated calcium phosphate solutions. The results, which paralleled the earlier studies, demonstrated that fluoride stabilizes the initial precipitate and reduces the subsequent formation of the first crystals, in favor of direct apatite formation.

The identification of the various calcium phosphate and apatite phases present in teeth and bones and formed in these synthetic systems, is in part dependent upon an accurate knowledge of their molecular structure and crystal architecture. Therefore, an important aspect of the mineralization research program is the structural characterization of standard hydroxy apatite and various substituted apatite preparations by infrared and Raman spectroscopy. Efforts during this past year centered on analysis of synthetic and naturally occurring carbonate-containing apatites. These studies indicated that 5-10% of the total carbonate in enamel replaces hydroxide ions in the crystal lattice, while the remaining 90-95% is believed to occupy vacant phosphate positions. These assignments have been borne out in initial studies of thermally prepared synthetic carbonate apatites. Because of difficulties in obtaining sufficient pure material for complete characterization, a specially designed reaction vessel is being constructed to prepare these synthetic samples, and these investigations will be continued during the coming year.

In a study initiated this year through a contract with the National Bureau of Standards, related physical techniques are being utilized to identify and characterize in situ the first formed enamel mineral. While much of the work has been involved with determining the appropriate preparative techniques, some interesting preliminary results have been obtained. Using the Raman microprobe on 5 um frozen sections of the apical end of the rat incisor, an analysis of the molecular species present in an area as small as 5 μm in diameter is possible. The data suggest that the initial enamel mineral may be low in phosphate and high in carbonate; as mineralization progresses, phosphate accumulates and a typical apatite spectrum is detectable. X-ray microanalysis and ion-microprobe analysis of comparable regions of the incisor are now being undertaken, and a quantitative assessment of magnesium and calcium in this region of enamel will be correlated with the phosphate and carbonate concentrations.

Research on the matrix proteins of fetal bovine enamel and dentin has primarily been concerned with the refinement of the extraction procedures, and compositional and electrophoretic characterization of the proteins. A sequential dissociative extraction procedure employing, in the presence of protease inhibitors, 4M guanidine followed by 4M guanidine-0.5 M EDTA,

is utilized for both enamel surface scrapings and the remaining dentin-rich tooth remnants. The enamel proteins extracted by this method fall into two major classes: (1) 12-13 proteins of the amelogenin (fetal) type, with molecular weights of 11-40,000 Daltons; and (2) three proteins, representing 12-15% of the total, of the enamelin (mature) type, with molecular weights of 49,000, 56,000, and 72,000 Daltons. These findings are significant because the molecular weights of the amelogenins are higher than those previously reported, probably due to the non-degradative conditions employed, and the presence of mature enamelin-like proteins in fetal matrix suggests that they may be conserved during enamel maturation. Extraction of the dentin matrix yielded three major proteins with molecular weights of 53,000, 65,000 and 96,000 Daltons. The 96,000 molecular weight protein was similar to dentin phosphoproteins isolated by other methods, with aspartic acid and serine/phosphoserine accounting for 60% of the amino acid residues, but contained higher levels of other amino acids, suggesting that it may actually occur as a larger non-degraded molecule than previously realized. Additional purification and characterization of these matrix proteins will be continued during the coming year, with the ultimate goal of defining their biogenesis and role in tooth mineralization.

Biochemical analysis of the factors involved in the growth and differentiation of cartilage and bone is essential to an understanding of normal skeletal development and structure as well as the numerous pathologic conditions affecting these tissues. The use of an experimental *in vivo* model of matrix-induced endochondral bone formation has allowed biochemical dissection of the various stages of cartilage and bone development. An important finding was that the level of the enzyme ornithine decarboxylase (ODC) is correlated with the proliferation of mesenchymal cells three days after implantation of the demineralized bone matrix particles, and with the proliferation of osteoprogenitor and endothelial cells on day eight. Thus, along with $^{35}SO_4$ and ^{45}Ca incorporation and alkaline phosphatase activity, ODC activity serves as a useful biochemical marker of the progress of differentiation in the developing bone plaques. Using these markers, this system has been utilized to determine the effect of corticosteroids, adrenalectomy, ingested fluoride and magnesium on the differentiation and mineralization of cartilage and bone.

The well-defined sequence of developmental changes in these induced plaques is also amenable to morphological studies, which can yield significant correlations between structural and chemical features. The sequential appearance and disappearance of the four different types of collagen in the developing plaques were studied by immunofluorescence techniques. The results correlated well with the known distribution of the various collagen types in other tissues. The ability of the demineralized bone matrix particles to induce cartilage formation *in vitro* is also being explored. Preliminary results indicate that rat periosteal cells will differentiate into chondroblasts when cultured with the particles. This *in vitro* system may prove advantageous for studies of cell surface-extracellular matrix interactions.

Additional studies of cartilage cells and matrix components were carried out using a transplantable rat tumor which produces well differentiated cartilage. This rat chondrosarcoma has proved to be particularly useful, as cells in vivo and in vitro appear similar to normal cartilage cells and synthesize an extracellular matrix of similar composition but lower density than that of hyaline cartilage. The decreased matrix density has been taken advantage of in a study comparing the structure of proteoglycans extracted from the matrix by different procedures. Measurements of monomer length yielded an average of 249 nm, and the separation between adjacent monomers on the hyaluronic acid backbone varied from 23-59 nm. Importantly, no differences were noted between proteoglycan aggregates isolated by associative procedures and those isolated by dissociative-reassociative procedures, nor between proteoglycans from the tumor and other cartilages. This tumor system will be utilized for electron microscopic radioautographic and immunocytochemical studies of proteoglycan synthesis, assembly and structure.

Secretory processes are common to almost all cells, thus the study of transport, packaging, storage and secretion of exportable proteins in exocrine cells yields information applicable to many cellular systems, including those producing extracellular matrices such as tooth, bone and cartilage. While progress has been made in all areas under investigation, the most significant findings relate to the following areas: (a) quantitative analysis of electron microscope radioautographs after injection of the glycoprotein precursor ^{3}H-fucose has confirmed the synthetic capabilities of the salivary gland striated duct cells. The results of these studies suggest that these cells actively synthesize glycoproteins for renewal of their extensive cell membrane, as well as glycoproteins for secretion into the saliva. Thus, in addition to modifying the electrolyte composition of primary saliva, the striated ducts appear capable of altering the organic content as well; (b) conditions for dissociation and short term culture of differentiated acinar secretory cells of the rat pancreas, parotid and exorbital lacrimal glands have been established, and these cells are now being used for a variety of experiments on certain basic aspects of the secretory process. The endocytosis and fate of the plasma membrane after exocytosis of secretory granules are being followed, using electron dense tracers and by radioautography after labeling of the plasma membrane with ^{125}I. Future investigations will include studies on the involvement of microtubules and microfilaments in various aspects of the secretory process, and the localization and distribution of adrenergic and cholinergic receptors on the plasma membrane; (c) in a collaborative study, the structural and cytochemical properties of the Golgi apparatus and GERL were examined in neurosecretory neurons of the supraoptic nucleus. Under normal conditions, the cytochemical properties of these cells parallel those previously established for a number of exocrine cells, and the neurosecretory granules are formed from GERL. However, under conditions simulating dehydration in which these neurons increase their production of vasopressin, there is an alteration in the localization of acid phosphatase and thiamine pyrophosphatase activities, morphological changes occur in

GERL and the Golgi apparatus, and the neuroaecretory granules form directly from the Golgi saccules. Comparable studies of other secretory cells will be carried out to determine if similar structural and functional alterations in these organelles can be induced by physiological or pharmacological stimulation.

In addition to the progress made on the individual research projects, as reviewed above, the past year has also witnessed a number of significant events for the Laboratory as a whole. In March of this year, the Third International Symposium on Tooth Enamel was held in Washington, D.C. Drs. John D. Termine and Marie Nylen served as organizers of the Symposium, and their efforts contributed greatly to the success of the meeting. Additionally, the efforts of the secretarial staff of the Laboratory, particularly Mrs. Patricia Youmans, played an essential role in the organization and smooth running of the conference. Several members of the Laboratory were honored this past year for their contributions to the NIH research effort and for significant advances in their own research field. Dr. E.D. Eanes was elected as a Fellow of the American Association for the Advancement of Science, and also received the NIH Director's Award for his studies of calcium phosphate chemistry and mineralization processes. Mrs. Betty Ho was awarded the NIH Merit Award for her contribution to the Laboratory's research program. Dr. Arthur R. Hand received the Basic Research in Oral Science Award of the International Association for Dental Research for studies of salivary gland structure and function.

Two significant personnel changes also occurred during this last fiscal year. Mrs. Elaine Lenk joined the Laboratory as a NIH Visiting Associate; she will collaborate actively with the rest of the Laboratory members, as well as with investigators from other NIDR laboratories, providing expertise in all aspects of electron microscopy. It is anticipated that this arrangement will significantly benefit the Laboratory's research efforts, and will foster closer cooperation and increased collaboration with the other NIDR laboratories. Secondly, a premanent successor to the position of Laboratory Chief was appointed in June of this year. Dr. Arthur R. Hand, who succeeded Drs. E.D. Eanes and J.D. Termine as Acting Chief, and served in that capacity for 11 months, was selected as the new Chief.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00026-11 LBS

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Ultrastructure and Cytochemistry of Secretory Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Ho, Betty	Biologist	LBS NIDR
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Laboratory of Biological Structure

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.75	1.41	2.34

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Basic mechanisms of the secretory process are studied in cells of the rat pancreas, salivary and lacrimal glands. Techniques utilized include light and electron microscopy, cytochemistry, radioautography, and biochemical determination of enzyme and cyclic nucleotide levels. Major areas of investigation are: (1) the role of the Golgi apparatus and GERL in secretory granule formation; (2) the structure and function of GERL and cyclic nucleotide levels in secretory cells of the beige mouse, a homologue of the human Chediak-Higashi syndrome; (3) the role of lysosomes in the intracellular degradation of secretory material; (4) the fate of endocytic vesicles formed during the removal of membrane from the luminal plasmalemma in vivo; (5) the route of access of extracellular material to the saliva; and (6) the structure, cytochemistry and distribution of peroxisomes.

NIDR CLASSIFICATION: 34200
34300

1. Project Description

Objectives:

The basic objective of this project is to obtain further knowledge of the structure and function of secretory cells and their organelle systems. Utilizing electron microscopic, cytochemical, radioautographic and biochemical techniques, cell ultrastructure is correlated with enzyme localization, quantification of cellular constituents, and glycoprotein synthesis and transport. Our efforts have concentrated on: (1) the role of the Golgi apparatus and GERL in the transport and packaging of secretory material; (2) the function of lysosomes in the degradation of secretory material and cellular membranes; and (3) the cytochemistry and functions of peroxisomes.

Methods Employed:

Tissues for morphological examination are fixed by vascular perfusion and prepared by standard techniques. Cytochemical incubations are carried out on 50-75 μm slices of fixed tissue. Radioautographs of tissue labeled with tritiated glycoprotein precursors are prepared by the method of Kopriwa (1973). The procedure of Nadler (1971) is utilized for grain counting and statistical analysis of EM radioautographs. Biochemical determinations of protein, DNA, amylase, peroxidase, and other enzyme activities follow standard procedures. Cyclic nucleotide levels are determined by radio-immunoassay.

Major Findings:

During the past year, our studies of transport and packaging in secretory cells have been concentrated in two main areas: the synthesis of glycoproteins by striated duct cells of rat salivary glands and islet cells of the rat pancreas, as shown by radioautography after injection of ^{3}H-fucose; and the effects of secretory stimulation on the structure and cytochemistry of the Golgi apparatus and GERL in lacrimal and parotid acinar cells and neurosecretory cells.

As reported last year, ^{3}H-fucose is readily incorporated by salivary gland striated duct cells. EM radioautographs demonstrated that between 5 and 20 minutes after intravenous injection, approximately 50% of the radioautographic grains were associated with the Golgi apparatus. By 40 minutes this value had fallen to 20%, and by two hours after injection the Golgi apparatus accounted for only 10% of the grains. In contrast, the percentage of grains associated with small (0.1-0.3 μm) apical granules and the plasmalemma increased from less than 5% each at 10 minutes to 25-30% each at two hours after injection. Grains over the apical granules were still numerous at four hours, but by 12 hours they were greatly reduced in number. The

plasmalemma was still reactive 30 hours after injection. These results suggest that the striated duct cells incorporate fucose into glycoproteins within the Golgi apparatus; some of these are secretory glycoproteins and are packaged and stored in the apical granules, while others are membrane glycoproteins which are incorporated into the extensive plasmalemma of these cells. The nature of the secretory glycoprotein is unknown, but it may be kallikrein, since this enzyme has been detected in striated duct cells by immunofluorescence, and kallikrein from some sources is known to contain fucose.

Examination of light microscope radioautographs of the rat pancreas indicated that certain cells in the islets of Langerhans readily incorporated ^{3}H-fucose. These heavily labeled cells had a distribution similar to that expected for the insulin-producing β-cells. As with striated duct cells, the labeling could indicate a membrane glycoprotein, as suggested by Bennett et al. (1974), or a secretory glycoprotein. In the case of a membrane glycoprotein, similar incorporation of isotope by all of the islet cells might be expected; on the other hand, no fucose-containing secretory glycoprotein is known to be produced by β-cells. Analysis of the EM radioautographs should indicate the source of the reaction and whether additional studies to determine the nature of the labeled material are warranted.

Studies of stimulated secretory cells were initiated with a biochemical analysis of the in vivo response of the rat exorbital lacrimal gland to pilocarpine, a cholinergic agonist. Measurements of cyclic nucleotide levels suggest an increase of cyclic AMP between one and five minutes, and an increase of cyclic GMP at about five minutes after injection; both cyclic nucleotides fell to control levels by 10 minutes. However, the standard errors for these time points were large, and these experiments are currently being repeated. The secretory response will be assessed by the discharge of the secretory enzyme peroxidase, and appropriate time points will be selected for morphological and cytochemical studies.

Neurons of the supraoptic nucleus elaborate the peptide hormones oxytocin and vasopressin. Preliminary observations suggested that these hormones may be packaged into neurosecretory granules in much the same way that exocrine secretory proteins are packaged by cells of the salivary and lacrimal glands. The production of vasopressin can be experimentally increased by adding 5% sodium chloride to the drinking water to simulate dehydration. Light and electron microscopic cytochemistry showed that the neurosecretory granules are indeed formed from GERL. During dehydration, the Golgi apparatus increases in size, while GERL decreases; under these conditions the neurosecretory granules appear to form directly from the Golgi

saccules. Additionally, there is a significant increase in the number of lysosomes present in the cells. When the animals are returned to distilled water the cells return to normal and the neurosecretory granules are again formed from GERL.

Previous studies by Essner and Oliver (1974) demonstrated that GERL is markedly enlarged in hepatocytes of the beige mouse, a homologue of the human Chediak-Higashi syndrome. Structural and cytochemical studies of parotid and lacrimal acinar cells in these mice indicated that GERL is morphologically normal. However, upon stimulation of the parotid by isoproterenol, exocytosis of secretory granules occurred much more rapidly in beige mice than in control animals. Since isoproterenol increases cyclic AMP levels, and previous studies by Janet Oliver et al. (1976) suggested that beige mice have lowered cyclic GMP levels, cyclic nucleotide levels were measured in 10 tissues from beige and C_{57} black mice. The only beige tissues showing significant differences from controls were the exorbital lacrimal gland, which had lower levels of both cyclic GMP and cyclic AMP; the parotid gland, which had a lower cyclic GMP level; and the duodenum, which had an increased level of cyclic GMP. Therefore, although some differences in cyclic nucleotide levels exist between various tissues, this probably does not represent the underlying disorder in the beige mouse. The anomalous response of the beige parotid gland to isoproterenol will be further studied by electron microscopy and assay of stimulated cyclic nucleotide levels.

Our previous study (1972) of the effects of starvation on the parotid gland, and the study of Johnson and Sreebny (1977) on starvation and liquid diet, suggested that lysosomes may play a role in the degradation of intracellular secretory material and glandular atrophy observed in these conditions. Our own studies of the effects of a liquid diet on the rat parotid were begun last year and continued through this year. One day after placing the animals on a liquid diet the acinar cells were filled with secretory granules, and the cellular content of amylase had increased by almost 70%. At two and three days there was a gradual reduction in the number of granules and amylase activity rapidly fell to about 50% of control (solid chow) values. An apparent increase in the number of cytochemically detectable lysosomes occurred by three days; these appeared to be mainly the residual body, secondary lysosome type. Large numbers of autophagic vacuoles and degenerating secretory granules, characteristic of starved animals, were not observed. Animals maintained on the liquid diet for up to three weeks had relatively normal appearing acinar cells, except they were smaller in size and contained considerably fewer granules than controls. Amylase levels declined to about 20% of controls by day seven and remained relatively stable for the remainder of the experiment. These results suggest that the reduction in secretory enzyme levels caused by a liquid diet may occur by a different mechanism

than during starvation. However, the increased numbers of lysosomes suggest that they may play some role in acinar cell atrophy. Lysosomal enzyme levels will be determined to correlate with the cytochemical observations. Additionally, the gland content of peroxidase, another secretory enzyme, will be assayed to determine if it parallels that of amylase.

Our studies of peroxisomes have concentrated on the duct cells of mouse salivary glands in order to confirm our previous observations. As reported earlier (1976), most of the duct cells contain large numbers of peroxisomes which are somewhat larger in size than peroxisomes in most tissues. They stain intensely for catalase activity, and appear to be "leaky" as a gradient of reaction product is frequently seen around the peroxisomes. The cytoplasm and nuclei of these cells also contain reaction product, but it is not clear whether this is due to leakage from the peroxisomes or represents a true cytoplasmic catalase. A few cells without cytoplasmic reaction product and smaller peroxisomes are also present. In the C_{57} black mouse, these "unstained" cells frequently contain crystalloids which are strongly reactive for catalase activity. The crystalloids are occasionally seen as rod-like extensions of peroxisomes, and several may be present in a single cell. These crystalloids have also been described by Hanker et al. (1977), and given the name "phi body". Their mode of formation and significance are unknown, but may relate to some metabolic difference between the C_{57} and other mouse strains. The peroxisomes in the ducts of the C_{57} are also much larger (up to 1 μm) than those in the C_3H, A/J and Swiss-Webster mice.

Significance to Dental Research:

These studies are expected to provide a better understanding of the structure and function of secretory cells. Secretory cells of the major and minor salivary glands provide the fluid environment of the oral cavity, and physiological or pathological changes in their function will greatly affect this environment.

Proposed Course:

During the coming year our studies of the Golgi apparatus and GERL will be focused in four main areas: (1) continuing the radioautographic assessment of glycoprotein synthesis and transport in salivary and lacrimal acinar cells and pancreatic islet cells; (2) structural and cytochemical studies of alterations in the Golgi apparatus and GERL during secretory stimulation and resynthesis of granules; (3) correlation of morphological changes with cyclic nucleotide levels during the accelerated secretory response of the beige mouse parotid gland; and (4) characterization of Golgi-GERL relationships in parotid acinar cells of young rats.

It is anticipated that the following new investigations will be initiated: (1) freeze-fracture studies of lacrimal and parotid acinar cells to characterize membrane specializations related to (a) secretory granule discharge and luminal membrane endocytosis, (b) changes in intercellular junction structure during stimulated secretion, (c) differences between the membranes of the endoplasmic reticulum, Golgi saccules and GERL, and (d) sites of close contact of nerves with the acinar cell plasmalemma; and (2) ultrastructural assessment of the effects of sialographic procedures on the rat submandibular gland.

The following studies are essentially complete and will not be continued: (1) cytochemistry of the Golgi apparatus and GERL in supraoptic neurons; (2) peroxisomes in salivary gland duct cells; and (3) the effects of liquid diet on the rat parotid gland.

2. Publications:

Hand, A.R., and Oliver, C.: Relationship between the Golgi apparatus, GERL, and secretory granules in acinar cells of the rat exorbital lacrimal gland. J. Cell Biol. 74: 399-413, 1977.

Hand, A.R., and Oliver, C.: Cytochemical studies of GERL and its role in secretory granule formation in exocrine cells. Histochem. J. 9: 375-392, 1977.

Hand, A.R., and Oliver, C.: Cytochemical studies of GERL and its role in secretory granule formation in exocrine cells. In Garrett, J.R., Harrison, J.D., and Stoward, P.J. (eds.): Histochemistry of Secretory Processes. London, Chapman and Hall, 1977, pp. 29-46.

Doty, S.B., Smith, C.E., Hand, A.R., and Oliver, C.: Inorganic trimetaphosphatase as a histochemical marker for lysosomes in light and electron microscopy. J. Histochem, Cytochem. 25: 1381-1384, 1977.

Oliver, C., and Hand, A.R.: Uptake and fate of luminally administered horseradish peroxidase in resting and isoproterenol stimulated rat parotid acinar cells. J. Cell Biol. 76: 207-220, 1978.

Hamosh, M., and Hand, A.R.: Development of secretory activity in serous cells of the rat tongue. Cytological differentiation and accumulation of lingual lipase. Dev. Biol. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00163-03 LBS

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Ultrastructure and Biosynthesis of Cartilage Proteoglycans

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hascall, Gretchen K.	Senior Staff Fellow	LBS NIDR
OTHER:	Waters, Judith F.	Biologist	LBS NIDR

COOPERATING UNITS (if any)

Dr. Robert Cranley, St. Agnes Hospital, Baltimore, Maryland

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Experimental Morphology Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.88	0.63	0.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project uses electron microscopy, histochemistry, and autoradiography to study the biosynthesis of cartilage proteoglycans and their relationship to collagen in cartilage matrix. Current studies include (1) electron microscopy of cartilage from mutant mice; (2) autoradiography of $^{35}SO_4$ incorporation into bone proteoglycans; (3) electron microscopy of cultured rat chondrosarcoma cells; and (4) measurements of purified proteoglycan molecules from rat chondrosarcoma.

NIDR CLASSIFICATION: 20323

1. Project Description:

Cartilage is a tissue in which cells, chondrocytes, synthesize and secrete proteoglycans and collagen to form an extensive extracellular matrix. The objectives of this project are: (1) to define the precise structure and localization of proteoglycans in the extracellular matrix; and (2) to determine how the chondrocyte synthesizes, packages, and secretes the proteoglycan and collagen for the matrix. Two experimental systems are utilized in this study: cultured rat chondrosarcoma cells, and mutant mice with cartilage abnormalities.

The Swarm rat chondrosarcoma forms a cartilage useful for model system research because the matrix is less dense than hyaline cartilage and allows better penetration of experimental reagents. In addition, these cells can be cultured so that they retain a chondrocyte appearance and rapidly synthesize new extracellular matrix (Dr. Vincent Hascall's laboratory, LB, NIDR, Z01 DE 00134-03). For these reasons I have chosen this system for extensive morphological studies on proteoglycan structure and biosynthesis.

Electron microscopy of the cells in the tumor, the cell pellet during isolation preparatory to culturing, and cultures after 18 hours show that all the cells are similar in structure to cartilage chondrocytes. The cytoplasm has large amounts of rough surfaced endoplasmic reticulum (RER) with distended cisternae filled with granular material, and an extensive Golgi apparatus, dispersed within the cytoplasm, associated with numerous condensing vacuoles and small smooth and coated vesicles. When the cells are fixed in the presence of ruthenium red, a strongly colored cationic molecule which binds to the anionic proteoglycans, stained granules similar to the extracellular proteoglycan matrix granules can be seen within the Golgi saccules and condensing vacuoles. The extracellular matrix of the tumor resembles cartilage matrix, with matrix granules and collagen fibrils. During isolation of the cells the matrix is completely removed, but after 18 hours in culture the cells have formed a new matrix which resembles the tumor matrix except that the newly formed collagen fibrils lie close to the cell surface.

The rat chondrosarcoma cultures rapidly take up $^{35}SO_4$ from the medium. Preliminary light microscope autoradiography shows that after a 5 minute pulse label is localized in a juxtanuclear position (presumably the Golgi apparatus), after a 5 minute chase the label is scattered in the cell and beginning to appear in the extracellular matrix, and after a 50 minute chase most of the label has disappeared from the cells and matrix. This confirms the biochemical results from Vincent Hascall's laboratory, which found that the cells rapidly synthesize and secrete proteoglycans, some of which remain in the matrix while

the rest are lost to the medium. I plan to do high resolution EM autoradiography with this system to try to determine which portions of the Golgi apparatus are involved in sulfate incorporation.

The current model of the proteoglycan molecule is a "bottlebrush" structure with many "filaments" of proteoglycan monomers attached to the central thread of hyaluronic acid to form an aggregate. Each "filament" is itself a small "bottlebrush" of chondroitin sulfate chains attached to a protein core. Previous studies on cartilage proteoglycans required the use of 4.0 M guanidine hydrochloride to dissociate the proteoglycan aggregates into monomers before they could be extracted from the tissue. The monomers were then dialyzed into 0.5 M guanidine to allow reaggregation. The assumption in all this was that the molecules reaggregated in vitro into the same form in which they existed in vivo. The tumor provided a way to test this assumption. Since the tumor matrix is less dense than cartilage matrix, tumor proteoglycans can be extracted directly in 0.5 M guanidine hydrochloride as intact aggregates. The purified aggregate molecules (obtained from Vincent Hascall) were spread by the Kleinschmidt technique for viewing in the electron microscope. I observed that these aggregates have precisely the same type of organization as aggregates formed in vitro. Measurements of 262 monomer portions of aggregates showed an average monomer length of 249 nm. The distance between monomers on the hyaluronic acid ranged from 23 to 59 nm; different aggregates had different spacings and, surprisingly, different areas of single long aggregates sometimes had different spacings. These molecules were the same size as proteoglycans from other cartilages, confirming the aggregate model of proteoglycan structure and the value of this tumor system for proteoglycan studies. In addition, comparison of the large size of the spread aggregates (averaging 500 x 2000 nm) with the size of Golgi vesicles (spheres about 300 nm in diameter) containing proteoglycan in tissue sections strongly suggests that aggregation occurs extracellularly. This confirms biochemical studies on aggregation by James Kimura in Vincent Hascall's laboratory.

Several laboratories are developing antibodies to different parts (the core, hook, and link areas) of the protein in the proteoglycan. I plan to label these antibodies with ferritin for immunocytochemical studies to see if different areas of the rough endoplasmic reticulum are responsible for synthesis of these components, and if different classes of Golgi vesicles are used to package the core-hook and link areas prior to their aggregation outside the cell. These antibodies, applied to the cell matrix, may also explain how matrix granules and interconnecting filaments, which represent proteoglycans condensed after conventional fixation and embedding techniques, are related to the aggregate structure.

Certain strains of mice have genetic abnormalities which result in shortened limbs. A study of the epiphyseal cartilage of these mice aimed at correlating morphological and biochemical abnormalities should provide information on how matrix structure affects function. The mouse "DMM" (disproportionate micromelia) has been studied as a team project with Dr. Kenneth Brown, LDBA, NIDR, studying the genetics (Z01 DE 00201 01), Dr. Hynda Kleinman, LDBA, NIDR, doing the collagen biochemistry (Z01 DE 00202 01), Dr. Jack Pennypacker, LDBA, NIDR, doing the proteoglycan biochemistry (Z01 DE 00203 01), Dr. Robert Cranley (pathologist, St. Agnes Hospital, Baltimore, Maryland) providing the light microscope histochemistry, and my own work on the electron microscope morphology. Our results show that the DMM epiphyseal cartilage has a columnar zone with very disorganized cells, and a greatly reduced proteoglycan component. However, by electron microscopy the extracellular matrix is almost normal in its organization with a moderately reduced number of matrix granules.

In a collaborative study with Dr. A.H. Reddi (Z01 DE 00204-01), autoradiography of $^{35}SO_4$ was used to follow proteoglycan synthesis in a matrix induced endochondral bone formation system. Subcutaneous implantation of powdered, demineralized bone matrix into rats results in formation of a nodule which shows a normal sequence of bone formation. At day 7 the nodule has cartilage, at day 11 ossification begins and at day 14 there is extensive bone formation. Autoradiography showed that on day 14, when bone formation is extensive, $^{35}SO_4$ was incorporated by osteoblasts and not by residual chondrocytes. These results, which indicate that osteoblasts are also capable of proteoglycan synthesis, are consistent with the observation that cartilage proteoglycans synthesized on day 7 are structurally and chemically distinct from bone proteoglycans synthesized on day 14.

Proteoglycans probably play significant roles in controlling collagen fibril formation, mineral ion transport, and cartilage mineralization. These current studies are considered significant steps in a broader program aimed at elucidating mechanisms of matrix formation, composition and mineralization.

2. Publications:

Reddi, A.H., Hascall, V.C., and Hascall, G.K.: Changes in Proteoglycan Types During Matrix-induced Cartilage and Bone Development. J. Biol. Chem. 253: 2429-2436, 1978.

Faltz, L.L., Reddi, A.H., Hascall, G.K., Martin, D.M., Pita, J., and Hascall, V.C.,: Characteristics of Proteoglycans Extracted from the Swarm Rat Chondrosarcoma with Associative Solvents. J. Biol. Chem. (in press).

PROJECT NUMBER (Do NOT use this space)

HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00178-03 LBS

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

A Study of the Early Mineralization Process in Rat Teeth

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Doty, Stephen B.	Research Biologist	LBS NIDR
OTHERS:	Worrell, Dorrette F.	Biologist	LBS NIDR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Experimental Morphology Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.81	0.27	0.54

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this study is to increase our understanding of the process of mineralization as it occurs during odontogenesis. Techniques of electron micro-microscopy and histochemistry are used to observe the mineralization process at the cellular level in incisors from mature rats and in molars from newborn rats grown in tissue culture for up to 3 weeks.

NIDR CLASSIFICATION: 10210

1. Project Description

Objectives:

The objective of this study is to increase our understanding of the process of mineralization as it occurs during odontogenesis and amelogenesis. This study combines techniques of electron microscopy, histochemistry and tissue culture to observe the mineralization process at the cellular level.

Methods:

Incisors from male Sprague-Dawley rats, weighing 100-250 grams, were prepared for electron microscopy and histochemistry by perfusion of the whole animal with fixative. Perfusion was via cardiac puncture and the use of 300 ml of 2.5% glutaraldehyde in 0.01 M cacodylate buffered perfusate over a 15-20 minute period. The incisors were removed and prepared for routine electron microscopy. For histochemistry, the incisors were demineralized, sectioned at 50 µm, incubated in various histochemical media, and also prepared for electron microscopy.

For tissue culture, first and second molars from newborn rats, 0-5 days old, were removed under sterile conditions using a dissecting microscope and fine jewelers forceps. The molars were removed into BGJb medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), fungizone (0.25 µg/ml) and vitamin C (0.09 mM), dissected free of surrounding tissue and cultured in supplemented BGJb medium with 20% horse serum at 37°C in a humidified atmosphere of 5% CO_2 in air. Tooth buds were cultured with and without 10% chick embryo extract in the medium. Tooth buds were grown either on Millipore filters in tissue culture cluster dishes or on Nucleopore filters in organ culture dishes. Molars in organ culture dishes were overlaid with 1% agar. Cultures were harvested at 1,2,5,7,14 and 21 days and processed for electron microscopy and histochemistry.

Major Findings:

The morphology of the mature rat incisor has been described by numerous authors at the light and electron microscope level and did not differ from that observed in this study.

Tooth buds from neonatal rats grown with 10% chick embryo extract in tissue culture cluster dishes appeared to remain quiescent for 4-6 days following their placement in culture. During this period, the tooth buds became flattened and covered with an extracellular matrix. After about 7 days of culture the tissue began to be more active as indicated by a larger zone of pre-dentin and the appearance of some

mineralization. Dentin formation continued, and enamel deposition occurred irregularly after 14 days in culture. The deposition of mineral in the dentin and enamel matrices appeared similar to that seen in vivo. Cultures of tooth buds from newborn rats (0-5 days) grown in organ culture dishes and over-laid with agar did not flatten, but retained their initial rounded shape. Cusp formation occurred in these molars (from 1-3 day old animals) after 5-7 days in culture.

Histochemical studies of the incisor revealed that alkaline phosphatase and alkaline inorganic pyrophosphatase activities were localized to the odontoblast membrane and cell surface. These activities were not found along the cell membranes of the ameloblast population during amelogenesis. Alkaline phosphatase was also found in the stratum intermedium but, to date, inorganic pyrophosphatase activity has not been seen at that site.

In the tooth buds, the appearance of alkaline phosphatase activity preceeded any mineral deposition. The earliest recognizable odontoblasts exhibited alkaline phosphatase activity even during the quiescent stage at the beginning of the culture period. The histochemical study of Golgi and lysosomal activity has not yet been completed.

Significance to Dental Research:

An understanding of the mechanisms involved in odontogenesis is fundamental to providing the best treatment for dental disease or correction of dental abnormalities. By studying odontogenesis at the cellular level and by comparing in vivo results to in vitro growth permutations, a clearer appreciation of the factors which control dental tissue growth and maintenance will be obtained.

Proposed Course:

The studies of the mature rat incisor were terminated after the PI left the laboratory in December, 1977. Culture of the rat molars will be continued by Mrs. D. Worrell.

Initial electron microscopic observations of the cultured tooth buds revealed inadequate preservation of fine structure. Therefore, various fixatives and buffers will be examined for their ability to preserve cellular detail in this system. Growth of tooth buds in vitro presents an opportunity to assess the effects of various experimental conditions or chemicals on tooth differentiation and structure. Initial experiments will examine the effects of anti-microtubular drugs on the secretion of dentin matrix.

Tooth buds from newborn animals (0-5 days) will be treated after various times in culture with colchicine, lumicolchicine and vinblastine. The effect of these drugs on cellular structure and dentin deposition will be assessed by light and electron microscopic cytochemistry.

2. Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00199-02 LBS

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

In Vitro Studies of Exocrine Gland Structure and Function.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Oliver, Constance	Senior Staff Fellow	LBS NIDR
OTHER:	Hand, Arthur R.	Chief, Laboratory of Bio. Str.	LBS NIDR
	Auth, Regina E.	Microbiologist	LBS NIDR
	Waters, Judith F.	Biologist	LBS NIDR
	Worrell, Dorrette F.	Biologist	LBS NIDR
	Washington, Michael	Student Trainee	LBS NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Experimental Morphology Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.64	0.80	1.84

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cell dissociation and short term culture (up to 1 month) methods have been established for rat exorbital lacrimal, parotid and pancreatic acinar cells. These cultures are being used to study various aspects of the secretory process. Initial emphasis is being placed on morphological, cytochemical and biochemical characterization of the cultured cells. Additionally, uptake of horseradish peroxidase and internalization of ^{125}I labeled cell membrane are being investigated in preparations of isolated pancreatic acinar cells.

NIDR CLASSIFICATION: 34300

1. Project Description

The primary objectives of this investigation are to establish procedures for short term culture of isolated exocrine gland acinar cells and to utilize these cultures in investigations of events involved in the secretory process.

Methods Employed:

Glands removed from 100 g rats were placed in oxygenated Ham's Nutrient Mixture F-12. Surrounding connective tissue, lymph nodes and fat were dissected free and the glands minced. The minced tissue was transferred to Spinner flasks and rinsed twice in calcium-magnesium free Hank's balanced salt solution. The tissue was then treated sequentially with 2mM EDTA and an enzyme solution containing collagenase and hyaluronidase. All procedures were done in an atmosphere of 95% O_2 - 5% CO_2. After dissociation the cell suspension was filtered through Nytex filters, and acinar cells separated by centrifugation through a ficoll gradient. Cells were cultured in Petri dishes in Ham's Nutrient Mixture F-12 containing penicillin (100 U/ml), streptomycin (100 µgm/ml), 20% heat inactivated rat serum and the appropriate secretogogue. Protein synthesis was assessed by determining the amount of ^{3}H-leucine incorporated into TCA precipitable protein.

For studies of membrane reutilization, isolated pancreatic acini were prepared by dissociating minced glands for one hour in a collagenase-hyaluronidase solution. Acini were then passed through Nytex filters and separated on a ficoll gradient. Suspensions of acini were iodinated enzymatically utilizing lactoperoxidase with glucose-glucose oxidase as an hydrogen peroxide generating system. Internalization and fate of ^{125}I labeled membrane was followed by light and electron microscope radioautography. Other acini were cultured in medium containing 1% horseradish peroxidase as a soluble phase marker. Protein synthesis was measured by ^{3}H-leucine incorporation.

Major Findings:

Initial emphasis has been placed on determining optimal conditions for dissociation and culture of acinar cells. The rat exorbital lacrimal gland has been used as a model for establishing dissociation and culture techniques because it is a pure serous gland, contains a high proportion of acinar cells to other cell types and has a readily demonstrable secretory peroxidase. Dissociation of rat exorbital lacrimal glands by alternate incubations in EDTA and collagenase-hyaluronidase solutions yielded a cell suspension which was approximately 90% acinar cells and had a viability of about 80% as determined by trypan blue exclusion. After isolation the single acinar cells rapidly reaggregated to form small acini of 5 - 20 cells. The cells retained their

original polarity upon reaggregation. When these isolated cells were cultured, they maintained good morphology for approximately 24 hours. After 48 hours, the apical portions of the cells became filled with secretory granules; lipid and lysosomes accumulated in the basal cytoplasm. Addition of 10^{-6} M carbamyl choline to the medium prevented these changes and allowed maintenance of the cells for up to 1 month. Initial measurements of protein synthesis showed that after 10 days in culture, the cells incorporated ^{3}H-leucine at the same rate as freshly isolated cells. Additionally, cells cultured for up to one week still retained cytochemically demonstrable peroxidase activity, and responded to stimulation with carbamyl choline. These same procedures proved successful for cultures of rat parotid and pancreatic acinar cells. Addition of 10^{-6} M isoproterenol and 10^{-5} M carbamyl choline, respectively, to the medium was essential for maintenance of differentiated acinar cells.

Because the pancreas contains no endogenous peroxidase, experiments on internalization of plasma membrane were conducted on isolated pancreatic acini. Initial experiments indicate that horseradish peroxidase is taken up from the cell surface in small endocytic vesicles and is transported to lysosomes. No reaction product could be found in Golgi saccules, immature or mature secretory granules. In order to mark the plasma membrane itself, pancreatic acinar cells were iodinated with ^{125}I. Preliminary observations show that in intact cells the majority of the label is located at the cell surface.

Significance to Dental Research:

In vitro studies of exocrine gland acinar cells should provide a greater understanding of both the controlling mechanisms and synthetic pathways involved in their secretory processes. Salivary gland secretions are absolutely necessary to maintain the health of the oral cavity. Therefore, knowledge of the normal secretory process is critical to our understanding of many pathological conditions which affect dental health.

Proposed Course of Project:

Cultured exocrine cells will be further characterized with respect to their cellular morphology and cytochemistry, protein synthetic capacity, and response to secretogogues. Additional studies will examine the distribution of membrane receptors, membrane reutilization, and the role of microtubules and microfilaments in the secretory process.

2. Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00012-16 LBS

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Infrared and Raman Spectroscopic Studies of Teeth and Bones and Related Synthetic Compounds

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Fowler, Bruce O. Research Chemist LBS NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Molecular Structure Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	1.0	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main objective is to determine compositional and structural details of the inorganic phase in teeth and bones. Infrared and Raman spectroscopy as well as chemical methods are employed in these studies. Methods are devised for the preparation of synthetic calcium apatites having controlled physical properties (crystal size and perfection) and chemical constituents (e.g., hydroxide, fluoride, chloride, carbonate, water and acid phosphate). The vibrational spectra of these apatites and related compounds are assigned and characterized. Isotopically enriched apatite analogs are prepared to facilitate spectral assignments. The spectroscopic assignments and supplemental spectral data (temperature dependency and polarization) are then utilized to establish compositional and structural details of the apatites in question which include: the type and geometry of constituent ions; the site or number of sites occupied by the ions; orientation of ions; chemical bonding and interactions of ions; and semi-quantitative estimations of the constituents present. The results for these controlled apatite systems are then related to the inorganic phase in calcified tissues.

NIDR CLASSIFICATION: 10210

1. Project Description:

Objectives:

The main objectives are to determine compositional and structural details of the inorganic phases in teeth and bones, with special emphasis on normal, abnormal, carious and chemically-treated human tooth enamel.

Methods and Approaches Employed:

Infrared and Raman spectroscopy and chemical methods are the primary tools of these studies. An understanding of the infrared and Raman vibrational spectra of various synthetic apatites and related compounds is necessary to determine corollary compositional and structural details for the inorganic phase(s) of hard tissue. Hence, these studies entail identification of the vibrational origin of infrared and Raman spectral bands for pure hydroxy-, fluor-, and chlorapatite, for mixed apatites containing hydroxide, fluoride, chloride, carbonate, acid phosphate, water and different cations, and for related calcium phosphates. The combined spectral data are then utilized to establish compositional and structural details of these apatites. These include the type of geometry of ions, orientation of ions, chemical bonding and interactions of ions, and semi-quantitative estimations of constituents present. Specialized spectroscopic techniques involving reflectance, polarization, low and high temperature, and high pressure devices are utilized in obtaining spectra. Methods are developed for the synthesis and purification of the compounds studied that require design and construction of specialized apparatus to maintain the rigid experimental conditions (e.g., high temperature and pressure) required to form apatites of controlled chemical and physical properties. Isotopically substituted analogs are prepared to facilitate assignment of spectral bands. The techniques are supplemented by chemical analyses to ascertain purity and chemical composition of the preparations.

Major Findings:

The proposed course of this year's project was altered to include Raman spectroscopic analyses of synthetic carbonate-containing apatites. Pertinent results at this stage were applied to tooth enamel, and studies were continued on preparation of synthetic carbonate-containing apatites. The major part of the carbonate component in mature human tooth enamel is thought to occupy two different lattice sites. About 5 to 10% of the total carbonate is attributed to replace hydroxide ions (site A). The location of site B, containing the major carbonate portion, is less certain; however, this site is generally assumed to be a vacant phosphate position. Raman bands observed for human tooth

enamel at 1105 cm^{-1} and part of the band at about 1070 cm^{-1} were assigned to the ν_1 modes of the carbonate ions on sites A and B, respectively. The Raman active ν_3 and ν_4 modes and the expected Raman active ν_2 modes of the carbonate ions were not detected in enamel spectra. These modes, ν_3, ν_4 and especially ν_2, have weak Raman intensities, and a high enamel fluorescent background using 5145 A excitation precluded their observation. Raman spectra of dentin did not indicate protein bands of significant intensities in the 1105 and 1070 cm^{-1} regions to cause band assignment conflicts.

Site A Carbonate in Enamel: Assignment of the enamel Raman band at 1105 cm^{-1} to carbonate on site A appears certain: (1) the wavenumber agrees with the Raman ν_1 carbonate band at 1106 cm^{-1} observed for synthetic $Ca_{10}(PO_4)_6CO_3$ analogs (CO_3 for two OH type replacement), (2) the weak intensity of the 1105 cm^{-1} band relative to the 1070 cm^{-1} band intensity agrees with expectations, (3) the 1105 cm^{-1} band increases in intensity with increasing carbonate in this site effected by carbon dioxide-thermal treatment, and (4) a higher wavenumber value for site A carbonate, 1105 cm^{-1}, than that of site B, about 1070 cm^{-1}, is expected by comparison with the higher average infrared ν_3 band wavenumbers for site A carbonate. The areas of several 1105 cm^{-1} enamel carbonate Raman bands (normalized to phosphate band areas) compared with those of synthetic $Ca_{10}(PO_4)_6CO_3$ analogs, containing 92% of the theoretical CO_3 for two OH replacement, indicated about 5 to 10% of the total enamel carbonate was in hydroxide positions. This agrees with Elliott's 5 to 10% values derived from infrared data.

Site B Carbonate in Enamel: The enamel Raman band centered at about 1070 cm^{-1} was attributed to carbonate on site B and to a ν_3 phosphate component. This band is broad and the separate carbonate and phosphate components have not yet been resolved. This assignment is based on band position and intensity correspondence with synthetic apatites: (1) various carbonate-containing apatites (carbonate not in hydroxide positions) have ν_1 Raman carbonate bands in the 1070 cm^{-1} region, and (2) the intensity (not attributed to crystal orientation affects) of the 1070 cm^{-1} enamel Raman band is greater than that expected for the ν_3 phosphate component.

Synthetic Carbonate Apatite Preparations: The products formed by reacting carbon dioxide with the $Ca_3(PO_4)_2$-CaO system at varying temperatures and pressures are being further studied because of their relevance to the carbonate components in tooth enamel. Previous infrared studies (NIDR Annual Reports: 1964-65), showed that carbonate thermally incorporated in the $Ca_3(PO_4)_2$-CaO system was chemically bound in at least two different sites (excluding $CaCO_3$) under the experimental conditions used. Carbonate was increasingly incorporated in the first site from none at $Ca_3(PO_4)_2$ (Ca/P = 1.50) to a maximum at about $Ca_9(PO_4)_6$-CaO (Ca/P = 1.67). For higher CaO contents

(Ca/P > 1.67), carbonate was predominately incorporated in a second site, and if the carbon dioxide pressure was high enough an additional phase of $CaCO_3$ was formed. Raman spectral analyses of these synthetic preparations have confirmed this. The major Raman bands for carbonate in the two different sites were identified and some uncertainties in weaker bands will be resolved using $^{13}CO_3$ containing preparations. The first site (site A above) corresponds to CO_3 for two OH replacement in $Ca_{10}(PO_4)_6(OH)_2$ and in tooth enamel. Chemical analyses of the thermally carbonated $Ca_9(PO_4)_6$-CaO composition gave values close to theoretical for $Ca_{10}(PO_4)_6CO_3$. Combined chemical, x-ray diffraction and spectroscopic analyses in this and other laboratories of the powdered $Ca_{10}(PO_4)_6CO_3$ material (type I carbonate apatite) show it has an apatite-type structure, an expanded a-axis relative to $Ca_{10}(PO_4)_6(OH)_2$, and indicate CO_3 incorporation along the hexad-axis channels. Infrared and Raman spectral bands observed for the second carbonate site in the Ca-PO_4-CO_3 material where Ca/P > 1.67 (type II material) are similar to those of the major carbonate component bands (site B above) in tooth enamel. Hence, chemical and physical characterization of the type II Ca-PO_4-CO_3 has bearing on understanding site B carbonate incorporation in tooth enamel. The chemical composition, stoichiometry and structure of the type II Ca-PO_4-CO_3 have not been established. This is because of possible incomplete reactions, additional phases and mainly because the reaction products were too small in size to effect physical separation for single-phase chemical analyses, and also too small for single-crystal spectroscopic and x-ray diffraction analyses. Consequently, in an effort to possibly obtain reaction products of adequate purity and of sufficient size for separation and subsequent chemical and physical analyses, attempts will be made to prepare the type II Ca-PO_4-CO_3 from a melt preparation which requires temperatures of about 1300°C and carbon dioxide pressures of about 150 atmospheres. To achieve these conditions, an apparatus consisting of an electrically heated furnace mounted within a carbon-dioxide-pressurized steel vessel has been designed. Features incorporated in this apparatus design to aid in successful sample preparation were: (1) inert apparatus materials to minimize sample impurities, (2) capacity for crystal separation from the melt under full pressure at 1300°C, (3) capacity for an anhydrous environment, (4) temperature measurement within sample and small temperature gradient across sample, (5) controlled slow sample cooling, (6) maximized sample container volume, with respect to acceptable temperature gradient, to yield sufficient sample quantities for single-run chemical and physical analyses, and (7) selection of structural components with substantial safety margins to avoid apparatus rupture under heat and pressure. This apparatus is nearly completed, and it will be utilized to prepare the type II Ca-PO_4-CO_3 for chemical and physical characterization.

Significance to Dental Research:

Characterization and assignment of the infrared and Raman bands of apatite containing biologically relevant ions and those of related calcium phosphates are essential in establishing corollary structural details for the inorganic phases of teeth and bones. The types and degree of incorporation of hydroxide, fluoride, chloride and carbonate ions, water, acid phosphate and different cations into biological apatites have bearing on the chemical, physical and biological properties of hard tissue.

Proposed Course of Project:

The preparation and characterization of specific biologically relevant synthetic carbonate apatites will be continued. Results will be related, where applicable, to the carbonate components in tooth enamel. Raman single-crystal symmetry species data on fluorapatite and hexagonal hydroxyapatite and both Raman and infrared powder data on fluorapatite, hexagonal and monoclinic hydroxyapatite, plus additional Raman data to be collected on hydroxide librational modes, will be prepared for publication.

Further work will continue on infrared and Raman external mode band assignments for fluorapatite and both the hexagonal and monoclinic forms of hydroxyapatite and chlorapatite using combined data from band symmetry species, isotopic band shifts, band temperature dependency, band intensity and mixed OH, F, Cl apatites.

2. Publications:

Fowler, B.O.: Infrared spectra of apatites. In Brown, W.E. and Young, R.A. (eds): Internat. Symposium on Structural Properties of Hydroxyapatite and Related Compounds. Gaithersburg 1968. New York, W.A. Benjamin (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00074-06 LBS

PERIOD COVERED
October 1, 1977 to September 30, 1978 NO1-DE-32418

TITLE OF PROJECT (80 characters or less)

Protein-Crystal Relationships in Mineralized Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Termine, John D.	Research Chemist	LBS NIDR
Other:	Miyamoto, Maureen S.	Staff Fellow	LBS NIDR
	Conn, Kathleen M.	Research Chemist	LBS NIDR
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COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Biological Structure

SECTION
Molecular Structure Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	1.6	1.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The biochemical and biophysical properties of developing skeletal and dental tissue proteins are being studied by several techniques. Dentin, bone and enamel matrix proteins are also investigated as to their functional and structural influences on active mineralization in biological systems. Special emphasis is placed on phosphoprotein biochemistry in these hard tissue matrix studies.

NIDR CLASSIFICATION: 10210
41321
41450
20230

1. Project Description:

The long range goals of this research project are twofold: (1) to study the structural and chemical properties of the inorganic and protein constituents of bones and teeth; and (2) to examine critically the changes that occur in these components during development, growth and disease states. Many of our procedures employ both model and in vitro systems as well as isolated biological entities. It is our hope that this multifaceted approach will provide a more thorough understanding of the biological mineralization process.

The structural techniques utilized in these studies include vibrational spectroscopy (infrared and laser Raman instrumentation), magnetic resonance spectroscopy (nuclear and electron spin), circular dichroism and x-ray diffraction. All the spectroscopic techniques chosen are designed to provide information on the functional groups and three dimensional environments associated with mineral binding sites in extracellular matrix macromolecules and encompass solution as well as solid state sampling technology. In vitro calcium phosphate formation from synthetic extracellular fluids is followed by powder x-ray diffraction (film) techniques, infrared spectroscopy and electron microscopy. Finally, routine chemical and biochemical procedures are used to study matrix proteins obtained from developing hard tissue.

Electron spin resonance spectroscopy experiments conducted in a collaboration afforded by an NIDR Contract (No. NO1-DE-32418) probed the mineral ion binding function of dentin matrix phosphoproteins using the paramagnetic vanadyl (VO^{2+}) and manganese (Mn^{2+}) ions as "reporter" groups. Vanadyl ions were found to bind to phosphoprotein carboxyl side chain groups to within 8 Å separation and could be displaced on a one-to one basis with added calcium ions. Langmuir adsorption isotherms for manganese indicated that this ion bound to organic phosphate side chain groups in both bovine molar and rat incisor dentin phosphoproteins. Both calcium and magnesium acted as competitors with manganese for these ion binding sites. Thus, both side chain carboxyl and organic phosphate groups actively bind mineral ions in dentin phosphoprotein molecules. Since these two functional groups are present on almost half of the total amino acid residues within dentin phosphoproteins, the potential activity of this protein species in dentin mineralization is readily apparent.

In the past fiscal year, ^{13}C nuclear magnetic resonance (NMR) spectrometry experiments were continued on fetal bovine enamel surface scrapings in a collaboration with Dr. D. A. Torchia, LB, NIDR. It was found previously that these mineralized tissue preparations, containing 20-25% protein, could be examined intact by ^{13}C Fourier transform NMR techniques and that developing enamel matrix proteins were rich in constituents exhibiting rapid segmental reorientation of their

peptide backbone carbons. These protein constituents exhibited rotational correlation times of 10 - 100 nanoseconds at 37°C. Those initial observations were confirmed and quantitated this past year in NMR experiments entailing both high-power nuclear decoupling and cross-polarization techniques. The results demonstrated that from 2/3 to 3/4 of the protein chains in developing fetal enamel matrix had peptide backbones similar in molecular mobility to those of globular proteins in free solution. Only about 1/4 to 1/3 of the protein chains in fetal enamel matrix exhibited restricted or anisotropic mobility such as that found in highly ordered fibrous proteins. Thus, the molecular characteristics of developing enamel matrices appear quite complex from a biophysical point of view. Any structural model for developing enamel must now take these mobility differences into account and still provide a reasonable basis for the orderly elaboration and alignment of enamel apatite crystallites, no mean task by any measure.

Last year, we reported initial progress in biochemical investigations on cell-free tooth matrices obtained from fetal calves 5 to 6 months in utero. Developing surface enamel was scraped from molars previously frozen in liquid nitrogen. After removal of the cusps, the underlying dentin-rich molar remnants were then frozen and crushed at liquid nitrogen temperature. Wherever possible, broad spectrum protease inhibitors were used in all standing solutions employed in our tissue preparation procedures, precautions warranted by the finding of neutral proteases in developing enamel tissue by ourselves and by several other laboratories over the past two years. In our ongoing collaboration on these enamel proteases with Dr. K. E. Keuttner, Rush Medical Center, Chicago, we recently discovered that in addition to the aforementioned neutral protease enzymes, fetal bovine enamel matrix contains lysozyme and other low molecular weight, cationic proteins having protease-inhibitor properties. These data suggest that a rather fine cell-modulated control of matrix proteolysis exists in developing enamel tissue, whereby proteolytic enzyme activity may be selectively counterbalanced by protease-inhibitor molecules. Similar proteolytic control mechanisms have been described for other organ systems.

During the past fiscal year, a sequential dissociative extraction scheme was devised to differentially extract tooth matrix proteins. The purpose of this extraction scheme was (a) to isolate in a non-degraded and enriched form those enamel and dentin matrix proteins most closely associated with the apatite crystallites of these tissues, and (b) to facilitate the identification of enamel or dentin specific proteins in preparations unavoidably containing both tissue species. In this extraction format, a frozen tissue powder (fetal bovine surface enamel scrapings or crushed dentinal remnants) was first extracted at 4°C and pH 7.4 in 4M guanidine containing a protease-

inhibitor cocktail. Molecular weight cuts of proteins (1) greater than 10,000 Daltons and (2) from 3,000 to 10,000 Daltons were then prepared from this extract by ultrafiltration. The crystal-rich residue from this initial extract was then treated further with 4M guanidine containing 0.5 M EDTA and a protease inhibitor cocktail. Ultra-filtration was again used to prepare the crude molecular weight cuts described above. Dissociative electrophoretic and chromatographic assay procedures were then used, along with amino acid analysis, to characterize the major protein species isolated in each instance.

For fetal bovine enamel scrapings, 80-85% of the total tissue protein and 75-77% of the total organic phosphate is isolated in the initial 4M guanidine extract, with over 95% of the protein extracted having a molecular weight of 10,000 or above. These proteins have a typical amelogenin amino acid composition, being quite rich in proline, glutamic acid, leucine and histidine. Amelogenins are only found in developing enamel, being absent from mature teeth. On SDS-urea P.A.G.E., the amelogenins isolated by our techniques ranged in molecular weight from 40,000-11,000 Daltons and consisted of 12-13 separate proteins. Ferguson plots (log R_f vs % polyacrylamide used in the electrophoretic SDS gel) for these proteins were linear, but did not match globular protein standards, indicating that their SDS - gel molecular weights were only apparent vs. known markers. Nevertheless, the SDS molecular weights for these amelogenin proteins extracted under non-degradative, dissociative conditions are considerably higher (1 1/2 to 2 times larger) than those reported for amelogenins prepared from fetal bovine tissues by other methods.

The sequential guanidine - EDTA extract of the fetal bovine enamel scrapings contained 12-15% of the total tissue protein and 23-25% of the total organic phosphate, with over 95% of the protein extracted again having a molecular weight of 10,000 or above. These proteins, surprisingly, had a typical enamelin or mature enamel protein amino acid composition, containing less proline, glutamic acid, leucine and histidine than amelogenin proteins and having considerably greater quantities of aspartic acid, threonine, serine, glycine, alanine, lysine and arginine. On SDS-urea P.A.G.E., these enamelin-like proteins contained three major species at molecular weights of 72,000, 56,000 and 49,000 Daltons. Ferguson plots for these proteins showed ideality of behavior vs. known globular protein standards, indicating that their observed SDS-gel molecular weights are probably correct. These proteins stained for organic phosphate in P.A.G.E. and were quite acidic, eluting from DEAE cellulose columns at ionic strengths of 0.45-0.55 in 7M urea buffer. This is the first time that proteins like these have been identified in fetal enamel tissue. Their presence in early enamel suggests that, in contrast to the amelogenins, they may be conserved during normal enamel maturation, perhaps gradually being converted to lower molecular weight, mature tooth enamelin proteins of similar composition.

Initial guanidine extracts of dentin-rich remnant preparations (see above) removed mostly enamel proteins of the amelogenin class, thereby providing substantial purification of the underlying crystal-bound dentin proteins. The sequential guanidine-EDTA extracts of these tissue preparations contained 40-45% of the total tissue protein and 91-94% of the total organic phosphate, with 90-95% of the total protein extracted having molecular weights greater than 10,000 Daltons. This second extract contained about 25% enamel contamination (enamelin class), based on amino acid composition data. Three major dentin-specific proteins were identified in this extract at molecular weights of 96,000, 65,000 and 53,000 Daltons on SDS-urea P.A.G.E. Ferguson analysis of these proteins showed considerable non-ideality and also indicated that they probably are of even higher molecular weight than that indicated on SDS-gels *vs.* known markers. All three of these proteins stained for organic phosphate on P.A.G.E. and eluted from DEAE-cellulose columns at ionic strengths of 0.50-0.65 in 7M urea buffer. The apparent 96,000 molecular weight protein was isolated as a single species based on SDS-urea gels and had a typical dentin phosphoprotein amino acid composition, with almost 60% of its total residues being either asparatic acid or serine/phosphoserine. In contrast to dentin phosphoproteins isolated by other methods, this protein preparation contained considerably higher levels of amino acids such as lysine, histidine, leucine and proline, suggesting that it may exist in a more mature non-degraded form than heretofore realized. The additional finding above that several potential phosphoproteins may exist in fetal bovine dentin also raises questions as to their initial biogenesis and structure/function relationships in dentin mineralization.

Considerable effort will be spent in the future to continue these biophysical and biochemical studies of tooth matrix proteins. NMR investigations will be directed toward establishing the role of phosphate in the structure-function relationships of dentin and enamel phosphoproteins. An attempt will be made to characterize further the high molecular weight dentin phosphoprotein and to determine its relationship to its lower molecular weight dentin analogues. Efforts will also be made (1) to purify the enamelin-like, fetal enamel matrix proteins, (2) to biochemically characterize them and (3) to determine their eventual fate in enamel maturation. Similarly, beginning attempts to isolate higher molecular weight amelogenin moieties from fetal bovine enamel will be undertaken in the near future. Finally, once purified tooth matrix protein components have been obtained, both *in vivo* and *in vitro* studies will be initiated to uncover their biological function and biogenesis.

Skeletal and soft tissue calcification diseases cut across a wide swath of medical and dental problems. Orthopedic diseases, arthritis, atherosclerosis, certain renal and endocrine disorders, for example,

all involve the structure and metabolism of the skeleton. Still further, is the need to understand thoroughly both the development and structure of mineralized tissues both to the caries, periodontal disease and craniofacial anomalies research objectives of the NIDR. The bones and teeth are composite tissues consisting of cellular, collagenous, glycoprotein, phosphoprotein and inorganic elements. As a consequence, their physiology and pathology are considerably more complicated than those of other tissues in the body. This research program is designed to study in detail the relationships that exist between the various constituents of hard tissues both from structural and functional points of view.

2. Publications:

Hassan, A.A., Termine, J.D., and Haynes, C.V.,Jr.: Mineralogical Studies of Bone Apatite and their Implications for Radiocarbon Dating. Radiocarbon 19: 364-374, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00088-05 LBS

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Chemical, Structural, and Morphological Studies on Calcium Phosphates.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Eanes, Edward D.	Chief, Molecular Structure Section	LBS NIDR
OTHERS:	Martin, Garland N. Jr.	Research Chemist	LBS NIDR
	Hailer, Arthur	Chemist	LBS NIDR
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COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Molecular Structure Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.08	1.0	2.08

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The formation and maturation of calcium phosphate prepared from physiological-like solutions is being studied with a number of physical-chemical and ultrastructural techniques including standard analytical chemistry procedures, electron microscopy and x-ray diffraction. Topics of present interes include (1) phase changes in aqueous calcium phosphate suspensions prepared by inoculating stable supersaturated solutions with apatite crystals; (2) effect ionic fluoride and magnesium on the formation, growth, and stability of precur phases to crystalline apatite.

NIDR CLASSIFICATION: 10210

4- 146

1. Project Description:

Objectives:

The purpose of this project is to study both the preparation from solution and the physico-chemical/ultrastructural properties of calcium phosphate salts, with particular emphasis on the formation and maturation of apatite from physiological-like solutions. The principal objective is to acquire a more complete understanding of biomineralization processes, with special emphasis on the physical-chemical aspects of mineral deposition in vertebrate hard tissue. During the year covered by this report, the principal undertakings were: (1) to study the effect of ionic magnesium on the formation and maturation of apatite crystals in aqueous suspensions at physiological pH and temperature; and (2) to investigate the formation, growth, and stability of non-apatitic calcium phosphates on apatite surfaces. In this latter undertaking, the effect of fluoride on these surface reactions was also investigated.

Methods Employed:

Synthetic calcium phosphate suspensions were prepared either by (1) spontaneous precipitation initiated through rapidly raising with KOH the pH of stable acidic solutions of calcium and phosphate ions to 7.4; or by (2) inoculating supersaturated solutions metastable at pH 7.4 with crystals of apatite. The suspensions were then aged at either 25° or 37°C, the pH being maintained at 7.4 with a pH-stat. Magnesium and/or fluoride ions were added either before initiating precipitation or at various stages during the aging process. Reaction kinetics were followed by monitoring solution calcium, total phosphate, hydrogen ion release, and fluoride consumption. Solid reaction products were examined chemically and by x-ray diffraction and transmission electron microscopy.

Major Findings:

Work done under this project during the past few years has shown that the maturation of calcium phosphate salts spontaneously precipitated from unstable supersaturated solutions at physiological pH and temperature is a complex process involving the sequential appearance of at least 3 distinct phases. The initial phase to separate from solution is an unstable amorphous calcium phosphate (ACP). The subsequent dissolution of this amorphous phase is accompanied by the formation of an intermediate crystalline phase similar in properties to octacalcium phosphate (OCP). This OCP phase, in turn, hydrolyzes into a stable apatitic end product. Additional studies have shown that, when in solution, small amounts of several classes of inorganic ions can profoundly affect the growth and stability of the various

solid phases formed during the precipitation reaction. As an example results obtained during the past year have shown that metal ions such as magnesium can extend the solution lifetimes of both the ACP and OCP precursor phases with the result that the appearance of the final apatitic phase can be delayed appreciably. This is in contrast to such anions as fluoride which, although effective ACP stabilizers, can greatly accelerate the conversion of OCP to apatite. Because of its stabilizing action on both intermediates, as little as 0.3 mM $MgCl_2$ can delay by a factor of two or more the nucleation of apatite.

During this past year a study was initiated to investigate the complex chain of precipitation events which occur when apatite crystals are introduced into supersaturated but metastable solutions at pH 7.4. Calcium and phosphate concentrations employed in these studies were similar to those found in biological fluids. Emphasis was placed on determining how fluoride influences the course of this apatite-induced precipitation process. Results showed that fluoride has an impact similar to that observed in spontaneously precipitated reactions. The initial accretions on the surface of the exogenous apatite crystals behaved like ACP in that they were stabilized by as little as 4.5 μM fluoride. The subsequent formation of the first crystals was, like OCP, greatly reduced by equally small amounts of fluoride. In fact, when fluoride levels approaching 13.5 μM were used, the OCP-like stage appeared to be completely suppressed in favor of direct formation of apatite.

Significance to Dental Research:

Hard tissue formation is a complex phenomenon involving many poorly understood processes, not the least of which is the deposition of mineral in the extracellular matrix. Because of the intimate association between mineral and matrix, it has proved difficult to study the purely physical-chemical dynamics underlying this deposition step *in vivo*. Consequently, to assess the importance of these physical-chemical factors it is necessary to thoroughly and systematically investigate the formation and growth of analogous calcium phosphate salts in physiological-like synthetic systems. The results obtained from such experiments indicate that OCP-like crystalline phases as well as amorphous phases may play important intermediary roles in the mineralization of hard tissue. The magnesium and fluoride experiments described above illustrate how small amounts of these anions may possibly alter the course of apatite formation *in vivo*. Such knowledge may allow us to better understand the nature of ion mineral interactions in teeth and consequently to more completely assess the anticaries property of such anions as fluoride.

Proposed Course of Project:

The studies on the effect of fluoride and magnesium on apatite formation and maturation will continue. Future emphasis will be directed toward examining how magnesium modifies the reactions that occur when stable supersaturated solutions of calcium and phosphate are seeded with exogenous apatite crystals. Additional studies will be initiated to examine the effect of fluoride on apatite dissolution and recrystallization under acidic conditions which commonly occur in oral fluids.

2. Publications:

Eanes, E.D., Meyer, J.L.: The maturation of crystalline calcium phosphate in aqueous suspension at physiologic pH. Calcif. Tiss. Res. 23: 259-269 (1977).

Meyer, J.L., Eanes, E.D.: A thermodynamic analysis of the amorphous to crystalline calcium phosphate transformation. Calcif. Tiss. Res. 25: 59-68 (1978).

Meyer, J.L., Eanes, E.D.: A thermodynamic analysis of the secondary transition in the spontaneous precipitation of calcium phosphate. Calcif. Tiss. Res. (in press).

Eanes, E.D., Meyer, J.L.: The influence of fluoride on apatite formation from unstable supersaturated solutions at pH 7.4. J. Dent. Res. (in press).

Eanes, E.D.: Enamel apatite: chemistry, structure and properties. In, Nylen, M.U., Termine, J.D. (eds): Third International Symposium on Tooth Enamel - Growth, Structure, and Function. J. Dent. Res. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00162-03 LBS

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Kinetic and Thermodynamic Characterization of Calcium Phosphate Precipitation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Meyer, John L.	Research Chemist	LBS NIDR
OTHER:	Hailer, Arthur	Chemist	LBS NIDR
	Selinger, H. Andrew	Biological Aid	LBS NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Molecular Structure Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.84	1.0	0.84

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to determine the thermodynamic and kinetic factors which regulate the nucleation, crystal growth and maturation of calcium, phosphate crystals. This is accomplished by estimating free ionic activities in solution for all species involved in the crystallization process and relating these terms to the observed precipitation steps. A further correlation is then made between the composition of the solution and the properties of the solid calcium phosphate phase in equilibrium with it. The effect of crystallization inhibitors on the precipitation of calcium phosphates is also being studied in order to elucidate their mode of action at crystal surfaces. Emphasis is placed upon inhibitors which occur naturally in physiological systems or which are common therapeutic agents.

NIDR CLASSIFICATION: 10210

1. Project Description:

Objectives:

A number of factors appear to regulate the nucleation, crystal growth and maturation of calcium phosphate precipitates under the conditions at which biological calcification occurs. It is the purpose of this investigation to elucidate the kinetic and thermodynamic parameters which control each of the major steps involved in calcification and thereby gain additional insight into the mineralization process itself. Emphasis is placed upon the solution phase and how its composition affects crystallization processes, although the isolated solid phases are also studied with conventional chemical, microscopic and spectroscopic techniques.

Methods Employed:

The calcium phosphate precipitations are performed under conditions of constant temperature and pH. From the analytical concentrations of the reactants, free ionic concentrations and chemical activities of each ionic species are calculated using known thermodynamic equilibrium constants and computed activity coefficients. A computer program has been developed to perform these calculations. Knowledge of the free ionic activities of each species involved in the precipitiation provides the necessary thermodynamic information for the correlation between events that occur in solution versus those that occur in the solid state. In addition, the kinetic pathways to the thermodynamic state for each precipitation step can also be studied once the concentration of each species is known.

Major Findings:

The spontaneous precipitation of calcium phosphate is characterized by the formation of an initial phase which is amorphous with respect to x-ray diffraction (amorphous calcium phosphate, ACP). If left in contact with solution, ACP transforms into a crystalline material with an apatitic-like x-ray diffraction pattern. It now appears that this first-formed crystalline material undergoes a secondary transition and the characteristics of this transition have been studied in some detail. Crystalline material isolated during this secondary stage differs significantly in its morphological properties from that of the subsequent apatite phase. A thermodynamic analysis has been made of this secondary transition and indicates that the first formed crystalline material has a solubility similar to that of octacalcium phosphate (OCP). In fact, the computed thermodynamic solubility product remains invariant in the pH range 7.00 to 8.60. The duration of the secondary stage is sensitive to pH and the transition appears

to occur by hydrolysis of the first formed OCP-like phase to a more basic apatitic phase with a tricalcium phosphate (TCP) stoichiometry. The crystalline material at the end of this transition has an invariant solubility product, in the pH range 7.00 to 8.60, when the TCP-like molecular formula is assumed. Changes in the solution chemistry which accompany the solid-to-solid transitions are consistent with the above conclusions. The results of this study are also consistent with those of a previous study which suggest that the stability of the amorphous calcium phosphate phase is dependent upon the instability of the solution phase with respect to OCP formation.

Significance to Dental Research:

A knowledge of the factors that influence calcium phosphate precipitation is required for a complete understanding of the physiological processes that result in hard tissue mineralization. A thermodynamic approach to the study of calcium phosphate precipitation under simulated in vivo conditions yields basic information that can be related ultimately to conditions that may exist in vital fluids in contact with the mineral phase. The combination of thermodynamic and kinetic methods can provide a better description of those dynamic processes resulting in physiological and pathological calcifications or decalcifications within the body.

Proposed Course of Project:

Experiments designed to characterize the entire course of the precipitation of calcium phosphate from the initial formation of ACP to its eventual transformation to crystalline hydroxyapatite will continue. Solutions in contact with the solid phase will be analyzed by chemical thermodynamic methods to obtain information on the nature of the various calcium phosphate phases formed. All crystalline materials will be analyzed by conventional techniques to assure that the thermodynamic analysis results are consistent with the properties of the solid phase. Crystal growth inhibitors are known to affect crystallization of calcium phosphates but a detailed analysis of their effects on all the various phase transitions has not yet been made. Because of the potential biological significance of mineralization inhibitors on intra- and extracellular calcification, future work will concentrate on these substances with particular emphasis on their mechanism of action at crystal surfaces.

2. Publications:

Eanes, E.D. and Meyer, J.L.: The maturation of crystalline calcium phosphates in aqueous suspensions at physiological pH. Calcif. Tiss. Res. 23: 259-269, 1977.

Meyer, J.L. and Eanes, E.D.: A thermodynamic analysis of the amorphous to crystalline calcium phosphate transformation. Calcif. Tiss. Res. 25: 59-68, 1978.

Meyer, J.L. and Eanes, E.D.: A thermodynamic analysis of the secondary transition in the spontaneous precipitation of calcium phosphate. Calcif. Tiss. Res., in press.

Eanes, E.D. and Meyer, J.L.: The influence of fluoride on apatite formation from unstable supersaturated solutions at pH 7.4. J. Dent. Res., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00204-02 LBS

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Collagenous Matrix and Bone Differentiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Biological Structure

SECTION

Molecular Structure Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.31	2.31	2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to investigate extracellular matrix-cell interactions employing an experimental system of matrix-induced endochondral bone differentiation. This experimental model further affords a method to undertake systematic studies on the biochemistry and physiology of endochondral bone formation. Subjects currently under investigation are: (1) transitions in collagen and proteoglycan types during bone development; (2) changes in ornithin decarboxylase activity during bone cell differention; (3) hormonal and nutritio influences on bone formation; (4) influence of fluoride on the rate of mineralization; (5) role of magnesium in bone formation; and (6) matrix-cell interacti in tissue culture.

NIDR CLASSIFICATION: 20220
20230
20324
41230

1. Project Description

Introduction and Background:

The origin and evolution of multicellular organisms were marked by theappearance and specialization of extracellular matrices. The extracellular matrix is predominantly composed of collagens, proteoglycans and glycoproteins. Of all the tissues in the body only bone, cartilage, tendon and tooth exhibit vast expanses of extracellular matrix. While we know that all extracellular matrices are products of cellular biosynthetic activity, we know very little about the interactions and feedback between matrix and cells. Our studies have concentrated on the area of collagenous bone matrix-cell interactions.

We have developed a useful experimental method to investigate several aspects of endochondral bone differentiation. Subcutaneous transplantation of demineralized rat bone or tooth matrix results in induction of bone formation locally. The sequential cellular changes are: (1) transient chemotaxis for leukocytes (days 1-2); (2) a more prolonged chemotaxis for fibroblasts (days 2-3); (3) cell proliferation (days 3-5); (4) chondrogenesis (days 5-7); (5) hypertrophy and calcification of cartilage (days 9-10); (6) osteogenesis and bone mineralization (days 11-14); (7) remodeling of the ossicle (days 14-18); (8) differentiation of hematopoietic bone marrow (days 18-24).

The induced bone and bone marrow persist indefinitely in a functional state. The temporal sequence is highly reproducible and we have extensively studied the factors influencing the sequence. This experimental model is the mainstay of our work and affords a system to dissect the main events in endochondral bone formation.

Experimental Methods:

Diaphyseal shafts are prepared from adult rats by standard techniques. Pulverized matrix particles of uniform size are then demineralized prior to implantation. The usual repertoire of standard biochemical laboratory techniques such as density gradient centrifugation, column chromatography and radioisotopic tracer methodology are extensively employed.

Transitions in Collagen Types During Endochondral Bone Differentiation:

The occurrence and distribution of several genetically distinct collagen types are now well established. It is reasonable to expect that the intricate cellular transitions observed during matrix-induced endochondral bone development would be accompanied by alterations in the type of collagen produced. The availability of purified, chemically

characterized collagen types has led to the production, isolation and immunochemical characterization of the collagen type specific antibodies. This development, in turn, resulted in the immunofluorescent localization of collagen types in tissue sections. Such immunohistological localization is especially useful in studies of the distribution of various types of collagen in complex tissues with several cell types, as in developing bone, where a biochemical approach be of limited utility. The localization of types I, II, III and IV collagens matrix-induced sequential development of cartilage, bone and bone marrow was studied in collaboration with Drs. S. Gay, R. Gay and E.J. Miller of the University of Alabama and Dr. J-M. Foidart of LDBA, NIDR. On day 3, type III collagen was localized as a fine network around the invading fibroblasts. On days 4-6 smaller amounts of type I were also detected around these proliferating cells. With the onset of chondrogenesis, type II collagen was detected in the cartilage matrix on day 6 and persisted until the early stages of bone formation. On day 9, during vascular invasion, type IV (basement membrane type) collagen was localized around proliferating endothelial cells. This was accompanied by osteogenesis on day 10 when type I collagen was demonstrated in the newly deposited bone matrix. On day 17 and thereafter type III collagen was localized as a fibrous array around nests of hematopoietic cells. The developing sinusoids in hematopoietic ossicles were intensely stained with antibodies to basement membrane (type IV) collagen.

Changes in Ornithine Decarboxylase During Cartilage and Bone Differentiation:

The occurrence of the polyamines putrescine (1,4 diaminobutane), spermidine and spermine during growth and differentiation of tissues is well known. The enzyme ornithine decarboxylase (EC 4.1.1.17; ODC) catalyzes the key step of decarboxylation of ornithine to putrescine and is elevated in many proliferating tissues. As a part of our continuing study of the role of cell proliferation in differentiation we have investigated the changes in this enzyme in matrix-induced plaques during endochondral bone development. There was a peak in ODC activity on day 3 corresponding to early proliferation of mesenchymal cells in close contiguity to the implanted collagenous bone matrix. A smaller peak of enzyme activity was observed on day 8 and was correlated with proliferation of osteoprogenitor stem cells and vascular endothelial cells. Induction of ODC represents one of the early responses to implanted bone matrix and has been used as a marker for mesenchymal cell proliferation on day 3.

Hormonal and Nutritional Influences on Bone Formation:

The described experimental model is amenable to an investigation of the regulatory role of steroid and polypeptide hormones on discrete

stages of endochondral bone differentiation. Our previous experiments established the critical role of pituitary growth hormone for osteogenesis. We have begun a systematic study of the influence of corticosteroids on bone formation. It is well known that one of the undesirable side effects of corticosteroid therapy in clinical medicine is osteoporosis. The precise mechanism underlying this is not known. Our results demonstrate that corticosteroids such as dexamethasone inhibit drastically the observed increase in ODC activity in day 3 plaques. This effect is restricted to corticosteroids; androgens and estrogens are devoid of this inhibitory effect. Conversely, adrenalectomy increases the ODC activity.

The influence of dexamethasone is transient; withdrawl of steroid administration on day 3 results in the prompt recovery of ODC activity on day 7. Dexamethasone diminished the incorporation of $^{35}SO_4$ into proteoglycans in chondrogenic plaques on day 7. Administration of dexamethasone on days 7 through 10 impaired osteogenesis and mineralization as assessed by ^{45}Ca incorporation. However, dexamethasone treatment on days 10 and 11 did not influence the mineralization in day 12 plaques. These results indicate that the corticosteroids act mainly on proliferation of mesenchymal cells prior to chondrogenesis and osteogenesis. Further studies are planned to demonstrate steroid receptors.

Fluoride has received considerable attention as a factor influencing bone and tooth mineralization. Despite extensive studies on cariostatic effects of fluoride, there is a relative paucity of information on the cellular effects of fluoride in mineralizing tissues. We have studied the influence of ingestion of 50 and 100 ppm fluoride in the drinking water on matrix-induced endochondral bone formation. The plasma fluoride levels were elevated to 25-30 μM in fluoride-treated rats, as opposed to a control level of 2-5 μM. The induction of endochondral bone in the fluoride-fed rats was compared to control rats with special reference to mineralization. These experiments revealed that fluoride retarded the rate of mineralization as indicated by ^{45}Ca incorporation but did not affect the extent of mineralization. Experiments currently in progress are aimed at investigating the cellular cyclic nucleotides and the nature of newly deposited bone mineral in fluoride supplemented rats by x-ray diffraction analysis.

The precise role of magnesium in mineralization is not known. Earlier studies have observed decreased bone growth in magnesium deficient rats. In order to ascertain the influence of magnesium on the matrix-induced endochondral bone formation, two groups of rats were fed the same basal diet with 50 ppm (deficient) and 1000 ppm (control) of magnesium. The rats were fed with these diets for seven days prior to implantation. Calcification of hypertrophied cartilage was delayed

in experimental rats. The bone formation and mineralization was monitored by alkaline phosphatase activity and ^{45}Ca incorporation. The results revealed that the rate and extent of mineralization was impaired in magnesium-deficient rats. Additional experiments are being conducted to understand the mechanisms involved.

Matrix-Cell Interactions in Tissue Culture:

In order to fully understand the molecular events underlying matrix-cell interactions occurring in vivo, an experimental method of cartilage induction in vitro would be useful. This problem was explored in collaboration with Drs. I. Binderman and R. M. Greene, LDBA, NIDR. We have compared the influence of bone matrix powder on rat mesenchymal cells derived from limb bud, skin and periosteum. Chondrogenesis was monitored by alcian blue staining, specific immunofluorescence for type II collagen and electron microscopy. We observed polygonal chondrocytes in the deep crevices on the undersurface of the bone matrix powder when periosteal cells of rat were cultured with rat bone matrix. It would appear that the geometry of the particle is crucial and further experiments are in progress to define the experimental system in tissue culture in order to enhance its utility for specific studies on matrix-cell surface receptor interaction.

Significance to Dental and Medical Research:

A detailed knowledge of bone induction by cell-free collagenous bone matrix has immense implications for fracture healing, and other orthopedic diseases and in the realm of oral implants. In cancer, impaired matrix-cell interactions lead to metastases. Our experimental model represents a prototype for studying matrix-cell interactions and may shed light on the mechanisms involved in normal physiology and in pathogenesis.

2. Publications:

Reddi, A.H., Gay, R., Gay, S., and Miller, E.J.: Transitions in collagen types during matrix-induced cartilage, bone and bone marrow formation. Proc. Nat. Acad. Sci. USA 74: 5589-5592 (1977).

Huggins, C.B., and Reddi, A.H.: Reticulo-endothelial cells in matrix-induced fibroblast-transformation ossicles. In "Search & Discovery". A tribute to Albert Szent-Gyorgy. (B. Kaminer ed.) Academic Press. N.Y. pp 273-278 (1977).

Reddi, A.H., Hascall, V.C., Hascall, G.K.: Changes in proteoglycan types during matrix-induced cartilage and bone development. J. Biol. Chem. 253: 2429-2436 (1978).

Rath, N.C., and Reddi, A.H.: Changes in ornithine decarboxylase activity during matrix-induced cartilage, bone and bone marrow differentiation. Biochem. Biophys. Res. Comm. 81: 106-113 (1978).

Faltz, L.L., Reddi, A.H., Hascall, G.K., Martin, D., Pita, J., and Hascall, V.C.: Characteristics of proteoglycans extracted from the Swarm rat chondrosarcoma with associative solvents. J. Biol. Chem. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00251-01 LBS

PERIOD COVERED
October 1, 1977 to September 30, 1978 Y01-DE-80027

TITLE OF PROJECT (80 characters or less)

Microprobe Analysis of Developing Rat Enamel

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Casciani, Francis S. Staff Fellow LBS NIDR

COOPERATING UNITS (if any)

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SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this study are the characterization and identification of the phosphate and carbonate phases which occur during the mineralization and maturation of rodent incisor enamel. Phosphate and carbonate phases localized in the tissue during development are analyzed using microprobe techniques. The Raman microprobe, capable of identifying molecular species, and elemental microprobe analyses (x-ray microanalysis and ion microprobe analysis) used in conjunction allow the correlation of data obtained from these two types of complementary analyses. This data then, may be interpreted to discern the distribution patterns of calcium, phosphate, magnesium and other ions at mineralizing sites in developing enamel.

NIDR CLASSIFICATION: 10210

1. Project Description

Objectives:

The objectives of this study include the following: (1) to discern the accumulation and describe the precise distribution of calcium, phosphate and magnesium in developing dental enamel during the mineralization and maturation of the tissue; and (2) to correlate this mineralization pattern with the varying stages of ameloblast function.

Methods:

During the preliminary stage of this project, most effort has been expended in sample preparation. Samples consist of thin (5-10 μm) sections of rat incisor dentin and enamel. Various methods of sample preparation have been utilized including (1) fixation with glutaraldehyde and embedding in plastic, (2) mounting in a hydrocarbon (Nujol) film, (3) sectioning of frozen unfixed tissue followed by air-drying or freeze drying of thin sections. Each of these has been examined from the point of view of providing histologically well characterized samples which offer reproducible results. The merits of each of these thin section preparation schemes continue to be investigated. To this point, it appears that fresh frozen air-dried or freeze dried thin sections are the most useful in all micro examinations employed in this study.

Raman microprobe analysis, x-ray microanalysis and ion probe analysis were made possible through the use of these instruments under an Interagency Agreement Y01-DE-80027 between NIDR and NBS.

Whole lower rat incisors are removed from the mandible with all cell layers intact immediately after sacrificing (60-100 g Sprague-Dawley rats). The incisor is frozen in liquid nitrogen and the apical end mounted for cryostat sectioning in the longitudinal axis of the tooth to allow the examination of pulp, odontoblasts, predentin, dentin, enamel and ameloblasts. The protocol which will be employed in future investigations, to know precisely the region of analysis, is the following: (1) 5 μm frozen sections, from incisors oriented as above, are transferred to the appropriate Raman microprobe substrate (either lithium fluoride or sapphire) and air dried or lyophilized. Alternate serial sections are stained employing conventional histological procedures. (2) Photomicrographs of the sections are recorded. (3) Raman microprobe data are collected on specific areas (normally 5 μm in diameter) of the section. (4) The same sections may then be carbon coated for x-ray microanalysis and ion probe analysis when necessary. (5) These sections may be further stained for histological study.

Major Findings:

Raman Analyses: All analyses performed have been limited to the first 5 mm of the apical end of the incisor. Within this region the ameloblast initiates secretion of enamel matrix which forms the inner enamel. 5 μm areas in this region have been scanned with the Raman microprobe. This method is capable of determining relative phosphate concentration based on the intensity of the ν_1 P-O stretching mode. In the initial enamel matrix, the absence or very weak intensity of this phosphate band suggests that the initial mineral phase may be a phosphate-deficient phase. A band more intense than the phosphate band occurs at 1120 cm^{-1}, a region of the vibrational spectrum commonly associated with carbonate stretching modes which may indicate a greater concentration of a carbonate phase. These data corroborate the recent findings of Robinson et al. (Third International Symposium on Tooth Enamel, 1978). In those studies it was noted that the first enamel matrix secreted by the ameloblasts had high concentrations of both magnesium and carbonate and relatively low concentrations of calcium and phosphate.

As mineralization proceeds, phosphate accumulates and the mineral phase becomes typically apatitic. Based on the Raman spectra of small areas in which phosphate is in low concentration, the data obtained suggest that an amorphous calcium phosphate is absent in this region.

X-ray Microanalysis and Ion Microprobe Analysis:

Comparable regions of the rat incisor have been analyzed by x-ray microanalysis. Initially, difficulties were encountered with sample preparation. It has been determined that the ideal thickness for samples with this method is 1 μm or less. Presently freeze-dried incisors are further dehydrated in 100% ethanol followed by propylene oxide and embedding in epoxy resin. This embedding method permits the cutting of 1 μm sections with histologic integrity. Preliminary x-ray microanalysis of the outer 1-5 μm areas has revealed significant quantities of magnesium, along with calcium and phosphate suggesting that if the initial phase is magnesium-carbonate rich and calcium-phosphate poor this preparation includes more than the first stage of mineralization.

In order to determine the effect of calcium on magnesium and vice-versa in the x-ray microanalysis experiments a sample of Huntite ($Mg_3Ca(CO_3)_4$) has been obtained. This sample, along with samples of calcium and magnesium phosphate embedded in plastic in the same manner as the hard tissue, will allow Mg/Ca ratios to be determined in the presence of both carbonate and phosphate. Some very preliminary data suggests that the sensitivity of magnesium analyses may differ depending on whether

it is present in a phosphate or carbonate phase. These plastic embedded and sectioned samples will be used as standards for calcium, magnesium and phosphorus analyses.

X-ray microanalysis will be compared with the ion-microprobe analysis, which has the advantage over x-ray microanalysis, that, in addition to analyses of calcium, phosphorus and magnesium, carbonate may also be quantitated.

Proposed Course: In vivo methods will be utilized to study the effect of altered tissue formation on this initial high carbonate, low phosphate region in developing rodent incisor enamel. This direction is chosen primarily as an attempt to morphologically isolate this non-phosphate mineral phase. If an initial non-phosphate mineral phase is involved in the mineralization of enamel, then its isolation may be accomplished by severely limiting phosphate intake. Therefore, assuming that such a diet will have little or no effect on the development of the carbonate phase, greater quantities of the carbonate phase may be more accessible for study thus permitting a more distinct spectrum in the Raman microprobe experiments without phosphate interference. Isolation of this phase in this manner would also imply a primary stage in mineralization which is not phosphate dependent. This sample would also allow one to obtain Mg/Ca ratios with the x-ray microprobe without phosphate interference.

Experiments are also planned to study the effects of high dietary fluoride on this carbonate phase. The ability of fluoride to reduce acid solubility (especially in dental caries) may well be an alteration of this carbonate rich phase. It is well known that carious enamel has lower magnesium and carbonate content.

Ion probe analyses are to be initiated to determine magnesium: carbonate ratios. Similar analyses are planned in mineralizing dentin.

2. Publications:

None

Annual Report of the Laboratory of Microbiology

and Immunology, National Institute of Dental Research

The research programs in this Laboratory have remained virtually unchanged since last years report, however, notable progress has been achieved in several areas. In the narrative to follow the major research findings and their significance are summarized according to the sectional activities within the Laboratory.

The MICROBIOLOGY SECTION continues to conduct a broadly based research program designed to provide new information on the individual microorganisms that comprise the oral flora and their relationships with one another. A major objective of this program is to delineate fundamental principles that will serve as a knowledge base for formulating rational approaches to the control of oral diseases that result from complex interactions between the resident microbial flora and its host.

The Section has a long-standing interest in pathways of carbohydrate metabolism operative in oral streptococci. A conspicuous void in our knowledge of this area, however, is the means by which sucrose is transported into the cell. This past year experiments were performed to study the problem in Streptococcus mutans. S. mutans translocates sucrose by means of a phosphoenolpyruvate-dependent phosphotransferase system. Using a mutant that is unable to hydrolyze the product of the sucrose phosphotransferase system, it has been possible to isolate the compound and characterize it chemically as sucrose phosphate. The phosphate group is located on the glucosyl moiety of the molecule. Subsequent metabolism of intracellular sucrose phosphate requires an enzyme capable of hydrolyzing it to glucose-phosphate and fructose. Such an enzyme has not been previously reported in oral streptococci and its presence in strains of S. mutans is currently under investigation.

Another problem dealing with sucrose utilization by the oral streptococci concerns the mechanism of secretion of the extracellular enzyme glucosyltransferase (GTase). This enzyme produces high molecular weight, adherent polymers of glucose from sucrose which are important in the colonization of tooth surfaces by cariogenic streptococci. Last year it was shown that GTase production by both S. salivarius and S. mutans 6715 growing in a chemically defined medium was highly dependent upon the presence of Tween 80 in the growth medium. It is now possible to demonstrate the stimulatory effect of the surfactant on GTase production with non-growing cell suspensions of S. salivarius. Using this model system, it has been found that production of the exoenzyme is completely dependent upon the de novo synthesis of a short half-life mRNA and does not involve translation of a preformed, stable mRNA. Moreover, the antibiotic cerulenin, which inhibits an early stage of fatty acid synthesis, is an effective inhibitor of GTase production in the resting cell system. These and other preliminary data suggest that membrane lipids and/or a post-translational modification of GTase that requires fatty acid synthesis may be involved in cellular secretion of this enzyme.

The Section's molecular biology program continues to provide much needed information on the function and mechanisms of transfer of plasmids in various oral bacteria. For example, it has recently been shown that a plasmid carried by certain species of Lactobacillus codes for lactose utilization by these organisms. Perhaps the most far-reaching advance in this area, however, has been the discovery that plasmid transfer between species of Streptococcus can occur by a process formally analogous to conjugation. An antibiotic resistance plasmid from Streptococcus faecalis introduced into Streptococcus sanguis by transformation has been transferred to a variety of other oral streptococci including S. salivarius and S. mutans. The transfer requires cell-to-cell contact between donor and recipient and is insensitive to DNase. Another striking finding is that streptococcal transconjugants can also act as donors for the intergeneric transfer of the antibiotic resistance plasmid to species of Lactobacillus. These results may have significant in vivo implications. The incidence of tetracycline resistance among the cultivable oral microflora in patients undergoing tetracycline therapy for periodontal disease is over 100 times greater than in patients not receiving this treatment. In addition, several of the tetracycline resistant isolates have been shown to carry a plasmid. It seems likely that conditions in plaque, where bacteria are in close contact, might favor the transfer of genetic information by a conjugative-like process. To what extent the observed increase in antibiotic resistant bacteria in patients receiving antibiotic therapy is actually explicable in these terms represents an area for future study.

Our studies on organisms that may be involved in initiating periodontal pathology have also produced interesting new results. In a clinical study we have examined the Actinomyces flora in patients with mild gingival inflammation and those presenting frank pocketing with accompanying bone loss. The predominant Actinomyces population in the former group consists of facultative anaerobic species (A. viscosus and A. naeslandii), whereas in the latter group the obligatorily anaerobic species A. israelii predominantes. This distribution of Actinomyces shows an interesting correlation with certain in vitro studies being conducted in collaboration with the Humoral Immunity Section. It is well known that one of the earliest colonizers of the tooth surface in developing plaque is S. sanguis. We have found that A. viscosus and A. naeslandii (but not A. israelii) can attach to S. sanguis. This is quite specific in the sense that the Actinomyces species will not aggregate with other streptococci such as S. mutans or S. salivarius.

Another interesting organism that has been isolated from human periodontal pockets is a slender gram negative, anaerobic rod that resembles Leptotrichia sp. The organism has never been isolated from any other location in the mouth. In vitro studies have revealed an unsuspected specificity of this organism for attachment to the root surface of the tooth. Electron microscopy has further shown that the organism possesses unusual cell surface organelles which may be involved in its attachment to the root cementum.

Two major lines of research continue to be pursued by investigators in the CELLULAR IMMUNOLOGY SECTION. Endogenous hormone-like mediators that are produced by macrophages and lymphocytes are being intensively investigated. These mediators are produced by lymphoreticular cells in response to a wide variety of antigens, mitogens, adjuvants and pharmacologically active substances. Such mediators can be recovered from supernatants of human and mouse lymphoid cell cultures undergoing a variety of *in vitro* immunological reactions. Studies aimed at delineating the regulation of production as well as characterization and purification of these soluble biologically active agents are being emphasized. This approach has revealed that many different mediators are produced and that a single molecular species may have a number of apparently distinct biological activities. For example, a human macrophage product termed Lymphocyte Activating Factor or LAF is found to have a M.W. of 60,000 and 15,000 daltons, respectively. Human LAF is a potent mitogen for mouse thymocytes and also enhances the thymocyte proliferative response to lectin mitogens. In addition, the higher M.W. LAF is associated with the capacity to increase the expression of differentiation antigens on thymocytes and can activate human T lymphocyte proliferation as well as lymphokine production. In contrast, the lower M.W. (15,000) mitogenic LAF activity produced in the mouse is associated, and perhaps identical with, an activity that promotes T helper cell dependent antibody production. Other closely related studies have revealed that lectin activated mouse splenic lymphocytes produce a lymphokine which co-chromatographs sequentially on Sephadex gels, DEAE cellulose, hydroxylapatite and phenyl sepharose. This lymphokine has three distinct immunological activities; it is mitogenic for mouse thymocytes, it enhances T helper cell activity and induces killer cell functions.

One major problem that has been encountered in these studies is that although these mediators are highly active biologically, and behave as polypeptides, they can be recovered in only minute amounts. Consequently, attempts to produce antibodies to some of these factors have been hampered because of insufficient protein which is required for immunization purposes. The mechanisms controlling production of certain mediators are therefore being studied. Inhibition of prostaglandin synthetase by indomethacin increases production of interferon and colony stimulating factor by mononuclear leukocytes. It has also been found that the generation of free radicals by cultured cells interferes with *in vitro* lymphocyte activation and antibody production. The addition of reducing agents such as 2-ME or glutathione or the free radical scavenger, superoxide dismutase, provides another means of increasing mediator production. In addition, a mouse macrophage cell line P388D$_1$ is available which produces high levels of apparently normal LAF activity. This cell line is currently being used to produce LAF in greater quantities for more extensive purification purposes. Cell lines or hybridomas that produce other mitogenic lymphokines will be employed in future studies. Such approaches will hopefully provide sufficient amounts of these potent activities to permit their biochemical identification and replication.

The second major focus of this section involves a study of the mechanism underlying the action of endotoxin, the biologically active principle or lipopolysaccharide (LPS) derived from the cell wall of gram negative bacteria. Evidence is accumulating which suggests that many of the biological effects of endotoxin are mediated by an initial interaction of LPS with receptors on lymphocytes or macrophages. Using an adoptive transfer system with LPS unresponsive C3H/HeJ mice, it has been found that a bone marrow derived cell type(s) is responsible for LPS-induced adjuvanticity, lethality, tumor necrosis and stimulation of acute phase proteins such as the SAA protein in amyloidosis. Data from a more sophisticated *in vitro* model system suggest that the adjuvant property of LPS requires both LPS sensitive T lymphocytes and macrophages. This hypothesis is supported by the finding that the genetic control of these *in vivo* effects of LPS is identical to the control of the effects *in vitro* of LPS on B lymphocytes and macrophages. Furthermore, evidence using CBA/N hybrid mice suggests that the macrophage may bear most of the responsibility for the biological effects of endotoxin. This question will be pursued using a variety of experimental systems with known or suspected alterations in macrophage function including chronic infection with BCG, use of germ free mice, athymic ("nude"), or endotoxin tolerant mice.

It is now well established that the sensitivity of mice to LPS is controlled by a single gene that is located on the fourth chromosome of the mouse. However, in addition to controlling endotoxin sensitivity and perhaps coding for LPS receptors on the cell surfaces, it has been found that this gene controls a number of other biological processes that do not involve the exogenous administration of endotoxin. Thus, LPS unresponsive C3H/HeJ mice differ from normal mice in a number of ways. They possess (1) increased resistance to the lethal effects of *Herpes simplex* virus, (2) decreased resistance to lethal infection with *Salmonella* and *Klebsiella*, (3) increased thymocyte reactivity to the effects of LAF and (4) decreased tumoricidal capacity. These findings suggest that either endotoxin (or some molecular equivalent) plays a vital role in the maintenance of normal host immune function and resistance or that the LPS gene is one important regulator of these functions.

Studies have been initiated to examine the effect of LPS on macrophages and the induction of soluble mediator production by these cells. LPS stimulates macrophages to produce a number of inflammatory molecules including LAF, colony stimulating factor (CSF), interferon (IF), tumor necrosis factor (TNF) and prostaglandins. Prostaglandins, which have been found to enhance LPS induced production of the enzyme collagenase by macrophages, have been found to suppress the production of CSF and IF. Whether these differences reflect alternate metabolic pathways within individual macrophages or whether they reflect differences in macrophage subpopulations is currently being investigated. Since the production of these endogenous mediators reflects the total host response to endotoxin these studies will help clarify the mechanism of action of endotoxin in gram negative infections as well as those systems that regulate host resistance.

A major emphasis of the HUMORAL IMMUNITY SECTION is the investigation of the immunological regulation of connective tissue metabolism. These studies are concerned with both the catabolism and anabolism of connective tissue elements in response to products of the cellular immune system and humoral mediators such as the complement system. The degradation of collagen by collagenase can be initiated by immunological mechanisms. Thus, macrophages stimulated with mediators derived from lymphocytes synthesize and secrete this enzyme. This production of collagenase is dependent on the stimulation of prostaglandin synthesis by the activated macrophages as shown by the finding that prostaglandin levels are markedly enhanced in cultures of activated cells and inhibitors of prostaglandin synthesis also inhibit collagenase production. This latter effect can be overcome by the exogenous addition of prostaglandins. The mechanism by which prostaglandins regulate collagenase production involves modulation of cyclic nucleotide levels within these cells. An increase in cAMP occurs in the macrophages subsequent to the enhancement of prostaglandin synthesis by the macrophages. Direct evidence for the role of cAMP has been provided by the finding that collagenase production can be restored by the exogenous addition of dibutyryl cAMP to cultures in which both prostaglandin synthesis and collagenase production have been blocked by indomethacin. Stimulation of cells by lymphokines is a prerequisite for the production of collagenase since neither exogenous prostaglandins nor dibutyryl cAMP alone trigger enzyme production. Subsequent to this activation step, prostaglandin levels are enhanced resulting in an increase in cAMP levels followed by the production of collagenase.

Destruction of connective tissue may also result from activation of the complement system. Fetal rat bones in organ culture release calcium in the presence of antibody reactive with a cell surface antigen and complement. Prostaglandins are also implicated in this degradative event since inhibition of their synthesis also inhibits calcium loss from the bones. Macrophages, cells which probably originate from a similar stem cell to the osteoclast which is involved in bone resorption, can be stimulated by antibody and complement to produce prostaglandins. Thus a monocytic cell is implicated in the complement mediated resorption of bone. Complement is known to affect the permeability of cell membranes and recent studies have demonstrated that activation of this mediator system results in a marked increase in the incorporation of the prostaglandin precursor arachidonic acid into prostaglandins in the bone tissue and macrophage cultures. In the bone cultures, subsequent enhancement of bone destruction occurs. The role of complement in bone destruction may be twofold. In addition to its effect on cell permeability, which may enhance the accessibility of prostaglandin precursors, it may also induce the release of prostaglandin precursors from cell membranes.

In addition to the degradative effects of immunologic reactions on connective tissue, products of activated lymphocytes and macrophages can also stimulate connective tissue synthesis. Fibroblasts not only proliferate in response to these mediators but also synthesize enhanced levels of collagen. Relevant to these investigations is the recent finding that hepatic granulomas from mice infected with Schistosoma mansoni stimulate fibroblast proliferation. This provides a possible explanation for the formation of the fibrotic lesions observed in these animals. Thus,

immunologic mechanisms may contribute both to the initial breakdown of connective tissue fibers and to the subsequent fibrosis observed in chronic inflammatory lesions.

Defective function of immunologically competent cells apparently contributes to the abnormal connective tissue metabolism observed in osteopetrosis. Lymphocytes from affected mice of two strains bearing this genetic defect are less responsive to the proliferative effects of mitogens than are those of their normal littermates indicating a lymphocyte defect. The opposite effect is observed in a strain of osteopetrotic rats. Lymphocytes from the osteopetrotic rats proliferate more than do those of their normal littermates. This may be attributed to the finding that macrophages from the affected animals synthesize lower levels of prostaglandins than the macrophages from normal littermates. Since removal of a macrophage rich cell population from the osteopetrotic rat spleens reduces the proliferative response to that of the normal littermate spleens, the primary defect in this model system may be macrophage related.

In continuing collaborations with the Laboratory of Oral Medicine and the Laboratory of Developmental Biology and Anomalies, it has been demonstrated that synthetic chemotactic peptides can be coupled to immunoglobulins without the loss of biological potency. In brief, the chemotactic factor was covalently coupled to anti-herpes antibody. The chemotactic factor fully retained its ability to induce migration of cells while the antibody lost none of its virus neutralizing activity. This highly imaginative work offers the way for rational approaches to specific immunotherapy.

The CLINICAL IMMUNOLOGY SECTION is studying immunological mechanisms in oral inflammatory reactions with particular emphasis on leukocyte functions in human periodontal diseases. The chemotactic responsiveness of polymorphonuclear and mononuclear leukocytes from patients with juvenile periodontitis is being examined. Polymorphonuclear leukocyte chemotaxis in response to C5a, a bacterial factor from E. coli and the synthetic peptide fMet-Leu-Phe was found to be normal in this group of patients. However, there was a reduced capacity of mononuclear leukocytes to respond chemotactically to the constituents of human dental plaque. In studies of histamine release from peripheral blood basophilic leukocytes of patients with varying degrees of gingival inflammation it was found that cell responsiveness was a property of the basophil and histamine release from these cells correlated better with the degree of inflammation than with the severity of bone loss. This suggests that during the acute inflammatory response in gingival disease basophils or mast cells are intrinsically more susceptible to degranulation with activating substances either derived from complement or from bacteria.

Investigations are continuing on the use of histamine release from human basophils as a model for inflammation and exocytosis. Recently, it has been postulated that secretion of granules from cells is dependent on the extracellular osmotic pressure and occurs due to an exchange reaction

mediated by the concentration of Cl^- in the medium. This hypothesis was tested by replacing different anions and cations in the basophil medium. Histamine release by an IgE mediated system occurred in sugar solutions and had only an absolute requirement for Ca^{++}. The results do not support the hypothesis that histamine release is an osmotic phenomenon dependent on the Cl^- concentration. In other studies with human basophils it was possible to define the conditions under which the cells can be "desensitized", i.e. cells can be made unresponsive to an immunological stimulus. This phenomenon shows no immunological specificity, occurs at high but not low antigen concentrations and is temperature and time dependent. It appears to be related to the generation of cell turn-off signals. Utilizing the human basophil histamine release system, mathematical models of the cell activation-desensitization are being developed and tested in collaboration with Dr. C. Delisi of the Laboratory of Theoretical Biology, NCI. The model relating the number of multiple bound antigens on the cell surface indicates that the number of active signals required to initiate release is small and on the order of 5-10. The lack of histamine release at high antigen concentrations is not simply due to lack of cross bridging of IgE molecules but due to desensitization signals.

Recently studies have concentrated on developing a cultured mastocytoma cell line for studies on exocytosis. A subline from the rat basophilic leukemia cell line has receptors for IgE and can be activated for release by either the use of anti-IgE or specific antigen. On cloning this subline, both histamine releasing and non-releasing clones have been obtained. Studies at present are attempting to compare the mechanisms of histamine release from these cultured cells to those from normal cells. Whether the defect in these cells is simply at the level of transduction of the signals at the cell membrane or due to cellular defects is unknown at the present time.

Clinical studies with Sjogren's patients continue to stress the role of genetic factors in the pathogenesis of this disease. Lymphocyte typing has shown that patients seem to carry two B cell alloantigens. Families of patients with Sjogren's syndrome are presently being typed to further define the genetics of these two antigens and their relationship to the disease process. Utilizing sensitive assay procedures, it has been found that Sjogren's patients have a high frequency of circulating immune complexes and several of these patients have developed immune complex glomerulonephritis. These findings suggest that the pathophysiology of Sjogren's disease may be based upon an autoimmune etiology with more generalized organ involvements than previously recognized.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE-0045-08 LMI

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Macrophage and Lymphocyte Derived Mediators that Activate T and B Lymphocytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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LAB/BRANCH

Laboratory of Microbiology and Immunology

SECTION

Cellular Immunology

INSTITUTE AND LOCATION

National Institute of Dental Research, NIH, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.25	1.75	1.50

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Mediators are produced by human macrophages and lymphocytes that have a number of in vitro immunological effects. Macrophages can be activated by a wide variety of agents including phorbol myristic acetate, muranyl dipeptide, "transfer factor", activated lymphocytes and lymphokines to produce a lymphocyte activating factor (LAF). LAF activity elutes in two peaks off BioGel P_{100} columns at 60,000 and 14,000-20,000 daltons. This LAF containing high MW peak augments immunological reactions by 1) stimulating or enhancing the proliferation of mouse thymocytes or human peripheral T cells, 2) increasing the expression of Ly-1 antigen on mouse thymocytes, 3) activating human lymphocytes to produce another lymphokine and 4) others have shown that LAF increases helper cell promotion of antibody responses. In contrast, antigens can induce lymphocytes to produce a factor (LMF) which activates human B cell proliferation. This factor over-laps the lower MW LAF peak. Thus, LAF and LMF can amplify immunological reactions by augmenting lymphocyte proliferation, differentiation and thus promoting lymphocyte functions.

NIDR CLASSIFICATION: 20314

PHS-6040
(Rev. 10-76)

OBJECTIVES:

The role of mediators produced by human macrophages and lymphocytes in the afferent limb of the immune response is being investigated. These endogenous nonspecific mediators appear to be essential in activating and amplifying lymphocyte mediated immunological reactions. Investigations of the nature and mechanism of action of these mediators in in vitro tissue culture models may provide the requisite understanding to permit eventual therapeutic manipulations of the signals initiating and augmenting immunologically induced inflammatory reactions.

METHODS:

1. The mitogenic effect of supernatants of cultured human mononuclear cells and lymphocytes is assayed on mouse thymocytes and human thymus derived (T) and bone marrow derived (B) lymphocytes.

2. These supernatants are also tested for their capacity to activate lymphokine production, to induce increased expression of lymphocyte differentiation markers and to promote helper function of T cells.

3. The effects of mediators partially purified by Bio Gel P_{100}, DEAE Sephadex and hydroxylapatite chromatography on lymphocyte proliferation and differentiation is being tested.

4. The role of cyclic nucleotides in the mechanism of action of these mediators is under investigation.

MAJOR FINDINGS:

Activated macrophages and lymphocytes produce more lymphocyte activating factor (LAF) and lymphocyte derived mitogenic factor for B cells (LMF) respectively, than resting cells. There are a number of interesting agents that induce macrophages to produce LAF. For example, a heat labil serum factor which may be a component of the alternate complement pathway may activate macrophages. Dialyzable extracts of leukocytes that contain transfer factor (skin test converting) activity also activate macrophages to produce LAF. However, the transfer factor activity can be separated from the factors in the leukocyte extract that induce LAF production. A cocarcinogen, phorbol myristic acetate (PMA) is the most potent inducer of this macrophage growth factor activity. Muramyl dipeptide (MDP), a synthetic analogue of mycobacterial cell wall also induces macrophages to produce LAF. Studies using the mouse macrophage cell-line P388D_1 reveal that LAF is produced only if both T lymphocytes and MDP are added to the macrophages indicating that lymphocyte-macropha interaction is required for successful stimulation by MDP. In fact, the most important pathway of macrophage LAF production depends on activated lymphocytes or lymphokines (presumably macrophage activating factor, MAF Thus, any agent that activates lymphocytes can indirectly also induce macrophage LAF production. In fact, we observed that lymphocytes contaminated by only a few percent macrophages would still produce mitog

MAJOR FINDINGS (continued):

factors with the two chromatographically characteristic peaks of activity at 50,000-70,000 and 14,000-22,000 daltons. We have also succeeded by using antigenic rather than mitogenic stimulants in activating lymphocytes to produce a factor which is mitogenic for human B cells. This LMF eluted in the 16,000-22,000 dalton range off BioGel P_{100} columns and therefore overlaps the low MW LAF.

In addition to their lymphoproliferative effects for mouse thymocytes, the pooled 50,000-70,000 dalton fractions of human MNL supernatants eluted from BioGel P_{100} columns were also capable of activating human T lymphocytes to proliferate and to produce a lymphokine that was chemotactic for human monocytes. These pooled fractions also rapidly induce mouse thymocytes to express much more Ly-1 but not Ly-2 antigens on their cell surface as determined by the Fluorescence Activated Cell Sorter. Thus, LAF or activities associated with it promote lymphocyte differentiation, and function as well as proliferation.

We have also continued our studies of abnormal production of macrophage mitogenic factors. 1. Macrophages from C3H/HeJ mice that are genetically unresponsive to endotoxin, in contrast with responder mice, did not produce LAF when incubated with endotoxin. Macrophages from F_1 progeny of responder and non responder mice produced intermediate levels of LAF activity. 2. Macrophages from some patients with Hodgkin's disease or Tuberculosis that are anergic were found to a) suppress lymphocyte transformation; b) to have impaired LAF production and c) to produce more PGE_2 than normal. However, these observations are inconstant and although PGE_2 can inhibit LAF production, both these abnormalities are more probably a late complication rather than a causative factor in the disease process.

SIGNIFICANCE TO DENTAL RESEARCH:

The central roll of cell mediated and humoral immunity in periodontal disease has been documented. Macrophages as well as lymphocytes and plasma cells participate in chronic gingival inflammation. In vitro tissue culture studies can be used to dissect the cellular interactions and to study the exogenous and endogenous signals that contribute to this inflammatory state. Thus, both unfractionated gingival plaque and cell wall extracts of Actinomycoses viscosus are potent activators of in vitro LAF production. This "monokine" may therefore augment inflammation in periodontal disease. Studies of LAF and lymphocyte derived mitogenic factors may eventually provide the means of therapeutic intervention in inflammatory reactions.

PROPOSED COURSE:

Studies designed to characterize and separate the monocyte and lymphocyte derived mitogenic factors chromatographically will be pursued. Attempts to produce antibodies to these mitogenic factors have been initiated.

PROPOSED COURSE: (continued)

Such antibodies can be used to facilitate further purification of these factors using affinity columns, to determine the effects of inhibiting these mediators, and to develop sensitive radioimmunoassays for detecting and identifying the mediators. Agents and conditions that regulate the production of these amplification factors should be determined. The mechanism of action of these mediators must be clarified. It has been established that LAF does not stimulate adenyl cyclase to produce cAMP, but the possibility that LAF activates guanyl cyclase and elevates cGMP is being studied. Furthermore, the levels of cAMP in several subpopulations of human T lymphocytes may differ significantly which may be an indirect consequence of factor induced differentiation. Elucidation of amplifying effects of endogenous mitogenic factors on immunological reactions may provide the keys to pharmacological intervention in inflammatory reactions of the host.

PUBLICATIONS:

1. Koopman, W. J., Farrar, J. J., Oppenheim, J. J., Fuller-Bonar, J., and Dougherty, S.: Association of a low molecular weight helper factor(s) with thymocyte proliferative activity. J. Immunol. 119:55-60,197

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3. Cohen, S., Pick, E., and Oppenheim, J. J. (Eds.): In The Biology of Lymphokines. Acad. Press, N. Y. In press.

4. Oppenheim, J. J., Mizel, S., and Meltzer. M.: Comparison of lymph and monocyte derived mitogenic factors. In Cohen S., Pick, E., and Oppenheim, J. J. (Eds.): The Biology of Lymphokines. In press.

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8. Baker, J. J., Wright, W. E., Chan, S. P. and Oppenheim, J. J.: Longitudinal effects of clinical therapy and the edentulous state on the transformation of lymphocytes from patients with severe periodontitis. Clin. Exp. Immunol. In press.

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11. Schechter, G. P., Wahl, L. M. and Oppenheim, J. J.: Suppressor macrophages in human disease. A Review in Proc. of VIII Internat. Meeting of the RES Society. Pub. Plemum Press, 1978. In press.

12. Oppenheim, J. J. Lymphocyte interaction with macrophages: Induction of LAF production. In Proc. of VIII Internat. Meeting of the RES Society Press. 1978. In press.

13. Oppenheim, J. J., Togowa, A., and Wahl, S.: Immunological role of macrophage and lymphocyte derived amplification factors. In Proc. of 12th Leucocyte Culture Conf. in M. Quastel (Ed.). Acad. Press In press.

14. Oppenheim, J. J., Koopman, W. J., Wahl, L. M., and Dougherty, S. F.: Prostaglandin E_2 rather than lymphocyte activating factor produced by activated human mononuclear cells stimulates increases in murine thymocyte cAMP. J. Immunol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZOI DE 00131-04 LMI

PERIOD COVERED

October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Regulatory Role of Thymus-Derived Lymphocytes on the In Vitro Antibody REsponse

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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	Simon, Philip	Microbiologist	LMI	NIDR

COOPERATING UNITS (if any)

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LAB/BRANCH

Laboratory of Microbiology and Immunology

SECTION

Cellular Immunology Section

INSTITUTE AND LOCATION

National Institute of Dental Research, NIH, Bethesda, Md.

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.00	1.00	2.00

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Culture supernatants from alloantigen- and Con A-stimulated mouse spleen cells contain T cell-derived factors which augment both humoral and cell mediated immune responses in vitro. The biochemical relationship between these factors and thymocyte mitogenic factor has been investigated. Thymocyte mitogenic factor (MW 38,000) has been found to be chromatographically inseparable, following a multiple step purification scheme, from the soluble mediator which enhances the antibody response of nude mouse spleen cells and from a mediator which restores the antigen-dependent cytotoxic response of mouse thymocytes. Thus, the data support the hypothesis that thymocyte mitogenic factor is a component in the activation pathways for both humoral (B-cell-mediated) and cellular (T-cell-mediated) immune responses. In addition, a second cytotoxic cell helper factor (MW 40,000) was similarly found to be inseparable from type II interferon. The finding of a second biochemically distinct cytotoxic cell factor (interferon) within the same supernatant illustrates the point that multiple helper factors with perhaps different mechanisms-of-action may function within the same activation pathway.

NIDR CLASSIFICATION: 20314

OBJECTIVES:

The principal objectives of this research project are: 1) to purify and characterize the mediators which regulate the antibody response; 2) to examine the biochemical relationship between these mediators and the factors which enhance cytotoxic lymphocyte responses and 3) to examine the mechanism-of-action of the immunoregulatory lymphokines.

METHODS:

The in vitro response of murine spleen cells was conducted in standard Mishell and Dutton cultures and examined for antibody-forming cells with the hemolytic plaque-forming cell (PFC) assay of Jerne and Nordin. Standard assays for the presence of cytotoxic lymphocytes (by ^{51}Cr release) and for the presence of Lymphocyte Activting Factor (LAF) were employed in some of the experiments. Chromatographic analyses of the soluble factors were conducted using standard procedures for gel filtration, hydroxylapatite, and hydrophobic chromatography.

MAJOR FINDINGS:

Previously, we demonstrated that one of the "helper" factors (for antibody production) in human allogeneic leukocyte culture supernatants was identical to the macrophage- or monocyte-derived thymocyte proliferative product which has been designated Lymphocyte Activating Factor. This observation suggested the possibility that a single mediator might serve to augment both B cell- and T-cell mediated immune responses. Because a thymocyte proliferative factor might be expected to enhance the generation of thymus-derived cytotoxic lymphocytes, it was of considerable interest to examine the biochemical relationship between T cell-derived thymocyte mitogenic factor and the helper factors active in the induction of hemolytic plaque-forming cell (PFC) responses and cell-mediated cytotoxic responses.

Lymphocyte-derived thymocyte mitogenic factor (TMF)-containing supernatants were generated by stimulating mouse spleen cells with 2.0 µg/ml Con A for 24-40 hrs. These spleen cell culture supernatants were also tested for and found to contain: 1) T cell-replacing factor (TRF) which restores the capacity of nude mouse spleen cells to make antibody in response to the thymic-dependent antigen, sheep erythrocytes (SRC), 2) killer cell helper factor (KHF) which enhances the alloantigen-specific cytotoxic lymphocyte response of mouse thymocytes and 3) type II immune interferon. All of the activities were found to be precipitated by ammonium sulfate between 50-70% saturation. Precipitated supernatant was fractionated by gel filtration on Sephadex G-100. The TMF and TRF activities co-eluted as identical peaks at an apparent MW of 35,000-38,000. The IF eluted as two peaks; one minor peak at approximately 90,000 daltons, and the major peak at MW (approximately 40,000-45,000 daltons) slightly greater than TMF and TRF. The KHF activity eluted as a broad peak which spanned both the IF and TMF/TRF peaks. Fractions encompassing the molecular weight range of 30,000-45,000 daltons were rechromatographed on Sephacryl S-200 and then subjected to hydroxylapatite chromatography. TMF, TRF, and one KHF

all eluted at 0.025M sodium phosphate as a narrow peak with a shoulder on the trailing edge. All of the IF and a second KHF co-eluted at 0.12M sodium phosphate. The hydroxylapatite fractions containing TMF and its associated TRF and KHF activities were further shown to co-elute during hydrophobic chromatography on phenyl sepharose. The IF and its associated KHF were shown to co-elute following affinity chromatography on blue sepharose.

The results of this work indicate that a single lymphocyte-derived factor (TMF) may serve to augment both cell-mediated and humoral immune responses.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

Periodontal disease is characterized by an inflammatory response within the gingival tissue. Histologically, a plasma cell infiltrate predominates in the chronic periodontal lesion. Moreover, dental plaque and its microbial constituents are potent adjuvants and appear to contribute to the exacerbation of the chronic inflammatory lesion. These results suggest that the local humoral immune response may be contributory to the pathogenesis of this disease.

The humoral immune response as well as cell mediated responses are regulated by complex multi-component networks which include immunoregulatory lymphokines produced by both macrophages and lymphocytes. Studies on the biochemical characterization and mechanism of action of the active factors should therefore contribute to our understanding of the mechanism by which lymphocytes are activated and participate in inflammatory reactions.

PROPOSED COURSE:

In the succeeding year a number of studies will be undertaken in order to: 1) test the possibility that TMF-associated TRF is functioning to enhance antibody synthesis indirectly through an activation of residual pre-T cells within the B cell population; 2) examine the intracellular biochemical changes in thymocytes that have been activated by TMF; 3) determine the cells of origin and target cells of the TMF-and IF-associated KHF. In addition, further purification and characterization studies on the TMF and IF will be performed including a survey of lymphocyte cell lines and hybridomas for their capacity to produce either one or both of the factors.

PUBLICATIONS:

1. Koopman, W. J., Farrar, J. J., and Fuller-Bonar, J.: Evidence for the identification of lymphocyte activating factor as the adherent cell-derived mediator responsible for enhanced antibody synthesis by nude mouse spleen cells. Cell. Immunol. 35: 92-98.

2. Farrar, J. J., and Koopman, W. J.: Characterization of macrophage- and lymphocyte-derived mitogenic factors and their effect on the antibody response in vitro. In "Biology of the Lymphockines" (ed. S. Cohen, E. Pick, and J. J. Oppenheim) Academic Press, New York. 1978, In press.

PUBLICATIONS (continued):

3. Farrar, J. J., Simon, P. L., Koopman, W. J., and Fuller-Bonar, J.: Biochemical relationship of thymocyte mitogenic factor and factors enhancing humoral and cell mediated immune responses. J. Immunol. 1978. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00167-03 LMI

PERIOD COVERED
October 1, 1977 - September, 1978

TITLE OF PROJECT (80 characters or less)
The Biological Actions of Bacterial Endotoxin In vivo and In vitro.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.25	3.75	0.50

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The LPS-unresponsive C3H/HeJ mouse strain has been utilized to help elucidate the mechanism of action of bacterial endotoxin (LPS). These mice possess a defect in the LPS-responsiveness of their lymphocytes, macrophages and fibroblasts that is due to a mutation in a single, autosomal co-dominant gene. The biological effects of LPS are probably mediated by an initial interaction of LPS with lymphocytes and/or macrophages since C3H/HeJ mice can be rendered susceptible to LPS-induced lethality, adjuvanticity and tumor necrosis by the adoptive transfer of bone marrow cells from LPS-sensitive mice. These effects may be mediated by a number of biologically active compounds such as prostaglandins, lymphocyte activating factor and tumor-necrosis factor. The LPS gene may play a vital physiological role since it appears to control: 1) susceptibility to Herpes simplex virus and S. typhimurium; 2) immune reactivity of thymocytes and 3) the ability of macrophages to kill tumor cells.

NIDR CLASSIFICATION: 20314

OBJECTIVES:

The major objectives of this project are: 1) to understand the mechanisms by which bacterial lipopolysaccharides and other related products produce lymphocyte activation. 2) To evaluate the role that oral bacterial lipopolysaccharides play in the initiation and continuation of the chronic inflammatory process that results in periodontal disease.

MAJOR FINDINGS:

The C3H/HeJ mouse strain is unresponsive to all the in vivo and in vitro effects of bacterial endotoxin (LPS). Our studies have concentrated on understanding the cause of LPS-unresponsiveness in this mouse strain and utilizing these mice as a model to elucidate the mechanism by which LPS induces its biological effects. We previously found that the B lymphocytes of these mice were intrinsically unresponsive to LPS. Furthermore, we found that it was possible to render these mice susceptible to the lethal effects of LPS by the adoptive transfer of spleen cells from a histocompatible but LPS-responsive mouse (C3H/HeN). This finding strongly suggested that endotoxicity was being mediated by an effect of LPS on some lymphoid cell. Our efforts have focused on determining the type of lymphoid cell involved in mediating endotoxicity, and trying to determine the mechanisms by which this occurred. Our major findings have been:

1) The macrophages of C3H/HeJ were found to be intrinsically unresponsive to LPS, as judged by a failure to be activated by LPS or to exhibit a cytotoxic effect. Macrophage LPS-sensitivity was found to be controlled by a single, autosomal codominant gene that either was closely linked or identical to the gene controlling B-cell LPS responsiveness.

2) In addition to finding that C3H/HeJ macrophages are unresponsive to LPS, several other findings have served to strengthen the role of macrophages as one of the important cells mediating endotoxicity. First we found that the adoptive transfer of LPS-sensitive spleen or bone-marrow cells resulted in the presence of normal numbers of LPS-sensitive macrophages in the C3H/HeJ recipients. Secondly, we found that CBA/N mice, which possess a selective defect in B-cell LPS responsiveness, had normally LPS-sensitive macrophages. Since both types of mice are killed normally by LPS, these findings demonstrate that LPS-sensitive macrophages and not B cells are necessary for endotoxicity. Finally, we found that C3H/HeJ macrophages did not release prostaglandins in vitro when stimulated by LPS, while macrophages from normal mice did. This finding suggested that LPS-induced prostaglandin release might be one of the initiating events in endotoxicity.

3) The study on the effect of LPS on macrophages has been greatly aided by the development of several new, sensitive and reliable assays of macrophage function. One aspect of the cytotoxic effect of LPS on macrophages can now be quantified by measuring the inhibition of phagocytosis of ^{51}Cr labelled SRBC. Using this assay, we have found that the lipid A moiety of LPS mediates phagocytosis inhibition. However, LPS-induced killing appears to be due to some heat labile component, since boiled

MAJOR FINDINGS (continued):

LPS will no longer kill macrophages although it will still inhibit phagocytosis. LPS stimulated macrophages also release a soluble factor, lymphocyte activating factor (LAF) which is a potent mitogen or co-mitogen for thymocytes. Macrophages from C3H/HeJ mice are resistant to both phagocytosis inhibition as well as LAF production.

4) The potential role of lymphoid cells in LPS-mediated events was studied using the adoptive transfer model. LPS will cause the necrosis of subcutaneous tumors in mice. LPS will also induce the production of an acute phase protein, serum amyloid associated protein (SAA). Both these effects can be observed in LPS unresponsive C3H/HeJ mice after the adoptive transfer of LPS-sensitive bone marrow cells. These findings strengthen the hypothesis that all the effects of endotoxin are mediated by the initial effect of LPS on some lymphoid cell, presumably the macrophage.

5) We have also utilized the C3H/HeJ - C3H/HeN mouse model to elucidate the cellular basis of another LPS-mediated event, adjuvanticity. Using an in vitro model of adjuvanticity, it was found that three cell types were required for this effect, B cells, T cells and macrophages. However, by using various combinations of relatively pure LPS-sensitive or resistant cell populations we determined that only the macrophages and T cells had to be LPS-sensitive. These findings suggest that adjuvanticity is mediated by a direct effect of LPS on T cells and macrophages and not on B cells as has been proposed by others.

6) Progress has been made on defining the physiological relevance of the LPS gene. We have identified four distinct biological processes that appear to be controlled by the LPS gene and that do not require the exogenous administration of LPS. As previously reported, C3H/HeJ mice are resistant to the lethal effects of herpes simplex virus. In addition, we have made the following observations:

1. C3H/HeJ mice are extremely susceptible to lethal challenge with S. typhimurium while C3H/HeN mice are resistant.
2. Thymocytes of C3H/HeJ mice are very sensitive to the effects of LAF in vitro in contrast to C3H/HeN thymocytes.
3. Macrophages of C3H/HeJ mice cannot be rendered tumoricidal with BCG. Formal genetic studies have demonstrated that macrophage tumoricidal capacity is controlled by the LPS gene. Analysis of the other effects is proceeding. These findings strongly suggest a vital role for the LPS gene in maintaining host defense mechanisms and the homeostatic control of the immune system.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

Bacterial endotoxins are ubiquitous in nature. Because of their unique ability to activate B lymphocytes, it is postulated that they may be essential to the normal maturation of the immune system. Furthermore, because of their diverse properties, they have been implicated as causative factors in certain pathological conditions such as gram negative shock. Our findings are therefore important to biomedical research for

SIGNIFICANCE TO BIOMEDICAL RESEARCH: (continued)

two reasons. First, they help us to understand the pathways by which B lymphocytes are induced to grow and divide. Secondly, they help us to understand the pathogenesis of the toxic effects of LPS, and suggest possible modes of therapeutic intervention.

One of the major goals of the NIDR is to understand the etiology of periodontal disease (PD) and to explore methods to prevent or eliminate this condition. Since PD is due to chronic inflammatory responses in the gingiva, and since endotoxins from oral flora are abundant in the mouth, it is important to understand how these endotoxins activate lymphoid cells to incite and prolong the immune response that results in PD.

PROPOSED COURSE:

1. The role of macrophages in mediating the effects of LPS will be pursued. Having perfected our techniques for the adoptive transfer of lymphoid cells, we will now attempt to transfer specific cell populations such as macrophages, B cells and T cells to determine the role of each in LPS mediated lethality, adjuvanticity and tumor necrosis. We will also utilize experimental animal systems with known alterations in macrophage function to determine the effect of these changes on sensitivity to LPS. These include: BCG treatment; induction of endotoxin tolerance and the use of germ-free mice.
2. The role of specific macrophage products in these events will be studied. These include the role of LAF in the adjuvant response, and the role of tumor necrosis factor (TNF) in the induction of tumor necrosis by endotoxin.
3. We will try to complete the formal genetic analyses that will help establish the biological relevance of the LPS gene. We will then try to determine the phenotypic expression of this gene in each of the previously described situations.

PUBLICATONS:

1. Kirchner, H., Hirt, H. M., Rosenstreich, D. L. and Mergenhagen, S. E.: Resistance of C3H/HeJ mice to lethal challenge with herpes simplex virus. Proc. Soc. Exp. Med. Biol. 157:29-32,1978.

2. Rosenstreich, D. L., Wahl, S. M., and McMaster, P.R.B.: The role of B-lymphocytes in cell mediated immunity. II. Delayed hypersensitivity induced by DNP-Ficoll in DNP-KLH immunized guinea pigs. Cell. Immunol. 38:116-123, 1978.

3. Ruco, L. P., Meltzer, M. S. and D. L. Rosenstreich.: Macrophage activation for tumor cytotoxicity: control of macrophage tumorcidal capacity by the LPS gene. J. Immunol. In press.

PUBLICATIONS (continued):

4. Rosenstreich, D. L.: The biological function of the LPS gene in "Biology of the Inbred Mouse." (ed. S. Morse) Academic Press, New York, N.Y. In press.

5. Rosenstreich, D. L., Vogel, S. N., Jacques, A., Wahl, L. M., Scher, I. and Mergenhagen, S. E.: Differential endotoxin-sensitivity of lymphocytes and macrophages from mice with an X-linked defect in B-cell maturation. J. Immunol. In press.

6. Rosenstreich, D. L., Vogel, S. N., Jacques, A. R., Wahl, L. M. and Oppenheim, J. J.: Macrophage sensitivity to endotoxin: genetic control by a single codominant gene. J. Immunol. In press.

7. Mergenhagen, S. E., Rosenstreich, D. L., McGhee, J. R., and Wahl, L.M.: Potential role of lymphoreticular cells in the host response to endotoxin. Proc. of the 4th European Immunology Conference. Annals. Immunologiae Hungaricae. In press.

8. Urbaschek, R., Mergenhagen, S. E., and Urbaschek, B.: Failure of endotoxin to protect C3H/HeJ mice against lethal X-irradiation. Infect. and Immun. 18: 860, 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE-00209-02 LMI

PERIOD COVERED: October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Biological and Biochemical Characterization of Lymphocyte Activating Factor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mizel, Steven B.	Senior Staff Fellow	LMI	NIDR
OTHER:	Rosenstreich, David L.	Medical Officer, Research	LMI	NIDR
	Mizel, Diane	Chemist	LMI	NIDR
	Weedon, Lynda L.	Biologist	LMI	NIDR
	Oppenheim, Joost J.	Medical Officer	LMI	NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Microbiology and Immunology, NIDR

SECTION
Cellular Immunology

INSTITUTE AND LOCATION
National Institute of Dental Research, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.75	1.25	1.50

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mouse macrophage cell line P388D$_1$, in conjunction with the potent inflammatory and tumor promoting agent, phorbol myristic acetate (PMA) have been used as a model system for 1) production and characterization of Lymphocyte Activating Factor (LAF) and 2) the analysis of the factors which regulate LAF production and secretion. PMA is a potent stimulant of LAF production by P388D$_1$ cells. The ability of several PMA analogs to enhance LAF production is consistent with their inflammatory and tumor promoting activities. The LAF produced in response to PMA is a 15,000 m.w. protein which is quite resistant to a variety of degradative and denaturing agents. LAF may exist within macrophages as a relatively inactive high molecular weight precursor which is converted to the 15,000 m.w. form upon activation. LAF activity in P388D$_1$ cells has been observed with m.w. of 13,000, 26,000, 39,000, 50,000 and 220,000 daltons.

NIDR CLASSIFICATIONS: 20314

OBJECTIVES:

The major objectives of this project are: 1) to purify and characterize the macrophage-derived Lymphocyte Activating Factor (LAF) which is mitogenic for thymocytes and which appears to play a role in the antibody response of B cells through an activation of helpter T cells; 2) to prepare a monospecific antiserum to the purified LAF; and 3) to understand the mechanism(s) by which T lymphocytes or bacterial products such as lipopolysaccharide (LPS) or phorbol myristic acetate (PMA) enhance the production and release of LAF.

MAJOR FINDINGS:

In order to obtain large quantities of LAF for detailed biological, chemical and immunological characterization studies, we have used as a cell source the murine macrophage cell line, $P388D_1$. These cells produced significant amounts of LAF after stimulation with PHA-activated T cells or lipopolysaccharide. However, the usefulness of these stimulants is limited since it is not possible to routinely obtain the numbers of activated T cells required for large scale LAF production (20-100 liters of supernatant) and LPS not only interferes with concentration and chromatographic procedures, but is also heterogenous and may, in some cases, interfere with thymocyte proliferation and antibody formation assays. We have concluded that an optimal stimulant should possess the following basic properties: 1) potent LAF-inducer; 2) small molecule of well-defined structure; 3) free of thymocyte (LAF-target cell) mitogenic activity; and 4) easily removed from LAF-containing preparations. In an effort to define such a stimulant, we have investigated the ability of the tumor promoter and inflammatory agent, phorbol myristic acetate (PMA) to induce LAF production, since PMA is a small molecular weight molecule which has been shown to be a potent macrophage activator. Our major findings are as follows: 1) Phorbol myristic acetate markedly stimulated LAF production by $P388D_1$ cells as well as normal human and mouse macrophages. This effect was both time and concentration dependent. Maximal stimulation of LAF production was usually observed when $P388D_1$ cells were incubated with 1×10^{-6}M PMA for approximately 144 hours.

2) Several phorbol derivatives were tested for their ability to stimulate LAF production by $P388D_1$ cells. PMA (phorbol-12-0-tetradecanoyl-13-acetate), PDD (phorbol-12, 13-didecanoate) and PDB (phorbol-12, 13-dibenzoate) all stimulated LAF production. PMA and PDD exhibited comparable activity and were more effective LAF-inducers than PDB. In contrast, the parent compound, phorbol (at concentrations up to approximately 3×10^{-5}M) was inactive. The pattern of phramacological specificity exhibited by the phorbol derivatives in the stimulation of LAF production is interesting in view of the observation that PMA and PDD also exhibit similar activities as tumor promoters and irritants, whereas, PDB is less effective and phorbol is inactive.

OR FINDINGS (continued)

3) In previous studies we demonstrated that P388D$_1$ cells made small amounts of LAF spontaneously and higher amounts in response to activated T cells or LPS. In each case, the supernatant LAF exhibited a molecular weight of approximately 16,000 daltons when chromatographed on Sephadex G-75 or BioGel P$_{100}$. No other molecular weight forms of LAF were observed. It was therefore important to determine the molecular weight of the PMA-induced LAF. When a concentrated supernatant from cultures of PMA-stimulated P388D$_1$ cells was chromatographed on a Sephacryl S200 Superfine column, LAF eluted with molecules of molecular weight 15,000 daltons, thus confirming our previous observation that P388D$_1$ cells release a molecular weight species of LAF which is similar to that reported for the normal macrophage-derived LAF. In contrast to the uniformity of molecular weight exhibited by the LAF released from P388D$_1$ cells, several charge subsepecies of LAF have been resolved on DEAE cellulose. The LAF produced in response to PMA also exhibited charge heterogeneity. When the peak of LAF activity from the Sephacryl column was chromatographed on DEAE cellulose, major peaks of LAF activity eluted in the range of 30 and 50 mM NaCl, and a minor peak between 60 and 70 mM NaCl. In contrast to results with the T cell-induced LAF, no PMA-induced LAF activity was detected in the DEAE column breakthrough. Thus, the PMA-induced LAF more closely resembles the LPS-induced LAF which also does not exhibit activity in the DEAE column breakthrough. Using ammonium sulfate fractionation, batch DEAE chromatography, Sephacryl S200 gel filtration, hydroxyapatite, and hydrophobic chromatography on phenyl-Sepharose, we have achieved a 2000-fold purification of LAF.

4) We have also investigated the chemical nature of PMA-induced LAF using enzymes and a variety of protein denaturing agents. LAF appears to be a protein as evidenced by its sensitivity to treatment with pronase or papain. Interestingly, LAF is only sensitive to papain when LAF is in an open denatured form. This suggests that LAF is a very tight molecule which easily refolds after denaturation. LAF is not sensitive to trypsin or a chymotrypsin under nondenaturing conditions. LAF is insensitive to 8M urea, 0.1-0.5% SDS, reduction and alkylation, high salt, and pH 4-9. Since LAF is not sensitive to SDS we have determined the molecular weight of LAF on SDS polyacrylamide gels. A value of approximately 12,000 daltons was obtained. This is in good agreement with the value from gel filtration chromatography (15,000 daltons). In addition, this result indicates that LAF is a polypeptide chain.

5) We have recently initiated a series of experiments to obtain antiserum to LAF. We have injected rabbits with out most purified preparations of LAF and have bled these animals after boosting them with additional LAF injections. These sera are presently being tested for anti-LAF activity.

6) In addition to our studies on the purification of PMA-induced LAF, we are also using PMA as a probe to investigate the regulation of LAF production and secretion. We found that within 1 hr after addition of PMA to P388D$_1$ cells, the intracellular level of LAF in these cells increased approximately four-fold. This increase did not require new protein synthesis, suggesting the presence of an intracellular precursor of LAF which is activated and

MAJOR FINDINGS (continued)

released in response to PMA. This hypothesis was evaluated by examining the nature of the LAF within the activated $P388D_1$ cells. Interesting, several molecular weight species of LAF activity were observed with molecular weights of 13,000, 26,000, 39,000, 50,000 and 220,000 daltons We have focused our attention on both the 13,000 and 50,000 molecular weight species since they are present in the greatest quantities. By a variety of criteria the 13,000 m.w. species is similar to the LAF obtained in the culture supernatants of these cells. We have attempted to convert the 50,000 m.w. species to low LAF using 8M urea, SDS + 2-Mercaptoethanol, and salt. However, none of these treatments altered the molecular weight of the 50,000 m.w. species. Chemically, the 50,000 m.w. LAF is sensitive to pronase, but not to trypsin, chymotrypsin, urea, SDS or high salt. Thus, it is similar to low LAF in its chemical sensitivities. It is quite possible that this high molecular weight form of LAF is converted to low LAF by enzymatic cleavage. This possibility is currently under investigation.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

Although it is well accepted that the immune system plays a dominant role in a variety of inflammatory reactions, the underlying mechanisms are not well understood. Several projects which have been undertaken in the LMI/NIDR, are united by a common effort to understand the role(s) of lymphokines in immunological function. It is only through an understanding of macrophage and lymphocyte function at the cellular and subcellular level, that we will be able to rationally manipulate the immune system for therapeutic purposes. In addition, the understanding of the mechanism(s) by which PMA, a potent inflammatory agent, activates macrophages would provide a foundation for a more general understanding of inflammation, especially as it applies to periodontal disease.

PORPOSED COURSE:

1) Studies on the purification and characterization of LAF will be extended as large amounts of material become available. In this regard, we are currently evaluating a microbead carrier method for increasing cell yields by 50-500X. This would provide for more cells per unit culture medium, and thus more LAF per unit culture medium.

2) Studies on the preparation of antisera to LAF will be continued.
3) Studies on the regulation of LAF production will be continued. Results obtained with $P388D_1$ cells are now being used to make predictions as to the nature of LAF regulation in normal resident and activated peritoneal macrophages. In addition, we plan to continue our parallel studies on the regulation of LAF production by activated T cells in the hope that a common mechanism of macrophage activation will emerge.

4) The mechanism of action of PMA on LAF production will be studied in considerable detail. In view of the potent inflammatory properties of PMA, these studies may provide invaluable clues and models for the study of inflammation in periodontal disease.

PUBLICATIONS:

1. Mizel, S. B., Oppenheim, J. J., and Rosenstreich, D. L.; Characterization of lymphocyte-activating factor (LAF) produced by the macrophage cell line, $P388D_1$. I. Enhancement of LAF production by activated by activated T lymphocytes. J. Immunol. 120: 1497, 1978.

2. Mizel, S. B., Oppenheim, J. J. and Rosenstreich, D. L.: Characterization of lymphocyte-activating factor (LAF) produced by the macrophage cell line, $P388D_1$. II. Biochemical characterization of LAF induced by activated T cells and LPS. J. Immunol. 120: 1504, 1978.

3. Mizel, S. B., Rosenstreich, D. L., and Oppenheim, J. J.: Phorbol myristic acetate stimulates LAF production by the macrophage cell line, $P388D_1$. Cell. Immunol. 1978. In press.

4. Rosenstreich, D. L., and Mizel, S. B.: The participation of macrophages and macrophage cell lines in the activation of T lymphocytes by mitogens. Immunol. Rev. 40: 102, 1978.

5. Oppenheim, J. J., Mizel, S. B., and Meltzer, M. S.: Comparison of lymphocyte and mononuclear phagocyte derived mitogenic amplification factors. In Biology of the Lymphokines (ed. S. Cohen, E. Pick, and J. J. Oppenheim). Academic Press, New York, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00210-02 LMI

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunological Studies of a Plaque-Susceptible Rat Strain

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ito, Nobuyoshi	Visiting Associate	LMI-NIDR
COPI:	Rosenstreich, David L.	Medical Officer-Research	LMI-NIDR
OTHER:	Mergenhagen, Stephan E.	Chief, Lab. Micro.&Immuno.	LMI-NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Microbiology and Immunology

SECTION

Cellular Immunology Section

INSTITUTE AND LOCATION

National Institute of Dental Research, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two closely related strains of rats with differential susceptibility for plaque production have been developed from the Wistar-Kyoto Rat Strain. Plaque-susceptible rats develop large plaque deposits after two weeks on a soft diet, while plaque-resistant rats do not develop plaque at any time. The immunological status of these two strains has been investigated. The functional ability of both T and B lymphocytes was identical in adults from both strains. Serum immunoglobulin and salivary IgA levels were also identical in rats on a hard diet. However, plaque-susceptible rats exhibit elevated serum IgM after a prolonged period on a soft diet which produces gingival plaque deposits. This elevation is due to the plaque which since it is not present in plaque-susceptible rats fed a hard diet which remain plaque-free. These findings suggest that the abnormal accumulation of plaque in these rats is not due to an immunological abnormality.

NIDR CLASSIFICATION: 20314,20216, 20217, 20211

OBJECTIVES:

The major objectives of this project are: 1) to determine why plaque-susceptible rats accumulate gingival plaque deposits. 2) To utilize the plaque-susceptible rat as a model of periodontal disease.

MAJOR FINDINGS:

A colony of plaque-resistant (ODU-R) and plaque-susceptible rats (ODU-S) has been developed. We now maintain small colonies of each in our own animal facility and in addition, have a colony of plaque-susceptible rats maintained under germ-free conditions in the animal production unit.

Preliminary genetic analysis of the inheritance of plaque-susceptibility suggests polygenic control. No other rat strain was found to be plaque-susceptible except the WKY which exhibited slight plaque accumulation. In addition, we are using selective breeding to develop a gingivitis-susceptible substrain of the ODU-S rat.

An immunological analysis of plaque-resistant and plaque-susceptible strains was performed. The functional activity of T and B lymphocytes was determined by studying the response of spleen cells to a variety of T and B cell mitogens in vitro. No difference was found between plaque-resistant and plaque-susceptible rats. In addition, serum IgM, IgG and IgA, and salivary IgA levels were determined in these strains maintained on both soft and hard diets. No difference in immunoglobulin levels was observed in rats on hard diets (plaque-free) but plaque susceptible rats on soft diets did manifest slightly elevated IgM levels that increased with age. These findings suggest that the development of plaque-susceptible rats is not due to an intrinsic immunological abnormality. However, young (5-week old) ODU-S rats possess a marked defect in response to PHA. This defect appears to be due in part to excess numbers of splenic suppressor cells.

The relationship between inflammation and bone loss has also been investigated using the ODU-S strain. Some ODU rats lose their incisors approximately three months after treatment with 100 mg/kg of the immuno-suppressive agent, cyclophosphamide. Tooth loss is preceded by the return of splenic T cell responsiveness to PHA and the development of gingivitis. Those rats that do not develop gingivitis (ODU-R and any other strains) do not lose their incisors. Although preliminary, these findings support the role of activated T cells in bone loss.

IGNIFICANCE TO BIOMEDICAL RESEARCH:

Plaque is the major etiological agent of periodontal disease. The availability of closely related rat strains with such a marked difference in susceptibility to the development of plaque can serve as a powerful model for studying the cause of plaque accumulation. Study of these rats

may help explain why some patients develop plaque while others with similar levels of oral hygiene do not. In addition, these rats have the potential of becoming an important model for studying the etiology of periodontal disease.

PROPOSED COURSE:

1. Genetic Analysis. This study will be continued. The nature of the gene(s) controlling plaque-susceptibility will be determined by crossing plaque-susceptible and plaque-resistant mice. F_1, F_2, and backcross generations will be analyzed and the number and type of inheritance investigated. The gingivitis susceptible strain will be further developed and characterized.

2. The Immunological Basis of Periodontal Disease. These studies will be continued by Dr. Ito at the Osaka University Dental School.

PUBLICATIONS:

1. Ito, N., Mergenhagen, S. E. and Rosenstreich, D. L.: Immunological comparison of plaque resistant and susceptible rats. Amer. Assoc. for Dental Res. Abstr. 1977.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1-DE-00238-01 LMI

PERIOD COVERED

October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Regulation of Mediator Production in Endotoxin-Stimulated Macrophages

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Moore, Robert N.	Staff Fellow	LMI NIDR
OTHER:	Urbaschek, Renate	Visiting Associate	LMI NIDR
	Mergenhagen, Stephan E.	Chief, Lab. Micro. & Immun.	LMI NIDR

COOPERATING UNITS (if any)

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SECTION

Cellular Immunology Section

INSTITUTE AND LOCATION

National Institute of Dental Research, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	1.25	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The controlling mechanisms which regulate production of inflammatory mediators by endotoxin stimulated murine macrophages are currently under investigation. Research has concentrated upon two important mediators, interferon and granulocyte-macrophage colony stimulating factor (CSF), and evidence has been obtained indicating that prostaglandins may serve a regulatory role in production of both mediators. Addition of indomethacin, a potent inhibitor of prostaglandin synthesis, to endotoxin-stimulated macrophage cultures has been shown to enhance interferon production and to elevate the colony stimulating activity of culture supernatant fluids. Work currently in progress is designed to determine if prostaglandins do indeed serve to regulate interferon and CSF production and if this controlling influence is mediated via cyclic nucleotides.

NIDR CLASSIFICATION: 20314

OBJECTIVES:

The major objective of this project is to investigate the nature of regulatory mechanisms functioning in an inflammatory response to control production of biologically active mediators. Particular emphasis is placed on understanding the interaction between macrophages and bacterial endotoxin resulting in production of interferon and colony stimulating factor (CSF).

MAJOR FINDINGS:

While it is a well established fact that bacterial endotoxin stimulates leukocytes to produce biologically active substances, previous attempts to investigate interferon induction by endotoxin *in vitro* have been hampered by low yields of the mediator. A major portion of the first year's work in this project has been involved with ascertaining culture conditions required for maximal interferon production. As a result of experiments investigating parameters such as cell source, cell numbers, endotoxin dose and culture time, culture conditions have been established which yield interferon levels two to five times greater than those previously reported. Utilizing this *in vitro* system preliminary experiments have been performed to investigate pertinent factors which may be involved in regulating interferon production. With regard to this mediator our results to date are:

1. Resident peritoneal cells from mice were found to produce interferon in response to endotoxin challenge, however, the yields of interferon were erratic. Much better results were obtained with peritoneal exudate cells from mice injected four days prior with thioglycollate broth. These cells cultured at relatively high cell numbers (7-10 x 10^6/ml) with moderate doses of endotoxin (1-10 µg/ml) produced amounts of interferon equivalent to 100-150 NIH reference interferon units, activities far in excess of previously reported endotoxin-interferon titers. Peak levels were obtained 6-8 hr after endotoxin challenge after which time interferon titers rapidly decreased in the culture supernatants.
2. Addition of indomethacin to PEC at the time of endotoxin challenge resulted in a marked enhancement of interferon production. Interferon levels approaching twice that of control cultures were observed in indomethacin treated cultures. Since indomethacin itself did not significantly affect the antiviral activity of interferon, the observed differences clearly represent a modification in the culture system affecting the supernatant interferon titer. The presence of indomethacin did not prolong interferon synthesis but instead enhanced the rate of production apparently by interfering with some regulatory mechanism which functions normally to control the rate of interferon synthesis and/or secretion.

MAJOR FINDINGS (continued):

A similar evaluation has also been initiated with respect to endotoxin-stimulated CSF production. CSF, unlike interferon, was not produced by thioglycollate-induced PEC. When resident peritoneal leukocytes were challenged *in vitro* with endotoxin, however, detectable levels of this mediator were produced. Colony stimulating activity of the culture supernatant was also markedly enhanced when indomethacin was added to the cultures at the same time as the endotoxin stimulant. Subsequent experiments failed to detect significant levels of inhibitory material in culture supernatants not containing indomethacin, suggesting that CSF production like interferon production is regulated by a cellular control mechanism that is sensitive to the presence of prostaglandins.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

Macrophages exposed to inflammatory stimuli such as bacterial endotoxin are known to produce substances which have profound effects on surrounding tissues and other cells of the immune system. Little, however, is known of the control mechanisms which function to regulate production of these mediators. It is essential that these basic mechanisms be identified and characterized before a thorough understanding of processes existing in inflammatory responses can be realized.

The experiments described above represent an attempt to ascertain regulatory mechanisms for the production of two such important mediators, interferon and CSF. The results indicate that production of both factors is influenced by similar processes possibly involving prostaglandins, a third form of mediator produced by stimulated macrophages.

PROPOSED COURSE:

Further efforts will be made to determine cultural conditions required for optimal production of both interferon and CSF *in vitro*. These studies will provide valuable information with regard to factors influencing macrophage response to endotoxin and will also provide model systems which may be adapted for future investigation of other mediators.

Investigations of the enhancing effect of indomethacin on both interferon and CSF production will be continued. Experiments will be designed to determine if this effect is attributable to a direct action of indomethacin on prostaglandin synthesis and subsequent alterations in cyclic nucleotide levels or is due to some other activity of the drug. Based on knowledge gained in the *in vitro* studies attempts will be made to extend these findings to the response of intact animals to bacterial endotoxin. Particular emphasis will be placed on granulopoiesis and known activities of interferon.

PUBLICATIONS:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 DE-00242-01 LMI

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Role of Oxygen-Derived Free Radicals in Inflammation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hoffeld, J. T.	Dental Officer	LMI NIDR
OTHER:	Oppenheim, J. J.	Medical Officer	LMI NIDR
	Curl, T. L.	Co-Step Dental Officer	LMI NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Cellular Immunology Section

INSTITUTE AND LOCATION
National Institute of Dental Research, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.50	1.50	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

One in vitro model of the inflammatory immune response is the murine primary antibody response system. We have shown, during the past year, that the response in this system is dependent on the concentration of reduced glutathione in the serum used, and that the enhancement of this response by the addition of 2-mercaptoethanol (2-ME) is a result of its reaction with glutathione. One of the functions of glutathione in vivo is the neutralization of free radicals; therefore we investigated the role of free radicals in this in vitro model of inflammation. Oxygen-derived chemical free radicals (viz., superoxide, hydroxyl, and singlet oxygen) are produced in primary cell cultures, both by active generation by the cells, and by autoxidation of dead cells. These radicals are particularly damaging to cells in the culture. We have found that free radical scavengers such as 2-ME/glutathione and superoxide dismutase can enhance the response of the system to the same degree, independently, and are additive in their effects. The common enhancement of this system by various free radical scavengers provides an in vitro model for the damage to neighboring cells, caused by the generation of free radicals by immunocompetent cells in chronic inflammation.

NIDR CLASSIFICATION: 20314

OBJECTIVES:

1. Demonstrate the deleterious effects on mammalian cells, in vitro, of spontaneously generated oxygen-derived free radicals.

2. Define endogenous mechanisms of neutralization of these free radicals in fetal calf serum, growth medium, and leukocyte subpopulations.

3. Modulate immune response activity in vitro by altering either production or activity of these free radicals.

METHODS:

1. In vitro immune response - the primary in vitro antibody response culture system of Mishell and Dutton was used throughout these experiments.

2. Assays for free radical induced cell damage - Two different assays provided a measure of the amount of cell damage in the cultures by measuring the end products of membrane lipid peroxidation. These assays are the lipid soluble fluorescence assay and the thiobarbituric acid assay.

3. Assays for content of glutathione - The paired spectrofluorometric assays of Hissin and Hilf were used to determine the concentration of reduced (GSH) or oxidized (GSSG) glutathione in samples of fetal calf serum (FCS).

4. 2-Mercaptoethanol (2-ME) treatment of FCS - The method of Opitz, et al. was used to "activate" FCS with 2-ME, then remove the 2-ME by lyophilization.

5. Assay of immune response activity - At the end of the cell culture period, the number of cultured cells able to secrete antigen-specific antibody was measured by the plaque-forming cell assay of Jerne and Nordin.

MAJOR FINDINGS:

It has been known for a number of years that cells in culture exposed to oxygen are subject to membrane lipid peroxidation, due to the generation of highly reactive chemical free radicals (including superoxide, hydroxyl, and singlet oxygen). These radicals are generated both as byproducts of a large number of biochemical reactions in the cells and as a function of the release of free energy in cell membranes of dead cells undergoing autoxidation. Leukocytes, in particular the macrophages and polymorphonuclear neutrophils, generate large quantities of free radicals as a mechanism of killing phagocytosed cells. Several mechanisms have been demonstrated in vivo and in vitro which prevent widespread damage to neighboring cells.

MAJOR FINDINGS (continued):

Since the in vitro antibody response is so critically dependent on the commercial lot of FCS, and since no single factor had been shown to correlate regularly with this supportive ability, it seemed reasonable to hypothesize that the supportive function might be an endogenous free radical neutralizing mechanism. The fact that 2-ME significantly augments the response suggested that a thiol-containing compound might be subserving this function. Of the known endogenous, thiol-containing free radical neutralizing substances, the most efficient and widely distributed is glutathione. During the past year, we have shown that the concentration of GSH in FCS correlates directly and definitively ($p < 0.00025$) with the degree of support provided by FCS in cultures. The depletion of GSH in FCS by mild heating procedures, diminishes the response supported by that FCS; this diminished support can be restored by the addition of commercially-prepared GSH. Red cell lysate (depleted of membrane fragments) which contains physiological proportions of glutathione and its regulatory enzymes, glutathione peroxidase and glutathione reductase, can convert a poorly supportive FCS to one which supports a good response.

We have additionally shown that the enhancement provided by 2-ME is mediated by its interaction with glutathione. Dialyzed FCS fails to be enhanced by the addition of 2-ME. 2-ME treatment of the FCS dialysate followed by lyophilization (to remove 2-ME) and addition to dialyzed FCS restores the ability of that FCS to support a response with no 2-ME in the medium, to the level provided by the untreated FCS in the presence of 2-ME. Similarly, 2-ME treated, lyophilized GSH restores the ability of a serum depleted of GSH to support a response in the absence of 2-ME.

Several free radical scavengers have been shown to augment the primary antibody response in vitro (viz, vitamins C and E). We have shown that the glutathione redox system (also a free-radical scavenging system) could subserve the same function.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

The study of immune function in regard to numerous pathological processes has shown the reactive leukocytes to be either beneficial or deleterious, depending on a number of both defined and undefined parameters. One of these parameters, only recently defined, is the ability of these cells to both generate and neutralize oxygen-derived free radicals. Recent work in a number of laboratories has implicated free radicals as the mediators of tissue damage in several chronic inflammatory conditions. The study of the mechanisms of generation and neutralization of these reactive intermediates should provide useful insights into pathological processes such as periodontal disease and rheumatoid arthritis.

PROPOSED COURSE:

We propose to extend these *in vitro* findings to the area of periodontal disease, both by the use of animal models and by the study of human tissue. The use of animal models would permit pharmacologic manipulation of the free-radical scavenging capability of the host to determine the involvement of this mechanism in protection against the pathological process. Human tissues from sites of chronic inflammation will be analyzed for evidence of damage due to free-radical mechanisms.

PUBLICATIONS:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE-00046-08 LMI

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)
Mediator Production by Lymphocytes and Their Role in Inflammation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Wahl, Sharon M.	Research Microbiologist	LMI	NIDR
OTHER:	Wahl, Larry M.	Senior Staff Fellow	LMI	NIDR
	McCarthy, James B.	Biologist	LMI	NIDR
	Kennedy, John B.	Biologist	LMI	NIDR

COOPERATING UNITS (if any)
David Wyler, NIAID, Laboratory of Parasitic Diseases

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NIH, NIDR, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.25	0.75	1.50

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Research in this laboratory focuses on the unique mechanisms of activation of T and B lymphocytes and how these cells interrelate in cellular immune responses. Once triggered, these lymphocytes proliferate and produce biologically active lymphokines which may act on macrophages to initiate migration (chemotactic factor) and enzyme production (macrophage activation factor). Whether these and other signals for macrophage activation are translated via alterations in cyclic AMP and GMP levels is being investigated. In addition to lymphokines which trigger macrophage function, activated lymphocytes also release a soluble factor(s) which stimulates fibroblast proliferation and modulates collagen synthesis. Activated macrophages can also influence fibroblast function. By these mechanisms the immune system may control connective tissue metabolism in inflammatory lesions.

NIDR CLASSIFICATION: 20314

OBJECTIVES:

The ongoing research in this laboratory focuses on understanding the mechanisms by which thymus dependent (T) and bone marrow derived (B) lymphocytes are involved in cellular immune phenomena. This includes the identification and characterization of biologically active agents produced by activated lymphocytes which may modulate the function of both inflammatory and noninflammatory cells.

In addition to T and B lymphocytes, macrophages play a prominent role in immune responses. We are investigating how lymphokines and other agents activate these phagocytic cells to migrate and to increase their biochemical functions as primary perpetrators of the inflammatory response.

Connective tissue metabolism is markedly altered in many inflammatory lesions. The co-existence of inflammatory cells and connective tissue cells in such lesions led us to investigate whether lymphocytes and macrophages could influence fibroblast function.

METHODS EMPLOYED:

Methods utilized to investigate the aforementioned objectives include already published procedures for macrophage chemotaxis, lymphocyte culture, proliferation, lymphokine production, cyclic AMP and GMP determinations, fibroblast culture, and analysis of collagen formation.

MAJOR FINDINGS:

T lymphocytes when activated by appropriate specific antigens respond by proliferating and by producing a number of biologically active mediators referred to as lymphokines. Our recent findings have implicated B cells as coparticipants in cellular immune phenomena. In addition, in a unique situation involving the T independent antigen DNP-ficoll, B cells alone appear capable of initiating a delayed hypersensitivity response through the production of lymphokines such as chemotactic factor and MIF. Another lymphokine produced by activated T and B lymphocytes is macrophage activat factor (MAF) which stimulates macrophages to increase the synthesis of enzymes, including the enzyme collagenase. This enzyme production is dependent upon an increase in prostaglandin synthesis which in turn eleva intracellular levels of 3'-5' cAMP. That this elevation in cyclic AMP may control macrophage activation will be further explored by determining its role in modulating the production of biologically active mediators (i.e., FAF, see below) which is also a function of stimulated macrophages.

Inflammatory lesions are frequently characterized by marked alterations in the adjacent connective tissue. This led us to investigate a modulator role for immune cells in fibroblast function. We have demonstrated that guinea pig dermal fibroblasts can be stimulated to proliferate by supernat from DNP-OA activated T lymphocytes. Furthermore, these fibroblasts appe to increase collagen synthesis as a consequence of exposure to lymphocyte mediator(s). We have also found that macrophages when activated by vari

OR FINDINGS (continued):

means have the potential of releasing a soluble factor which initiates fibroblast proliferation. This mediator is produced early after activation of the macrophages and is not found in the culture fluid of nonstimulated cells. Preliminary characterization of this factor indicates that it is a molecule of 30-50,000 M.W. Thus, the immune system may be responsible for the alterations in connective tissue seen in inflammation.

In light of our findings that lymphoid cells can influence fibroblast function, we have explored an in vivo model of delayed hypersensitivity for possible interactions between lymphoid cells and fibroblasts. Infection of mice with Schistosoma mansoni results in granuloma formation in the liver which is then followed by hepatic fibrosis. The cause of this fibrosis, which results in death of the animals, is unclear. Our investigations suggest that the liver granulomas which form in response to the schistosomal infection stimulate fibroblast proliferation with the release of a soluble molecule(s) and thus may play a role in the development of hepatic fibrosis in S. mansoni infections. We are currently attempting to define the cellular source of this fibroblast stimulating mediator within the granuloma.

In related studies we are exploring macrophage function in irradiated dogs. In the early intervals following irradiation the dogs are monocytopenic but following transplantation with bone marrow or peripheral blood cells their monocyte levels begin to rise slowly. However, in preliminary studies it appears that these cells cannot function normally as measured by the chemotaxis assay. Thus, we hope to determine at what stage in the development of these cells from precursor form to mature monocyte that they develop the capacity (receptors, intracellular equipment, etc.) to migrate toward an appropriate stimulus. Other parameters of macrophage function will also be studied.

NIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

Cell-mediated immunity is a major component of the total immunologic response associated with certain pathologic conditions. While T cells were once considered the primary constituent of cell-mediated reactions, it is now evident that B cells can also be triggered to engage in these reactions. This is of consequence in lesions such as those found in periodontal disease where B cells are a predominant component. By identifying a role for B cells in such pathologic conditions it becomes feasible to alter their activity and thereby possibly modify the course of the disease.

Activated lymphocytes may be largely responsible for the production of chemotactic stimuli which attract macrophages to sites of infection or inflammation. Once localized at such a site these macrophages may be activated to produce a number of enzymes and to release soluble factors (prostaglandins, fibroblast activating factor, etc.), which influence

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH (continued)

other cells. If continuously activated as in chronic inflammatory lesions like periodontal disease and rheumatoid arthritis, these cells may contrib to the pathologic tissue destruction by the release of lysosomal enzymes, collagenase, and prostaglandins. Thus, through an understanding of the wa these cells are triggered, it may be possible to control or modulate their function.

Furthermore, the interaction of the immune system with components of the connective tissue system resulting in both destruction and construction of connective tissue may aid in the understanding of the mechanisms involved in excessive connective tissue destruction seen in chronic inflammatory disease as well as the excessive formation of collagen seen in fibromas and other related lesions.

PROPOSED COURSE:

Investigations are continuing into the mechanisms of induction of inflammation which in some cases may progress to chronic pathologic lesion The pathways by which lymphocytes are triggered to proliferate and to release the factors responsible for mediating inflammatory lesions require further study. Further characterization of the T cell mediator capable of mediating B cell activation in cellular immune reactions is planned.

Attempts will be made to further define the various parameters of macropha activation and how they relate to variations in the intracellular levels of 3'-5' cAMP. Such parameters include nonlysozomal enzyme release (e.g. collagenase), lysozomal enzyme release (e.g. lysozyme), phagocytosis glucose uptake, protein synthesis and the production of fibroblast activat factors. Furthermore, the differentiation of immature macrophages into functional cells will be explored in the bone marrow transplanted dog mod It is essential to understand the process of macrophage activation in ord to be able to control it in chronic inflammatory lesions such as occur in periodontitis.

Areas of study will also focus on the interaction between the lymphoid system and connective tissue components. Lymphocytes and macrophages app to be able to influence fibroblast growth and thereby may provide a pathw for normal connective tissue reconstruction following injury and/or for the markedly altered connective tissue changes which occur in various pathologic conditions. These studies will be pursued both in our in vitr model and also in the in vivo model of schistosomiasis. Characterization of the soluble mediators produced by the lymphocytes and macrophages will e correlated with the mediators released from active granulomas. Additiona the role of lymphocytes in modulating fibroblast function in the patho-physiologic processes associated with scleroderma in humans will be investigated.

JBLICATIONS:

1. Rosenstreich, D. L., S. M. Wahl, and P. R. B. McMaster. 1978. The role of B-lymphocytes in cell-mediated immunity. II. Delayed hypersensitivity induced by DNP-Ficoll and DNP-KLH immunized guinea pigs. Cell. Immunol. (In press).

2. Wahl, S. M., L. M. Wahl, and J. B. McCarthy. 1978. Lymphocyte mediated activation of fibroblast proliferation and collagen-production. J. Immunol. 120: In press.

3. Wyler, D. J., S. M. Wahl, and L. M. Wahl. 1978. Hepatic fibrosis in schistosomiasis. Egg granulomas secrete fibroblast stimulating factor in vitro. Science. In press.

4. Olsen, C. E., S. M. Wahl, L. M. Wahl, A. L. Sandberg, and S. E. Mergenhagen. 1978. Immunological defects in osteopetrotic mice. In Mechanisms of Localized Bone Loss. Supp. Calc. Tissue Abstracts. Information Reticulal. Inc., Washington, D. C. p. 389.

5. Wahl, L. M., C. E. Olsen, S. M. Wahl, A. L. Sandberg, and S. E. Mergenhagen. 1978. Prostaglandin regulated macrophage collagenase. In Mechanisms of Localized Bone Loss. Supp. Calc. Tissue Abstracts. Information Reticulal., Inc., Washington, D. C. p. 181.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00061-07 LMI

PERIOD COVERED October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Complement Activation and Inflammation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sandberg, Ann L.	Research Biologist	LMI	NIDR
OTHER:	Wahl, Larry M.	Senior Staff Fellow	LMI	NIDR
	Pazoles, Pamela	Chemist	LMI	NIDR
	Mergenhagen, Stephan E.	Chief, LMI, NIDR	LMI	NIDR
	Michalek, Suzanne, M.	Postdoctoral Fellow	LMI	NIDR

COOPERATING UNITS (if any)

Larry Raisz, University of Connecticut Health Center

LAB/BRANCH

Laboratory of Microbiology and Immunology

SECTION

Humoral Immunity Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.75	1.75	1.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Current investigations involve the mechanisms by which activation of the complement system results in the destruction of connective tissue. Immunoglobulins reactive with cell surface antigens in bone initiate complement activation via either the classical or alternative pathway with resultant destruction of bone in organ cultures. This pathological event is mediated by prostaglandins synthesized by the bone. Osteoclasts are involved in bone resorption and recent studies have demonstrated that a cell of similar origin, the macrophage, can be stimulated by antibody and complement to synthesize prostaglandins. One mechanism by which complement exerts these effects has been shown to be the enhancement of the incorporation of the prostaglandin precursor arachidonic acid, into prostaglandins. Current studies focus on the effects of hormones on complement mediated prostaglandin synthesis, the complement components involved in this reaction and the mechanism by which prostaglandins produce the ultimate destruction of connective tissue.

NIDR CLASSIFICATION: 20315

JECTIVES:

Several factors contribute to the destruction of connective tissue. One mechanism by which this may occur is through activation of the complement system. Current research in this area is directed towards defining the manner by which immunoglobulins and complement stimulate prostaglandin synthesis with resultant bone resorption. The cellular elements with which the immunoglobulins and complement interact, the mechanism by which this interaction induces prostaglandin synthesis, and the modulation of this process are under investigation.

THODS EMPLOYED:

Bone resorption is quantitatively assayed by determining the ^{45}Ca released into the media of fetal rat bone organ cultures. Prostaglandins are determined by specific radioimmunoassay. Antibody fractionations, their characterization and proteolysis, cell culture techniques and other techniques have been previously defined.

JOR FINDINGS:

Immunoglobulins reactive with cell surface antigens, activate complement in organ cultures of fetal bone with consequential destruction of that bone. Immunoglobulins which activate either of the two described complement pathways (classical or alternative) are effective in this regard.

Prostaglandins which are known to induce bone resorption, serve as mediators of this biological event. The addition of prostaglandin synthetase inhibitors eliminates complement dependent bone resorption. In addition high levels of prostaglandins are detected in the supernatants of cultures of bones containing antibody and complement.

It has been shown in recent studies that the cell involved in the synthesis of prostaglandins is probably of the monocyte series which includes the osteoclast, a cell participating in bone destruction as well as the macrophage. In vitro cultures of rat macrophages can be stimulated to produce prostaglandins by incubation with rabbit anti-rat erythrocyte immunoglobulins and complement. The prostaglandin production by these cells is inhibited by indomethacin which blocks prostaglandin synthesis. This effect on macrophages requires a complement system which is intact at least through the sixth component (C6) since the addition of complement from animals genetically deficient in this component is ineffective in supporting complement-dependent macrophage stimulation. This component is also required for bone destruction as determined by ^{45}Ca release and enhanced prostaglandin production by the bones in organ culture. The addition of purified C6 to the deficient serum restores activity in both the macrophage and bone cultures. Thus, complement dependent bone resorption may be attributed to stimulation of a bone cell of monocytic origin, perhaps the osteoclast.

MAJOR FINDINGS (continued)

The action of antibody and complement in this system is presumably a membrane effect. Recent investigations have demonstrated that complement activation results in a marked increase in the incorporation of arachidonic acid, the prostaglandin precursor, into prostaglandins in macrophage and bone cultures. This effect is also dependent on late complement components and can be blocked by inhibitors of prostaglandin synthesis. The enhanced synthesis of prostaglandins observed after treatment with arachidonic acid may be attributed to the induction of cell membrane permeability by complement. This would allow access of the prostaglandin precursor to the enzymes involved in prostaglandin synthesis .

SIGNIFICANCE:

These studies demonstrate that the humoral immune system may contribute to connective tissue destruction as shown by the detrimental effects of antibody and complement on bone. These reactions may contribute to the increased levels of prostaglandins demonstrated in inflammed gingivae as well as in the synovia of patients with rheumatoid arthritis in which connective tissue destruction is a characteristic finding.

PROPOSED COURSE:

Efforts will be initiated to further delineate the mechanistic aspects of complement dependent bone resorption. The effect of complement activation at a cell surface will be investigated in regard to its effect on the cell membranes of macrophages and the complement components required. Additional studies will investigate the effect of complement on the bones of osteopetrotic mice and rats in which a resorption defect exists. In vivo experiments will include the transplantation of various normal cell populations into these mice and in vitro studies are designed to investigate possible differences in the functional status of cell populations of these mice and normal animals in regard to complement dependent prostaglandin synthesis.

PUBLICATIONS:

1. Sandberg, A. L., Riasz, L. G., Goodson, J. M., Simmons, H. A. and Mergenhagen, S. E.: Initiation of bone resorption by the classical and alternative complement pathways and its mediation by prostaglandins. J. Immunol. 119: 1378, 1977.

2. Sandberg, A. L., Raisz, L. G., Goodson, J. M., Wahl, L. M. and Mergenhagen, S. E.: Complement dependent bone resorption. Calcif. Tissue Abs. Information Retreival Inc., Washington, D. C. 1978, pg.159.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00216-02 LMI

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Immunological Control of Connective Tissue Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Wahl, Larry M.	.5	Senior Staff Fellow	LMI NIDR
OTHER:	Olsen, Charles E.	1.0	Post-Doctoral Fellow	LMI NIDR
	Wahl, Sharon M.	.5	Research Microbiologist	LMI NIDR
	Sandberg, Ann L.	.25	Research Biologist	LMI NIDR
	Jones, John	1.0	Microbiologist	LMI NIDR
	McCarthy, James	.5	Microbiologist	LMI NIDR
	Winter, Christine	1.0	Microbiologist	LMI NIDR

COOPERATING UNITS (if any)

David Salomon - LDBA
Shigeto, Abe - LDBA

LAB/BRANCH
Microbiology and Immunology

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NIH/NIDR, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.00	2.25	1.75

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the role of the immune system in connective tissue metabolism. We have shown that the production of collagenase by activated macrophages is regulated by prostaglandins which are produced by these cells. An increase in the synthesis of prostaglandins is required prior to the production of collagenase by macrophages whether they are activated by endotoxin or lymphokines. The prostaglandins produced by activated macrophages appear to regulate collagenase production by modulating cAMP levels within these cells. Other studies dealing with the role of the immune system in bone resorption have revealed a lymphocyte proliferative defect in osteopetrotic mice. In another osteopetrotic animal model, the toothless rat, the affected animals have macrophages which are defective in their production of prostaglandins.

NIDR CLASSIFICATION; 20315

OBJECTIVES:

The purpose of the ongoing research project is the examination of the influence of the immune system on connective tissue metabolism. This involves the study of cellular mechanisms by which macrophages and lymphocytes are activated leading to the production of collagenase and lymphokines. Some of the factors under investigation which may be involved in the regulation of the metabolism of these cells are prostaglandins and cyclic nucelotides. The regulation of bone resorption is also being studied by utilizing osteopetrotic animal models to determine the cellular defect which may be responsible for the lack of bone resorption.

METHODS EMPLOYED:

The culture methods and the assays for collagenase, prostaglandins, cyclic nucleotides and collagen synthesis have been published.

MAJOR FINDINGS:

The activation of macrophages by either endotoxin or lymphokines results in the stimulation of prostaglandin synthesis. The same kinetics in prostaglandin production by macrophages is observed whether they are activated by endotoxin or lymphokines. We have shown that the prostaglandins produced by these activated macrophages regulate the collagenase production by modulation of the cyclic nucleotide levels within these cells. cAMP levels increase in the macrophages subsequent to the increase in prostaglandin and remain elevated for 24 hrs. Macrophage collagenase activity can be enhanced by adding either cAMP or prostaglandins to the cultures in the presence of an activator of these cells. Prostaglandins or cAMP when added alone to macrophage cultures do not stimulate collagenase production. Indomethacin, a prostaglandin synthetase inhibitor, blocks collagenase production in macrophage cultures. However, the enzyme activity can be restored by the exogenous addition of either prostaglandins or cAMP. It thus appears that endotoxin or lymphokines interact with the cell membrane of the macrophage resulting in the stimulation of prostaglandin which in turn modulates the cyclic nucleotide levels and collagenase is subsequently produced.

The role of the immune system in bone resorption is being evaluated in osteopetrotic mice and rats. Our results indicate that in two of the mouse models (micropthalmic and osteopetrotic) there is a lower proliferative response of the lymphocytes to various mitogens and no apparent defect in the macrophages. However, in the osteopetrotic rat (toothless) the lymphocyt are hyperproliferative in response to mitogens and their stimulated macropha produce lower levels of prostaglandins than the macrophages from normal littermates. Additionally, when adherent cells are removed from the rat spleen cells the lymphocyte proliferative response between affected and normal littermates is about the same. Thus, the primary defect appears to reside with the macrophage in the toothless rat whereas the two osteopetroti mouse models seem to have a lymphocyte defect.

IGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

Chronic inflammatory lesions such as periodontal disease and rheumatoid arthritis are associated with the destruction of connective tissue. Our present studies have focused on furthering the understanding of the cellular events leading to the degradation of connective tissue. These studies have indicated that prostaglandins have an essencial role in the production of collagenase by macrophages. Thus, any way in which the production of prostaglandins and the corresponding change in cyclic nucleotide levels may be modulated would potentially effect the degradation of connective tissue.

The results from the studies with osteopetrotic mice and rats indicate that there may be different defects in the immune system which may contribute to osteopetrosis. Evaluation of the manner in which these defects in lymphocytes and macrophages are associated with the pathological condition may also contribute to our understanding of the involvement of these cells in bone resorption.

)POSED COURSE OF STUDY:

The interactions between macrophages, lymphocytes and fibroblasts will continue to be explored in regard to their importance in connective tissue formation and degradation. This will involve examining the manner in which endotoxin or lymphokines interact with the macrophage to stimulate prostaglandin synthesis and make the cells more sensitive to prostaglandins and cAMP.

In the studies of the osteopetrotic animal models, the macrophage and lymphocyte functions and interactions will be examined further. It will be of interest to determine whether the defect in the ability of the macrophages from osteopetrotic rats (tl) to synthesize normal levels of prostaglandin has an effect on molecules produced by the macrophages or on lymphocyte function. Such molecules produced by macrophages might include enzymes such as collagenase or lymphocyte activating factor (LAF). The mitogenic defect in lymphocytes from osteopetrotic mice will be analyzed further to determine if there is a defect in the production of lymphokines.

'LICATIONS:

1. Wahl, L. M., Olsen, C. E., Sandberg, A. L., and Mergenhagen, S. E.: Prostaglandin regulation of macrophage collagenase production. Proc. Nat. Acad. Sci. 74: 4955-4958, 1977.

2. Rosenstreich, D. L., Jacques, A. R., Wahl, L. M., and Oppenheim, J. J. Genetic control of macrophage sensitivity to endotoxin. II. Control by a single autosomal dominant gene of macrophage susceptibility to endotoxin mediated killing and the production of lymphocyte activation factor. J. Immunol. 1978. In press.

PUBLICATIONS:

3. Sugimoto, M., Dannenberg, A. M., Jr., Wahl, L. M., Ettinger, W. H., Hastie, A. T., Daniels, D. C., Thomas, C. R., and Demoulin-Brahy, L.: Extracellular hydrolytic enzymes of rabbit dermal tuberculous lesions and tuberculin reactions collected in skin chambers. Amer. J. Path. 90: 583-608, 1978.

4. Wahl, L. M., Olsen, C. E., Wahl, S. M., Sandberg, A. L., and Mergenhagen, S. E.: Prostaglandin regulated macrophage collagenase. In Mechanisms of Localized Bone Loss. Special supplement to Calcified Tissue Abstracts. Information Retrieval, Inc., Washington, D. C.p. 181, 1978.

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7. Wyler, D. J., Wahl, S. M., and Wahl, L. M. Hepatic fibrosis in schistosomiasis Egg granulomas secrete fibroblast stimulating factor in vitro. Science, 1978. In Press.

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9. Wahl, S. M., Wahl, L. M., and McCarthy, J. B. Lymphocyte mediated activation of fibroblast proliferation and collagen production. J. Immunol. 1978. In press.

10. Mergenhagen, S. E., Rosenstreich, D. L., McGhee, J. R., and Wahl, L. Potential role of lymphoreticular cells in the host response to endotoxin. In Proceedings of the 4th European Immunology Meeting, Budapest, Hungary, 1978. Accepted for publication

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00254-01 LMI

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)
Adherence of Oral Actinomycetes to Oral Streptococci and to Mammalian Cell Surfaces

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Cisar, John O.	Senior Staff Fellow	LMI NIDR
COPI:	Kolenbrander, Paul	Senior Staff Fellow	LMI NIDR
OTHER:	Berg, Shelley	Biological Aid (Micro)	LMI NIDR

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Humoral Immunity Section and Microbiology Section

INSTITUTE AND LOCATION
NIH, NIDR, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.00	1.75	.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies are underway to examine the mechanisms by which species of oral Actinomyces adhere to oral Streptococcus species and mammalian cells. The actinomycetes may contribute to the etiology of periodontal disease and it is significant that many human strains carry a lactose inhibitable receptor which seems to account for both coaggregation with certain streptococci and hemagglutination with human erythrocytes. This receptor is associated with surface fibrils (i.e. fimbriae) on the actinomycetes and efforts are being made to identify a fibrillar antigen(s) which contains the binding site. Coaggregation of various strains of A. viscosus and A. naeslundii with S. sanguis strains appears to depend on a system of specific cell surface interactions which can be used to group these bacteria. This system may contribute significantly to the distribution of these microorganisms within the oral cavity and is being investigated.

NIDR CLASSIFICATION: 20315,10110, 10120, 10230

OBJECTIVES:

This project focuses on mechanisms which enable Actinomyces viscosus and A. naeslundii to adhere to various oral streptococci and to mammalian cell surfaces. These events may play an important role in the development of plaque and could also contribute to the ability of actinomycetes to inhibit the gingival cervice, adjacent to potential sites of inflammation. The long range objectives of this study are (1) to better understand the binding phenomena which account for adherence of actinomycetes to streptocc and mammalian cells; (2) to identify bacterial antigens which are directly involved in adherence and determine whether antibodies to these can effecti block these phenomena and (3) to consider the biological effects which resu from the binding of bacterial components to mammalian cells.

METHODS:

Binding of actinomycetes to streptococci and to human red blood cells is determined by coaggregation of bacterial strains and by hemagglutination respectively. Mild sonication of A. viscosus T14V cells is used to remove surface fibrils and these are partially purified by gel filtration. An assay developed to detect binding of the fibrils to S. sanguis 34 is discussed below.

MAJOR FINDINGS:

Actinomyces viscosus T14V and 20 of 26 other isolates of A.viscosus and a A. naeslundii coaggregate with S. sanguis 34 and hemagglutinate with neuraminidase treated A, B, or O erythrocytes. These interactions are inhibited by low concentrations of the disaccharide lactose which contains a β-linked, nonreducing galactosyl residue. Mild sonication of T14V cells releases the binding factor which is associated with high molecular weight cell surface fibrils. These do not directly agglutinate S. sanguis 34 cells but lactose inhibitable agglutination occurs when small amounts of antibody to T14V fibrillar antigens are added to the reaction mixture. These findings have resulted in the development of a microfilter assay for fibrils with coaggregation activity.

Coaggregation of A. viscosus and A. naeslundii with S. sanguis appears to be dependent on a system of specific cell surface interactions which are Ca^{++} dependent and involve heat labile receptors (85°C for 30 min) on one cell type and heat stable structures on the other. This has led to the grouping of coaggregation reactions into three types:

1) Those which involve heat labile receptors on the actinomycetes and heat stable structures on the streptococci. These are all inhibited by lactose.

2) Those which involve heat stable structures on the actinomycetes and heat labile receptors on the streptococci. These are not inhibited by lactose.

3) Those which involve heat labile receptors and heat stable structures, each present on both cell types. These are not inhibited by lactose but

MAJOR FINDINGS (continued):

become lactose inhibitable when heated streptococci are used in the coaggregation assay.

Studies with 26 isolates of A. viscosus and A. naeslundii and 15 isolates of S. sanguis suggest that groups of actinomycetes and streptococci can be defined by their patterns of coaggregation reactions. Significantly S. mutans (6 isolates, S. salivarius (4 isolates), A. israelii (3 isolates) and a rat strain of A. viscosus do not appear to participate in the coaggregation scheme.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

These studies provide initial evidence on the specific nature of the cell surface interactions which mediate adherence of actinomycetes to oral streptococci and to mammalian cells. It is possible that these phenomena may contribute significantly to the distribution of these microorganisms within the oral cavity. The actinomyces are thought to play a role in the etiology of root surface caries and periodontal disease. It is highly significant that many actinomcyetes of human origin appear to carry a lactose inhibitable receptor which as the dual potential for binding streptococci and human erythrocytes. The interaction with mammalian cells may represent an early event in the initiation of inflammation. Moreover, by understanding this adherence phenomenon and others at the molecular level, possible approaches toward inhibiting them may become apparent.

PROPOSED COURSE:

Studies on the cell surface fibrils of A. viscosus T14V are in progress to delineate their specificity and mechanism of binding to S. sanguis 34 and human erythrocytes. Efforts are being initiated to identify a fibrillar antigen which contains the binding site. These will focus on the abilities of antibodies, including secretory IgA, to inhibit coaggregation and hemagglutination. As adequate quantities of purified fibrils become available, they will be tested for their ability to activate various mammalian cells including lymphocytes, macrophages and fibroblasts..

Investigations are continuing to define groups of actinomycetes and streptococci by their respective patterns of coaggregation reactions These will be expanded to include clinical isolates of actinomycetes. Attempts will be made to correlate these groups with common cell surface antigens.

PUBLICATIONS:

1. Cisar, J. O., McIntire, F. C., and Vatter, A. E.: Fimbriae of Actinomyces viscosus T14V: Their relationship to the virulence-associated antigen and to coaggregation with Streptococcus sanguis 34. In McGhee, J. R., Mestecky, J., and Babb, J. L. (Eds.): Secretory Immunity and Infection. Plenum Press, N. Y. 1978. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00007-18 LMI

PERIOD COVERED

October 1, 1977 through September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies on the Regulation of Carbohydrate Metabolism in Oral Microorganisms

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Wittenberger, Charles L.	Research Microbiologist	LMI NIDR
COPI:	St. Martin, Edward J.	Staff Fellow	LMI NIDR
OTHER:	Beaman, Alfred J.	Biologist	LMI NIDR
	Wolf, Anitra, C.	Bio Lab Technician (Biochemistry)	LMI NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Microbiology and Immunology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

National Institute of Dental Research, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanisms by which various pathways of carbohydrate metabolism are regulated in oral bacteria continue to be under investigation. Currently, special emphasis is placed on resolving the means by which oral streptococci synthesize and secrete the extracellular enzyme, glucosyltransferase (GTase). It has been found that production of this enzyme by Streptococcus salivarius and Streptococcus mutans 6715 growing in a chemically defined medium is highly dependent upon the presence of Tween 80 in the medium. In the case of S. salivarius, the surfactant appears to act either directly or indirectly at the level of enzyme synthesis since 1) it dramatically enhances the differential rate of GTase production and 2) the stimulatory effect of Tween 80 is blocked by chloramphenicol. Synthesis and secretion of GTase in response to the surfactant has recently been achieved with nongrowing cell suspensions of S. salivarius.

NIDR CLASSIFICATION: 10140, 20212

OBJECTIVES:

It is the continuing general purpose of this project to examine fundamental mechanisms by which the biochemical activities of the microbial cell are regulated and to delineate, where possible, the molecular basis for such regulation. This report is a summary of studies that were oriented specifically toward 1) delineating the physiological function of two distinct glyceraldehyde-3-phosphate dehydrogenases present in certain oral streptococci and 2) resolving the means by which oral streptococci secrete the extracellular enzyme, glucosyltransferase.

METHODS EMPLOYED:

All are standard techniques routine to the type of studies herein described

MAJOR FINDINGS:

Multiple glyceraldehyde-3-phosphate dehydrogenases in oral streptococci. We reported previously the unusual finding that all Streptococcus mutans serotypes and a strain of Streptococcus salivarius possess two distinct glyceraldehyde-3-phosphate dehydrogenases (GA3PD). One enzyme is specific for NADP and serves to supply the cell with NADPH for reductive biosynthet reactions, while the other is specific for NAD and functions to form 1, 3-diphosphoglycerate for substrate level ATP formation. Since the two enzymes operate to fullfill different cellular needs, it was of interest to examine possible mechanisms by which their activities might be regulate

Our current results indicate that control of the two GA3PDs is complex and, in the case of the NAD-GA3PD, perhaps indirect. For example, the NAD-enzyme is unresponsive to a large number of ligands tested as possible effectors. These include intermediates of the glycolytic and hexose-monophosphate pathways, various amino acids and certain cations. The enzyme, is however, strongly inhibited by NADH. Because of the equilibriu constant of the reaction catalyzed by the NAD-GA3PD (0.53 M^{-1}) glyceraldehyde-3phosphate oxidation is dependent on the next enzyme in the pathway, 3-phosphoglycerate kinase and the NAD^+-specific lactate dehydrogenase for removal of the reaction products (1,3-diphosphoglycerat and NADH). It is to be noted that the lactate dehydrogenase from this organism has an absolute and specific requirement for fructose-1, 6-diphosphate for catalytic activity. Thus, restricted activity of 3-phosphoglycerate kinase, possibly via a high ATP:ADP ratio, and/or low lactate dehydrogenase activity due to a low cellular pool of fructose-1, 6-diphosphate, could lead to an accumulation of NADH. This would be expected to result in inhibition of NAD^+-GA3PD activity and thus favor oxidation of glyceraldehyde-3-phosphate (GA3P) by the NADP-enzyme. Since the $NADP^+$-GA3PD catalyzes an irreversible oxidation of GA3P to form 3-phosphoglycerate, it might be expected to function more or less independe of the 3-phosphoglycerate kinase. The NADP-enzyme is also insensitive to NADH. Indeed, it appears that the latter-enzyme is regulated in a more direct manner. It is inhibited by several compounds that serve as substr for biosynthetic reactions and which have no effect on the NAD^+-enzyme.

OR FINDINGS (continued)

These compounds include erythrose-4-phosphate, sedoheptulose-7-phosphate and 3-phosphohydroxypyruvate. The enzyme is also strongly activated by NH_4^+ and K^+ at concentrations that have no effect on the NAD-enzyme.

Synthesis and secretion of glucosyltransferase. Glucosyltransferase (GTase) is produced by a number of oral bacteria and is involved in colonization of tooth surfaces by certain cariogenic streptococci. The studies summarized here were designed to gain insight into the means by which GTase is secreted by the bacterial cell.

The production of extracellular GTase by S. salivarius growing in a chemically defined medium is highly dependent upon the presence of Tween 80 in the growth medium. The surfactant appears to stimulate GTase production by acting either directly or indirectly at the level of enzyme synthesis, since 1) it dramatically enhances the differential rate of GTase production and 2) the stimulatory effect of Tween 80 is blocked by chloramphenicol. Similar results have been obtained with the GTase produced by a strain of S. mutans.

In an attempt to reduce the complexity of the growing cell system, we have also studied the minimum requirements for synthesis and secretion of the enzyme. It has been found that cell suspensions produce GTase in response to Tween 80 under non-growing conditions in a medium containing only a mixture of amino acids, NH_4^+, Mg^{++} and an energy source (glucose).

The development of a resting cell system which synthesizes and secretes GTase in the absence of exogenous precursors of messenger RNA (mRNA) allowed us to determine whether S. salivarius might produce a large excess of stable GTase mRNA which could be translated in the absence of de novo mRNA synthesis. Such a model has been proposed by other investigators for exoenzyme production by Bacillus liquifaciens. This, however, does not appear to be the case for S. salivarius. Rifampicin, a potent inhibitor of DNA-dependent RNA polymerase, completely and instantaneously inhibits GTase production in the non-growing cell system. Thus, production of GTase is dependent upon the continued synthesis and translation of a short half-life mRNA. The fact that this occurs in the absence of exogenous RNA precursors indicates that the cells either have an endogenous pool of such precursors or are able to produce them from the metabolites added in amounts sufficient to sustain new mRNA synthesis for up to 60 minutes.

It has also been of interest to determine whether membrane lipids are in any way involved in GTase secretion. The antibiotic cerulenin, which inhibits an early stage of fatty acid synthesis in microorganisms, is an effective inhibitor of GTase production in both growing cells and the non-growing cell system. These and other preliminary data suggest that membrane lipids and/or a post-translational modification of GTase requiring fatty acid synthesis may be involved in cellular secretion of this enzyme.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

These studies on the regulation of central metabolism and exoenzyme secretion in oral microorganisms are undertaken with a broad view toward advancing our state of knowledge concerning the means by which the cell coordinates and controls its diverse biochemical activities. Such information is clearly of broad biological significance. More specifically, however, the microorganisms under investigation have been strongly implicated as etiological agents of oral diseases. It is further anticipated, therefore, that this work will lead to a more comprehensive understanding of how these bacteria successfully colonize and subsist in the complex oral ecosystem. Moreover, results from our studies on glucosyltransferase secretion by oral streptococci may well provide insight into possible means of artifically controlling its production.

PROPOSED COURSE:

In general, these studies on enzyme regulation and exoenzyme secretion in members of the oral microflora will be continued. Special emphasis will be placed on resolving the specific mechanism by which oral streptococc secrete the extracellular enzyme, glucosyltransferase. S. salivarius will be used extensively as a model system for studying the secretory process. A mutant approach to mechanism delineation will be employed. It is of high priority to develop a means for dissociating enzyme synthesis for enzyme secretion.

PUBLICATIONS:

1. Ciardi, J. E., Beaman, A. J., and Wittenberger, C. L.: Purification resolution and interaction of the glucosyltransferases of Streptococcus mutans 6715. Infect. Immun. 18: 237-246, 1977.

2. Wittenberger, C. L., Beaman, A. J., and Lee, L. N.: Tween 80 effect on glucosyltransferase synthesis by Streptococcus salivarius. J. Bacteriol 133: 231-239, 1978.

3. Marucha, P. T., Keyes, P. H., Wittenberger, C. L., and London, J.: A rapid method for the identification and enumeration of oral Actinomyces. Infect. Immun. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00022-13 LMI

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Comparative Physiology and Genetics of Lactic Acid Bacteria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	London, Jack	Research Microbiologist	LMI NIDR
COPI:	None		
OTHERS:	Chace, Nina M.	Chemist	LMI NIDR
	Celesk, Roger	Postdoctoral Fellow	LMI NIDR
	McCabe, Robert	Bio Lab. Tech. (Micro)	LMI NIDR

COOPERATING UNITS (if any)

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Microbiology and Immunology

SECTION
Microbiology

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.80	1.80	2.00

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new pathway found in certain strains of lactic acid bacteria for the metabolism of pentitols, namely, ribitol and xylitol has been described. Only the first two enzymatic steps, the pentitol phosphotransferase system and the pentitol phosphate dehydrogenase, appear to be novel. Following the oxidation of pentitol to pentulose phosphate the latter enters the ribose pathway and eventually joins the EMP pathway. In addition to elucidating the regulation of biosynthesis and function of these enzymes, attempts are being made to transfer the genetic information coding for pentitol utilization to appropriate pentitol nonutilizers.

NIDR CLASSIFICATION: 10110 and 10250

OBJECTIVES:

A. A study delineating the pathway by which the pentitols, ribitol, xylitol and D-arabitol are utilized by lactic acid bacteria is presently well underway. In addition to describing the pathway, the mechanisms responsible for regulating the biosynthesis and function of the pentitol dissimilating enzymes are also being investigated. The immediate goals of this project are:

1) To determine mode of regulation of enzyme biosynthesis and those products that serve as inducers.

2) Survey dental plaque and material from carious lesions to determine whether pentitol-utilizing lactobacilli occur in the human oral cavity.

3) To estimate effect of pentitol-sugar substitutes on ecology of oral microflora.

B. In relation to the above project, an attempt is being made to demonstrate the transfer of genetic information, specifically those genes coding for xylitol and ribitol utilization, by natural vectors between strains of lactic acid bacteria. Two systems, transformation and conjugation, are presently being developed to achieve this end. Of primary concern now are the following problems:

1) Optimization of cultural conditions to insure maximum frequencies for transformation and conjugation.

2) Determine effects of plasmid transfer during conjugation on the maintenance of native plasmids.

3) Attempt to transfer heterologous DNA via conjugation or transformation to various recipients.

C. The isolation and characterization of morphologically diverse gram negative bacteria from the periodontal pocket has also been initiated. Dr. R. Celesk has been studying a slender filamentous rod resembling Leptotrichia species and attempting to cultivate oral spirochetes. The primary objectives of this project are:

1) The development of reliable methods for the cultivation and characterization of the various spirochaetal morphotypes present in periodontal lesions.

2) A nutritional and biochemical characterization of the filamentous Leptotrichia-like organisms.

3) Determine mechanism of attachment of Leptotrichia-like organism to root surface of teeth.

METHODS EMPLOYED:

Conventional immunological, biochemical and bacteriological methods were employed for the studies described herein.

MAJOR FINDINGS:

A. Pentitol metabolism by lactic acid bacteria. Lactobacillus casei and Streptococcus avium are unique among lactic acid bacteria in that strains of each are capable of utilizing pentitols for growth. Studies with strains of L. casei revealed that ribitol or xylitol and arabitol are heterofermentatively converted to a mixture of lactate, acetate and ethanol. The ratio of ethanol to acetate varies with the O_2 content of the growth medium gas phase. Ribitol and xylitol are metabolized via the same route as ribose, all three substrates induce high levels of phosphoketolase and ribulose-5P-3'-epimerase which convert their common intermediate product, xylulose-5-P, to acetyl-P and glyceraldehyde-3-P.

Glucose appears to repress the synthesis of the pentitol transport system but has no effect on its function. Neither synthesis nor function of the second enzyme in the pathway, pentitol phosphate dehydrogenase, appears to be affected by glucose directly.

A number of ribitol-utilizing strains of L.casei have been isolated from dental plaque and carious lesions, indicating that these biotypes may not be as rare as previously thought. To date, no xylitol-utilizing strains have been isolated, but a search for these forms has not been rigorously pursued.

B. Intergeneric DNA exchange among lactic acid bacteria. The plasmid pAMβ1 was transferred by conjugation from a Lancefield group F Streptococcus (strain DL812) to pentitol-utilizing strains of Streptococcus avium and Lactobacillus casei. In the latter instance, the transfer represents the first demonstration of intergeneric DNA exchange in gram positive bacteria. The morphological differences between donor and recipient did not deter the exchange of plasmid DNA. Using the same cultural conditions, pAMβ1 could not be transferred into- or intraspecifically between lactobacilli. Special media and cultural conditions have been developed to enhance conjugation between lactobacilli.

Transfer of pAMβ1 from certain strains of streptococci to lactobacilli causes the latter to apparently lose their native plasmid(s). The reasons for this incompatability are not yet known, however, the system may be potentially valuable for determining the role of the native plasmid in L. casei.

Conventional transformation procedures are being adapted for the lactobacilli to transfer specific biochemical traits from one species to another.

MAJOR FINDINGS (continued)

C. Characterization of microorganisms from human periodontal lesions. A number of strains of slender filamentous gram negative rods resembling Leptotrichia sp. have been isolated from periodontal lesion suppurations. Although these forms are nonmotile, they are capable of circular movement following polar attachment to solid substrates. Clinical examination revealed that these bacteria are only found in deep periodontal pockets. Highly structured surface organelles derived from the organism's outer membrane appear to be responsible for their attachment. Recent in vitro studies suggest that the organism attaches specifically to the root surface areas of the tooth rather than the enamel-covered crown. These observations are supported by experiments comparing adherence of the organisms to powdered root material and hydroxyapatite spheres; the binding of these bacteria to the former material is twenty times greater than the latter.

Oral spirochetes are being isolated by the Millipore filter technique and various anaerobic media are being tested to determine whether they will support growth.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

The utilization of pentitols by lactic acid bacteria appears to be a rare trait, being limited to one species of Streptococcus, S. avium, and twenty percent of the Lactobacillus casei strains tested. However, if transfer of genetic material among lactic acid bacteria, especially interspecific transmission, occurs with any frequency, the use of pentitols as sugar substitutes will eventually select for populations of oral streptococci and lactobacilli able to utilize these compounds. The genetic studies described here should give some indication as to the relative efficiency of xylitol as a sugar substitute.

Whether the slender gram negative rods currently under investigation are related to periodontal disease is not yet known. However, their ability to colonize the cementum area of the tooth root structure assures their presence in the periodontal pocket in relatively large numbers. Should their cell walls contain endotoxin-like material, it seems likely that these bacteria could contribute to the massive immunological response observed in chronic cases of the disease.

PROPOSED COURSE OF RESEARCH:

A. 1) The pathway of xylitol and ribitol metabolism of S. avium requires elucidation. 2) The regulation of pentitol enzyme biosynthesis and functio will be defined with specific attention given to the nature of the inducer and the induction process. 3) A survey of the prevalence of pentitol-utilizing oral lactobacilli already underway will be continued.

B. 1) The effect of introducing pAMβ1 plasmid on the stability of native plasmid of L. casei will be investigated to assess the importance of those native plasmids. 2) Creation of a pAMβ1 plasmid will be attempted to obtain a vehicle for mobilizing chromosomal genes. 3) A completely reliable mating system is being developed that will permit liquid matings as well as filter pad matings.

PROPOSED COURSE OF RESEARCH (continued)

C. 1) Attempts to purify and isolate oral spirochetes will be continued.

2) The examination of mechanism of adherence of the Leptotrichia-like organisms will be expanded to include the role of biochemical determinants of the outer membrane layer in colonizaiton. 3) The biochemical, nutritional, and morphological traits of the Leptotrichia-like organisms will be described.

PUBLICATIONS:

1. London, J., and Chace, M. M.: New pathways for the metabolism of pentitols. Proc. Natl. Acad. Sci. 74: 4296-4300, 1977.

2. London, J.: Evolution of proteins in prokaryotes and bacterial phylogeny. T.I.B.S. 2: 256-258. 1977.

3. Marucha, P. T., Keyes, P. H., Wittenberger, C. L.,and London, J.: A rapid method for the identification and enumeration of oral Actinomyces. Infect. Immun. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZOI DE 00042-8 LMI

PERIOD COVERED

October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Utilization of Carbohydrates by Oral Bacteria

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Chassy, Bruce M.	Research Chemist	LMI NIDR
OTHER:	Porter, Emily	Chemist	LMI NIDR
	Hull, Eunice	Biological Aid	LMI NIDR
	Gibson, Evelyn	Biologist	LMI NIDR

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Microbiology and Immunology

SECTION

Microbiology Section

INSTITUTE AND LOCATION

National Institute of Dental Research, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.40	.90	2.50

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study is directed at examination of the early stages of carbohydrate metabolism in the gram-positive homofermentative oral microflora. Glucokinase and fructokinase from Streptococcus mutans have been purified to homogeneity and biochemically characterized. Since this species has been shown to transport sucrose via a PEP:PTS system, the physiological importance of these enzymes remains to be established. Sucrose-6-phoshate has been synthesized and its biological activity examined. The role of plasmids in potentiating carbohydrate utilization in these organisms is also under study. Techniques for the elimination of plasmids from lactobacilli have been developed. Using these techniques to generate plasmid-variants, it has been demonstrated that lactose metabolism in Lactobacillus casei DR1002 is plasmid-associated. The possibility that sucrose metabolism in L. casei DR1004 may be similarly plasmid-mediated is under investigation.

NIDR CLASSIFICATION: 10250

OBJECTIVES:

The oral streptococci and lactobacilli [illegible] found in the oral cavity. These organisms all require fermentable carbohydrate for growth and energy, and they all produce lactic acid. The ability to concentrate and utilize carbohydrates effectively, in an ecosystem where carbohydrates are frequently scarce, has probably been a major selective criterion for oral organisms. Some species, such as Streptococcus mutans and to a lesser extent Lactobacillus casei, have been implicated in the formation of carious lesions of the teeth. Fermentable carbohydrates, especially sucrose, are required for this process as well.

1) One objective of this work is to understand how carbohydrates, in particular sucrose, are dissimilated by various oral streptococci and lactobacilli. It is hoped that such knowledge will contribute to our understanding of both the evolution of these oral microbes and some specific factors which contribute to pathogenicity.

2) A second major objective has been to determine what role, if any, plasmids have played in the adaptation of oral bacteria to their ecosystem. Determination of the genetic potential of the cryptic plasmids that occur in some species of oral streptococci and lactobacilli is necessary in order to reach conclusions about the adaptive role hypothesized for these plasmids. Since plasmids have been implicated in coding for pathogenic potential in other microbes, that possibility is being examined for certain members of the oral microflora.

This laboratory has reported the presence of plasmids of unknown genetic potential in Lactobacillus casei strains. This aspect of the project is directed at determining what phenotypic traits are plasmid-coded in the L. casei strains known to contain cryptic plasmids. Ultimately, the relationship of these plasmids with plasmids of similar genetic potential isolated from the same genera as well as other genera will be investigated. Such studies should provide insight into the origin and evolutionary role of plasmids in the adaptation of the oral microflora to their environment.

MAJOR FINDINGS:

A. Purification and properties of glucokinase and fructokinase:
Studies in this laboratory have demonstrated the presence of both extra- and intracellular invertase as well as specific glucokinase and fructokinase in strains of S. mutans. This combination of enzymes would afford the cell an alternate pathway for the permeation of sucrose, glucose, fructose and mannose. Present research is directed at determining the role and relevance of these two alternate systems of carbohydrate mobilization. Glucokinase has been isolated from sucrose-grown cells of S. mutans SL-1 and OMZ 70. The enzyme has been purified to electrophoretic homogeneity by DE-52 ion-exchange, Sephacryl-200 gel permeation, and ATP-affinity gel chromatography. Gluckinase has been found to be highly specific for glucose and ATP. The reaction requires a divalent metal ion for activity; manganous ion is optimal. The

MAJOR FINDINGS (continued):

C. Plasmid-associated properties of lactobacilli: After testing a variety of known plasmid curing techniques, it was determined that a combination of acriflavin and mitomycin C could eliminate up to 100% of the plasmid DNA present in several strains of L. casei. Isolates of L. casei subsp. casei DR 1002 lacking the resident 23 mdal plasmid, pDR 101, consistantly lacked the ability to ferment lactose. No other changes in fermentation patterns were observed. Thus, it was concluded that lactose metabolism is plasmid-mediated in this strain. Spontaneous loss of pDR 101, as frequently occurs in other plasmid-bearing bacteria, has not been observed with this strain. It has been observed that lac- variants grow more slowly on glucose-containing media than the parent strain DR 1002 (20-25% doubling time). This finding may account for the inability to detect spontaneous plasmid- variants, since lac+ cells would predominate over lac- cells during culturing. The growth rate advantage of lac+ plasmid containing isolates also implies the presence of other, as yet undetermined, plasmid-coded functions for pDR 101.

Similar experiments have been conducted with L. casei subsp. rhamnosus DR 1004. Preliminary data indicate that sucrose metabolism can be lost at frequencies approaching 100% after treatment with plasmid "curing" agents. Spontaneous suc- variants have been isolated as well. In keeping with this finding, it has been observed that suc- cells grow more rapidly than parent sucrose + cultures. Whether the observed "curing" of sucrose metabolism is dependent upon loss of the 19 mdal plasmid found in L. casei DR 1004 is under investigation.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

The mechanism of carbohydrate utilization has doubtless been an important selective criterion in the emergence of oral microorganisms. These studies underscore the diverse and complex adaptation of these organisms to efficiently utilize carbohydrates. Ultimately, such studies should allow us to understand the "economy" of these organisms and may suggest changes that can be made in the oral environment that would favor shifts in the ecosystem to support a benign rather than a pathological microflora.

Considering the demonstrated role of plasmids in coding for the pathogenic potential of other bacteria, it would be of great value to know if such a relationship exists between plasmids and the known pathogenicity of some of the gram-positive members of the oral microflora. In addition, a study of the distribution, function and relatedness of these plasmids may contribute to our understanding of bacterial evolution, specialization, and adaptation to changing ecosystems.

AJOR FINDINGS (continued)

enzyme has a fairly low MW; it lies in the 32-49 kdal range depending upon the strain studied. No allosteric or other small molecule regulatory control has been noted, either by direct testing or from kinetic data. The kinetic mechanism has been demonstrated to be one common for kinases; ordered bi bi with glucose binding prior to ATP and ADP dissociating prior to glucose-6-phosphate.

Fructokinase has been purified by similar techniques. As has been observed for other bacterial fructokinases, fructose and mannose are both substrates for the enzyme isolated from S. mutans. Kinetic analysis of the enzyme is in progress.

B. Synthesis and biological activity of sucrose-6-phosphate: As is the case for most bacteria studied, oral streptococci possess a specific phosphoenolypyruvate-driven active transport system (PEP-PTS) for the transport of most carbohydrates they are capable of fermenting. A specific sucrose PTS has been demonstrated in S. mutans. Transport of sucrose by this mechanism would result in the generation of intracellular sucrose phosphate and would necessitate the presence of enzymes capable of its catabolism. The known intracellular invertases of S. mutans strains all have an unphysiologically high Km for sucrose; it is possible that this enzymic activity represents hydrolysis of sucrose as a secondary substrate and that the preferred substrate is sucrose phosphate generated by the PTS specific transport system. Even if this were not the case, some mechanism must exist for the dissimilation of sucrose phosphate. Prior to any attempt to find a sucrose phosphate invertase or hydrolyase, it was necessary to prepare sucrose phosphate. Three isomers of sucrose phosphate are possible; it is not known which is the form produced by the PTS system. Only one of the three isomers, α-D-glucopyranosyl-1 ⟶ 2-β-D-fructofuranosyl-6'-phosphate, has been synthesized. The synthesis requires UDPG: fructose-6-phosphate transglycosylase, an enzyme which can be isolated from wheat germ. The isolation of this enzyne from wheat germ was improved by the introduction of a DE-52 ion-exchange chromotography step to resolve the enzyme from contaminating phosphatases. Enzyme purified by this new procedure was used to generate substrate quantities of sucrose phosphate.

Radiochemically-labelled sucrose phosphate was prepared as well. After verification of structure, the sucrose phosphate was then tested as a substrate for the intracellular invertase purified from sucrose-grown extracts of S. mutans. Since neither crude extracts of S. mutans OMZ 70 or 6715-10, or purified invertase from OMZ 70 are active in catalyzing the hydrolysis of this isomer of sucrose phosphate, it is concluded that the physiologically active substrate must be one of the other two possible isomers.

PROPOSED COURSE:

1. To prepare other isomers of sucrose phosphate and determine if a sucrose phospho-invertase is present in S. mutans. The most available route to the physiologically relevant isomer will be to generate substrate quantities of sucrose phosphate using a sucrose PTS system.
2. To determine the physiological role of invertase, glucokinase and fructokinase.
3. To determine the specific step or steps in lactose metabolism that are plasmid-coded in L. casei DR 1002. Direct assays of lactose PTS. β-galactosidase, and phospho-β-galactosidase are of immediate interest.
4. To determine if the plasmids found in other L. casei subsp. casei strains carry lactose fermentation determinants. If this is the case, plasmid homologies will be evaluated using restriction enzyme mapping, DNA-DNA hybridization and heteroduplex analysis.
5. To determine if sucrose metabolism is plasmid-associated in L. casei DR 1004. Other plasmid-coded determinants will be evaluated. Of particular interest will be to determine if any cell wall or membrane components or antigenic determinants are lost or altered upon plasmid deletion.

PUBLICATIONS:

1. Donkersloot, J. A., Flatow, U., Gibson, E., and Chassy, B. M.: Characterization of glucosyltransferase-deficient, plasmid-containing mutants of Streptococcus mutans LM-7. Infection and Immunity, 1978. In press.

2. Chassy, B. M., Gibson, E., and Giuffrida, A.: Evidence for plasmid-associated lactose metabolism in Lactobacillus casei subsp. casei. Current Microbiology, 1978. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 DE 00043-08 LMI

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)
Physiological and Genetic Studies on Pathogenic Oral Microorganisms.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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OTHER:	Harr, Robert J.	Bio Lab Tech (Micro.)	LMI NIDR
	Flatow, Ursula	Microbiologist Tech.	LMI NIDR
	Chassy, Bruce M.	Research Chemist	LMI NIDR
	Hull, Eunice M.	Bio Lab Aid	LMI NIDR

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Microbiology Section

INSTITUTE AND LOCATION
National Institute of Dental Research, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.35	1.10	2.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Because it has been established that the enzyme glucosyltransferase (GT) contributes significantly to the cariogenicity of Streptococcus mutans, the multiplicity of this enzyme and the genetic locus (or loci) controlling its synthesis are under investigation. In particular, the proposals that GT is encoded by a plasmid or a lysogenic phage are being tested. Experimental data obtained so far with spontaneous GT-deficient mutants of S. mutans LM-7 do not support either of these proposals. To determine if the mutant phenotype is due to a mutation in the plasmid, plasmids from parent and GT mutant strains are being mapped with restriction endonucleases. The GT multiplicity problem is being analyzed by electrophoresis. It was found that serotype c and e strains only had a high-molecular-weight aggregate that synthesized an insoluble glucan. In contrast, serotype d and g strains showed distinct activities of lower molecular weight that synthesized insoluble and soluble glucans, respectively.

NIDR CLASSIFICATION: 10230, 10240, 10260

OBJECTIVES:

Animal studies have conclusively shown that certain oral microbes such as Streptococcus mutans can be pathogenic under a suitable set of conditions. Other, primarily epidemiological, evidence suggests that S. mutans may also be involved in the development of caries lesions in humans. Because of these considerations, the general objectives of the present study are to define, both biochemically and genetically, selected factors that contribute to the observed virulence of S. mutans. So far, the emphasis of our studies has been on the enzyme glucosyltransferase (GT). Whereas other investigators have partially characterized GT from strain 6715 (serotype g), little work has been reported concerning the enzyme from the commonly found serotype c and e strains. Consequently, one goal of our studies has been to characterize GT from strains C67-1 (serotype c), and LM-7 (serotype e) and to examine whether these activities occur in multiple forms, as is the case with strain 6715. The ability of S. mutans cells to agglutinate upon addition of dextran is also considered to contribute to its virulence. A second objective of the present study has been to examine whether this notion has merit and whether GT and the dextran-agglutination trait are associated, or genetically distinct traits. The other major thrust of this study is to explore the genetic background of the GT trait. In connection with what is mentioned above, one of the objectives of the project is to find out whether there is one GT gene or whether there are multiple genes. In addition, we wanted to critically examine whether GT is controlled by a plasmid or a lysogenic phage. Because these studies have shown that strain LM-7 spontaneously loses GT at a relatively high frequency, we have initiated efforts to learn more about mutation rates of this organism in general.

METHODS EMPLOYED:

Conventional methods or methods described in last year's report have been used.

MAJOR FINDINGS:

Whereas the extracellular GT from strain 6715 (serotype g) can be concentrated satisfactorily by ultrafiltration, the activities from strains C67-1 (serotype c) and LM-7 (serotype e) are largely lost during this operation, presumably due to irreversible adsorption to the ultrafiltration membrane. However, concentration by ammonium sulfate precipitation allowed enzyme recoveries greater than 50%. Upon electrophoresis and enzyme activity staining both C67-1 and LM-7 showed only a high molecular weight GT aggregate that synthesized an insoluble glucan. This was in contrast to strain 6715 which showed

MAJOR FINDINGS (continued):

multiple lower-molecular-weight activities synthesizing soluble and insoluble glucans respectively. Electrophoresis revealed that sodium dodecyl sulfate can dissociate the GT aggregate from strains C67-1 and LM-7 into lower-molecular-weight activities that also synthesized an insoluble glucan. However, the estimated molecular weight of the smallest enzymatically active form observed was still fairly high (about 500,000). No GT synthesizing a soluble glucan was observed after this detergent treatment. Thus, it appears that the GT from serotype c and e strains is fundamentally different from the GT in serotype g strains and that further study is warranted. Parenthetically it can be reported that these electrophoretic studies also revealed that the fructosyltransferase in serotype c and e strains can be separated into heterogeneous high-molecular-weight aggregates and several distinct lower-molercular-weight species.

With respect to the question whether the dextran-agglutination trait is required for the expression of virulence, it can be reported that this does not appear to be the case because, compared with 6715 cells, LM-7 cells agglutinated only very weakly. The finding that LM-7 mutants virtually devoid of GT also agglutinated very weakly proves that the ability to do so is genetically distinct from GT.

We reported last year that S. mutans LM-7 (serotype e) gives rise at relatively high frequency to GT-deficient mutants. However, studies in collaboration with B. M. Chassy have shown that this is not due to loss of the resident plasmid pAM7. The loss of GT also does not appear to be associated with the loss of a lysogenic phage encoding GT synthesis, because neither parent nor mutant strains lysed appreciably upon addition of mitomycin C. These findings appear to contradict those reported by others. To characterize the loss of GT biochemically, cell-free culture, fluids were examined by electrophoresis. The parent showed a high-molecular-weight aggregate that synthesized an insoluble glucan; this aggregate was absent in the mutants. The finding that sodium dodecyl sulfate can dissociate the aggregate with retention of at least some of the activity should facilitate future comparative studies between parent and mutant. Tests done in collaboration with P. Soprey have shown that LM-7 parent and GT mutant cells are antigenically distinct; the nature of this difference is being studied.

The finding that S. mutans LM-7 loses GT at a much higher rate than expected for a classical chromosomal mutation made it imperative to examine mutation frequencies in this organism more in general. Whereas, spontaneous mutations to melibiose fermentation, rifampin resistance and streptomycin resistance all occurred at typically low rates (about 1 in 10^8), a mutation from rough to smooth colonial morphology has been identified that occurred at a much higher rate (about 1 in 10^3).

MAJOR FINDINGS (continued):

The nature of this mutation and the effect on virulence will be examined. Preliminary evidence suggests that the change in colonial morphology is associated with a change in a surface antigen.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

It has been established that insoluble glucan synthesis is required for expression of the cariogenicity of S. mutans. A second reason to fully characterize this activity stems from the fact that other scientists are considering GT as a vaccine against dental caries. Because S. mutans cells are also being tested as a vaccine, identification of the surface antigens of these cells should shed light on the nature of the antigens that allow protection which in turn could lead to the development of a safer vaccine.

The observation that some S. mutans traits mutate at a much higher frequency than others is of considerable interest because this problem may well have wide biological significance.

PROPOSED COURSE OF STUDIES:

1. The GT from strain C67-1 will be examined further with emphasis on the isolation, purification, and characterization of a low-molecular-weight form of the enzyme.
2. Enzyme preparations from LM-7 parent and GT mutant strains will be compared after SDS treatment in order to pinpoint the protein that is missing in the mutants. In addition, we will attempt to establish the nature of the residual GT activity in the mutants. This approach will include the isolation and characterization of GT-positive revertants.
3. Plasmids from parent and GT mutant strains will be compared by restriction endonuclease mapping to determine whether the mutant phenotype is due to a plasmid mutation.
4. Since a strain of S. mutans LM-7 that does not contain a plasmid has been identified, it will be compared with the plasmid-containing strain in order to assign a phenotype to the plasmid. Attempts will also be made to cure this plasmid by introduction (via conjugation) of another plasmid.
5. The smooth and rough colonial morphology types will be characterized with emphasis on virulence, adhesion, GT, and surface antigens.
6. The possibility that GT is encoded by a lysogenic phage will be pursued by electron microscopy in collaboration with C. F. Garon (NIAID).

PUBLICATIONS:

1. McCabe, R. M., and Donkersloot, J.A.: Adherence of Veillonella species mediated by extracellular glucosyltransferase from Streptococcus salivarius. Infect. and Immun. 18: 726-734, 1977.

2. Donkersloot, J.A., Flatow, J., Gibson, E. and Chassy, B. M.: Characterization of glucosyltransferase-deficient, plasmid-containing mutants of Streptococcus mutans LM-7. Infect. and Immun. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DE 00096-05 LMI

PERIOD COVERED
October 1, 1977 - September 30, 1978

CT 0600112

TITLE OF PROJECT (80 characters or less)
Microbiological Features of Cervico-radicular Plaque in Healthy and Diseased Periodontium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Keyes, Paul H.	Dental Director	LMI	NIDR
OTHER:	Howard, Surya A.	Health Technician (Dental)	LMI	NIDR
	London, Jack P.	Research Microbiologist	LMI	NIDR
	McCabe, Robert M.	Bio. Lab Tech (Micro)	LMI	NIDR

COOPERATING UNITS (if any)
William E. Wright, Clinical Dental Services Section, NIDR

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Microbiology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.45	2.2	0.25

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Specimens of cervico-radicular bacterial mats are being obtained from persons with and without periodontal lesions. Samples are examined by phase-contrast microscopy, and assessed by fluorescent antibody techniques and cultural methods for suspected pathogens. Patients with lesions are being advised to use salts for their home-care oral-hygiene program and to take tetracycline hydrochloride if they are unable to eliminate white blood cells and motile bacteria from lesions. The response of soft and osseous tissues is being followed around teeth whose cervico-radicular spaces have been cleared of motile bacteria and white blood cells.

NIDR CLASSIFICATION: 10400

OBJECTIVES:

This project is designed to provide answeres to the following questions: 1) can phase contrast microscopy be used to distinguish differences in the microbial flora present in samples removed from healthy and diseased areas? 2) If bacterial complexes suspected of having a periodontophathic potential can be identified, what therapeutic measures can be used to suppress or eliminate them? 3) Are there distinguishable differences in the Actinomyces population present in samples removed from teeth having chronic destructive periodontitis and teeth having no discernable evidence of disease?

MAJOR FINDINGS:

Phase contrast microscopy has been used to assess the gross microbial flora present in samples from healthy and diseased periodontium. Results indicate that healthy gingival crevices do not harbor spirochetes, large motile rods or white blood cells. In contrast, large numbers of spirochetes, motile rods and white blood cells are consistently found in samples from root surfaces undergoing autogenous exfoliation. A somewhat less complex microbial flora is observed in older patients having marginal gingivitis.

It became apparent that the only patients suitable for studying marginal gingivitis are older persons (50 + years), because marginal gingivitis in younger persons may be the initial stages of chronic destructive periodontitis. It is unlikely that persons over 50 years of age with marginal gingivitis will experience chronic destructive periodontitis.

In patients with progressive destructive periodontitis, root surfaces of affected teeth are observed to be coated with bacterial mats. Phase contrast microscopy reveals that the surfaces of these mats are characterized by masses of motile microorganisms whose action appears to circulate and propel bacterial by-products and tissue fluids into circumradicular spaces. Removal of these bacterial coatings results in a cessation of the destruction process. Therapeutic measures tested and found to be effective in controlling the microbial population include local treatment with various inorganic salt solutions and administration of tetracycline.

Another aspect of this project has been an attempt to evaluate the possible involvement of Actinomyces species in human periodontal pathology. A fluorescent antibody technique developed last year for identifying and enumerating Actinomyces in plaque has been used to monitor the Actinomyces population in patients. Two groups were studied; one with mild gingival inflammation and another presenting frank pocketing with accompanying bone loss. The predominant Actinomyces population in plaque samples from the former group consists of facultative areobic species (A. viscosus and A. naeslandii). In samples from the latter group, however, the obligately anaerobic species A. israelii predominates. It remains to be established whether there is a cause or an effect relationship between the observed shift in Actinomyces population and the disease state of the patients.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

These studies are designed to explore relatioships between the microbial flora and the disease state of the host. It is anticipated that information derived from such studies will ultimately be useful in formulating rational approaches to the diagnosis and treatment of periodontal disease.

PUBLICATIONS:

1. Marucha, P. T., Keyes, P. H., Wittenberger, C. L., and London, J. A rapid method for the identification and enumeration of oral Actinomyces. Infect. Immun., In press.

2. Keyes, P. H., Wright, W. E., and Howard, S. A. The use of phase contrast microscopy in the diagnosis and treatment of periodontal lesions. An initial report I. Quintessence International Dental Digest. Jan., 1978, pp. 51-56.

3. Keyes, P. H., Wright, W. E., and Howard, S. A. The use of phase contrast microscopy in the diagnosis and treatment of periodontal lesions. An initial report II. Quintessence International Dental Digest. Feb. 1978, pp. 69-76.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE-00174-03 LMI

PERIOD COVERED

October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

The Role of Plasmids in the Pathogenesis, Ecology and Taxonomy of the Oral Microflora.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	LeBlanc, Donald J.	Research Microbiologist	LMI NIDR
COPI:	St. Martin, Edward J.	Staff Fellow	LMI NIDR
OTHER:	Lee, Linda N.	Chemist	LMI NIDR
	Lewis, Barbara S.	Microbiologist	LMI NIDR

COOPERATING UNITS (if any)

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Dr. Robert J. Hawley, Georgetown University

LAB/BRANCH

Laboratory of Microbiology and Immunology

SECTION

Microbiology

INSTITUTE AND LOCATION

National Institute of Dental Research, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.35	1.10	1.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An erythromycin-resistance plasmid, pAM^{β}_1, from a strain of Streptococcus faecalis was shown to mediate its own transfer, by a mechanism resembling conjugation, among several species of streptococci including certain strains of S. mutans, S. sanguis, and S. salivarius. Evidence has also been obtained which suggests that this 17 megadalton plasmid is a composite molecule consisting of two independent replicons with molecular weights of 10×10^6 and 7×10^6. Studies on patients treated for periodontal disease with tetracycline (Tc) suggested that this antibiotic selects for a highly resistant oral microflora. The Tc-resistant trait has been shown to be plasmid-mediated in some of these isolates, and one strain of S. sanguis transferred the resistance to a strain of S. mutans during cell-to-cell contact. The ability of Lancefield group N streptococci to metabolize lactose is known to be governed by plasmids. The lactose plasmid of one strain of S. lactis was also shown to influence the biochemical pathway by which galactose is utilized by this organism.

NIDR CLASSIFICATION: 10230 - 10250 - 10280 - 10110

OBJECTIVES:

The primary long-range objective of this project continues to be an elucidation of the role of bacterial plasmids in the pathogenesis, ecology and taxonomic status of the oral microflora, particularly the streptococci. This report summarizes the results of studies aimed at: 1) establishing potential mechanisms of genetic transfer among the oral streptococci; 2) determining the effects of administering antibiotics for treatment of periodontal disease on the development of resistant forms of oral streptococci; 3) comparing the molecular properties of plasmids in different streptococcal species; and 4) continuing an examination of lactose plasmids in the group N streptococci to establish a model system for examining plasmids coding for metabolic traits among streptococci in general.

METHODS EMPLOYED:

All procedures used in these studies were standard microbial or molecular biological techniques. In a few instances methods commonly employed for the study of plasmids from gram-negative bacteria were applied in the examination of streptococcal plasmids with minor modifications.

MAJOR FINDINGS:

Mechanism of Genetic Transfer Among the Oral Streptococci. The β plasmid of Streptococcus faecalis strain DS5, which codes for resistance to the antibiotics erythromycin (Em) and lincomycin (Lm), was one of the first plasmids identified in a member of the genus streptococcus. Previous work in this laboratory showed that the β plasmid could be introduced into the Challis strain of S. sanguis, as well as a Lancefield group F streptococcus, S. anginosus-constellatus by transformation. We have now shown that the latter strain, once carrying the β plasmid, can serve as an excellent donor of this plasmid and readily transfer the resistance markers to a variety of lactic acid bacteria including three species of oral streptococci, S. mutans, S. sanguis and S. salivarius. The process resembles conjugation in that the transfer is insensitive to DNase and requires cell-to-cell contact. S. sanguis and S. mutans transconjugants were shown to have acquired a plasmid with physical properties identical to the β plasmid. Furthermore, the presence of the β plasmid in S. mutans and S. sanguis confers upon these organisms the ability to serve as β plasmid donors in intraspecies matings. The frequency of plasmid transfer in intraspecies matings is generally 50- to 100-fold greater than in interspecies matings.

Studies on the Influence of Selective Pressure on the Oral Microbial Plasmid Pool. The effects of antibiotic therapy on the oral microbial flora are presently being investigated. Studies on patients treated for periodontal disease with tetracycline (Tc) suggest that this antibiotic selects for a highly resistant (> 25 μg Tc/ml) bacterial population. Less than 1% of the flora from sub-gingival plaque samples,

MAJOR FINDINGS (continued)

cultivable on an enriched medium under anaerobic conditions, is resistant to high concentrations of Tc when obtained from patients with no recent history of Tc treatment. Following two weeks of treatment with Tc, between 10 and 100% of the population exhibits high levels of resistance to this antibiotic. This population remains relatively stable for at least 2-3 months after the cessation of treatment. Of 80 separate Tc-resistant isolates from 10 patients, more than 90% have been streptococci with S. sanguis II (20), S. sanguis I (16), S. salivarius (15) and S. mitis (9) representing the majority of these strains. None of the Tc-resistant strains isolated thus far have been identified as S. mutans.

Plasmids have been isolated from Tc-resistant stains identified as S. sanguis and S. mitis, and curing experiments suggest a plasmid origin for Tc resistance in some of these strains. Attempts to transfer Tc-resistance plasmids to competent cultures of the Challis strain of S. sanguis and the group F S. anginosus-constellatus strain, by transformation, were unsuccessful. However, a Tc resistant strain of S. sanguis II has recently been shown to serve as a donor of the resistance trait when incubated with an S. mutans recipient strain on membrane filters. Attempts to demonstrate the presence of plasmid DNA in transconjugant isolates are currently in progress.

Molecular Properties of the β Plasmid in Different Streptococcus Host Strains. The β plasmid can be isolated from the S. faecalis DS5 strain as a 17 megadalton covalently closed circular (CCC) molecule which is stable upon storage for months. Previous work in this laboratory showed that when β plasmid DNA was isolated from a Challis transformant strain, the covalently closed circular form behaved as a 15.5 megadalton molecule which was rapidly converted to a linear form. When a β plasmid transformant of the group F strain was grown in the absence of Em, the plasmid could be isolated in a form which was identical to the β plasmid isolated from S. faecalis, in size, conformation and stability. More recently, we have found that if the group F strain was grown in the presence of Em the plasmid was present in three species, all CCC molecules, with molecular weights of 17×10^6, 10×10^6 and 7×10^6. These three species and their relationship to each other, are currently being investigated.

Plasmid-Associated Lactose and Galactose Metabolism by Streptococcus Lactis. S. lactis strain DR1251 utilizes lactose via a lactose-specific phosphoenolpyruvate-dependent phosphotransferase activity (lac-PTS) which transports the carbohydrate into the cell and also phosphorylates in the 6-carbon position of the galactose moiety. Following hydrolysis of the lactose-phosphate by a phospho-β-galactosidase (P-β-gal), the galactose-6-phosphate and glucose products are further metabolized via the tagatose-6-phosphate and EMP pathways, respectively. We have found that free galactose is also metabolized by the tagatose-6-phosphate pathway following transport and phosphorylation of the substrate by the

MAJOR FINDINGS (continued)

lac-PTS activity. The lac-PTS has been shown to have an apparent Km of 5×10^{-5}M for lacrose and 2×10^{-2}M for galactose. PTS activity for both lactose and galactose, as well as P-β-gal activity, are present at high levels when the organism is grown on either lactose or galactose. S. lactis strain DR1251 loses the ability to grow on lactose at a high frequency when incubated at 37°C with glucose as the growth substrate. Loss of ability to metabolize lactose is accompanied by the loss of a 32 megadalton plasmid. Lac- isolates are still able to grow on galactose, but are deficient in both lactose and galactose PTS activity, as well as P-β-gal activity. Galactokinase, a key enzyme of the Leloir pathway, phosphorylates galactose in the 1-carbon position. The activity of this enzyme is approximately 5-fold greater in galactose-grown lac^- cultures than in galactose-grown lac^+ cultures. Accompanying the increase in galactokinase activity is a shift from a predominantly homolactic to a heterolactic fermentation. These results suggest that although S. lactis strain DR1251 metabolizes galactose primarily via the tagatose-6-phosphate pathway, using a lactose PTS activity to transport this substrate into the cell, loss of this transport system does not result in a gal^- phenotype. Rather lac^- derivatives of this strain may still metabolize galactose via the Leloir pathway.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

The contributions of plasmids to the pathogenesis, ecology and taxonomic status of several groups of bacteria, particularly the Enterobacteriaceae, have been well-documented. It is expected that these extrachromosomal elements will be shown to play similar roles among the oral microflora. The studies on the influence of selective pressure on the plasmid gene pool among oral bacteria are expected to provide insight into the extent of ecological diversity of the oral microflora. In vitro and in vivo plasmid transfer experiments will help to provide information on the mechanisms of plasmid dissemination among the inhabitants of the oral environment. It is further expected that the overall comparative studies on plasmids among streptococci will contribute to a better understanding of these elements in general.

PROPOSED COURSE:

The studies on conjugal transfer of plasmid DNA will continue with efforts to optimize mating conditions. Such parameters as mating temperature, pH, the influence of mono- and divalent cations, and the best growth stages for mating will be evaluated. Efforts to mobilize chromosomal DNA, using known transmissible plasmids, will constitute a major effort over the next year. Success in this latter endeavor will permit genetic analysis of many streptococcal gene functions, as well as their regulatory properties. The influence of Tc treatment on the total resistant flora of 26 patients has now been determined. More than 400 separate isolates,

PROPOSED COURSE (continued)

resistant to high levels of this antibiotic, have been obtained. These isolates will be further examined for: 1) identification of their taxonomic status; 2) resistance to other antibiotics; 3) the role of plasmids in the resistance phenotypes; and 4) the transmissibility, or mobilizability, of the resistance traits. Efforts will also be made to determine the relatedness of the antibiotic resistance plasmids to each other, as well as detecting the presence of transposable resistance elements among these plasmids.

Preliminary data suggest that sucrose metabolism and the production of the small peptide antibiotic, nisin, by the group N streptococci may be plasmid coded. Experiments will be conducted to confirm these results and to identify the plasmids involved. Plasmid-coded enzymes for sucrose metabolism and nisin production will also be examined.

PUBLICATIONS:

1. LeBlanc, D. J., Cohen, L. and Jensen, L.: Transformation of Group F Streptococci by plasmid DNA. J. Gen. Microbiol. 106:49-54, 1978.
2. LeBlanc, D. J., Hawley, R. J., Lee, L. N. and St. Martin, E. J.: "Conjugal" transfer of plasmid DNA among oral streptococci. Proc. Natl. Acad. Sci. U.S.A. 1978. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZOI DE 00208-02 LMI

PERIOD COVERED
October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)
A genetic analysis of metabolic pathways in various members of the oral microbial flora.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Edward J. St. Martin	Staff Fellow	LMI	NIDR
OTHER:	Robert G. Goldfarb	Biological Aid	LMI	NIDR

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Microbiology Section

INSTITUTE AND LOCATION
National Institute of Dental Research, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.80	.80	1.00

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Several key reactions present in Streptococcus mutans have been examined using mutant analysis techniques. Two enzyme systems for ammonia assimilation have been identified: glutamate dehydrogenase and the coupled reactions of glutamine synthetase and glutamate synthase. The primary pathway for sucrose utilization has also been determined. Catabolism of sucrose was found to be initiated by a highly efficient phosphoenolpyruvate-dependent sucrose phosphotransferase reaction followed by hydrolysis of sucrose phosphate by an intracellular sucrose phosphate hydrolase. In addition, the regulation and function of ethanol dehydrogenase was examined. This fermentation enzyme was found to be required for metabolism of mannitol and sorbitol.

NIDR CLASSIFICATION: 10250

OBJECTIVES:

The general objective of this project is to use mutant isolation and analysis techniques to examine the function and regulation of several key metabolic reactions present in cariogenic microorganisms. More specifically, we have undertaken the study of ammonia assimilation, sucrose catabolism and regulation of fermentation products in S. mutans.

METHODS EMPLOYED:

Standard microbiological techniques were employed.

MAJOR FINDINGS:

Ammonia assimilation. We have previously reported that several strains of S. mutans are capable of growth in a chemically defined medium without the addition of an exogenous supply of amino acids. An enzyme, glutamate dehydrogenase, that is capable of fixing free ammonia to form amino acids was detected in these strains. However, examination of glutamate dehydrogenase negative mutants revealed that they were still capable of growth in the absence of amino acids. Using these mutants, we have identified a second method for ammonia assimilation in S. mutans. This pathway is composed of two enzymes, an ATP driven glutamine synthetase and a NADH coupled glutamate synthase, that function together to fix free ammonia for the biosynthesis of amino acids. Preliminary experiments suggest that the levels of only one enzyme, glutamate synthase, is regulated by the availability of amino acids.

Sucrose catabolism. S. mutans contains several enzymes that are capable of hydrolysing sucrose. However, the low specific activity of these enzymes coupled with their relatively poor affinity for the substrate sucrose suggest that they play only a minor role in the catabolism of sucrose. We have identified a phosphoenolypyruvate-dependent sucrose phosphotransferase activity (suc-PTS) in S. mutans. This activity is induced by growth on sucrose and has an apparent Km for sucrose of 7×10^{-5}M. Preliminary experiments suggest that the product, sucrose phosphate, is phosphorylated on the glucose moeity.

We have isolated mutants that are missing either Suc-PTS activity or the ability to hydrolyse sucrose phosphate by their inability to grow when 5mM sucrose is provided as the only source of carbohydrate. The primary route of sucrose utilization is therefore via this highly efficient Suc-PTS reaction and intracellular sucrose phosphate hydrolase.

Fermentation enzymes. The primary fermentation product of carbohydrate metabolism by S. mutans is lactic acid. However, less acid products such as ethanol, acetate and formate can be produced. We have found that the levels of two enzymes that lead to ethanol production, acetaldehyde dehydrogenase and ethanol dehydrogenase (EDH), are elevated when calls were grown on limiting glucose concentrations or on the polyols mannitol and sorbitol. We have isolated mutants of S. mutans that have simultaneou lost the ability to use both polyols and found that one such mutant was missing EDH activity. The ability to utilize polyols could be restored

MAJOR FINDINGS (continued)

in the mutant if an exogenous electron acceptor such as oxygen or pyruvate was provided. The finding that EDH is required for polyol fermentation will permit the isolation of several new classes of mutants to help elucidate the regulation and function of these fermentation enzymes.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

The cariogenic potential or oral microorganisms resides in their ability to effectively colonize and proliferate on dental surfaces where they produce acid fermentation products from available carbohydrates. The use of mutant analysis will permit us to determine which of the biological reactions possessed by these microbes might be important to their successful colonization of a competitive and changing environment, such as the oral cavity, and permit expression of their pathogenic potential.

PROPOSED COURSE OF STUDIES:

The study of ammonia assimilation in S. mutans will continue with a kinetic analysis of the enzymes glutamine synthetase and glutamate synthase in order to compare their ability to fix ammonia with the results previously obtained with glutamate dehydrogenase. The effect that ammonia levels and added amino acids have on the regulation of these enzymes will also be determined.

Work on the pathway of sucrose utilization will now focus on the sucrose phosphate hydrolysing enzyme. In order to examine this reaction, substrate levels of sucrose phosphate will have to be prepared. Preliminary experiments indicate that sucrose phosphate hydrolase negative mutants can be used to synthesize sucrose phosphate.

Our finding that ethanol dehydrogenase negative mutants are unable to metabolize polyols will now permit the isolation of several new classes of mutants. Both negative and constitutive regulatory mutants of ethanol dehydrogenase will be sought. Such mutants will be used to examine the regulation and function of these secondary fermentation enzymes.

PUBLICATIONS:

1. LeBlanc, D. J., Hawley, R. J., Lee, L.N., and St. Martin, E. J.: "Conjuval" transfer of plasmid DNA among oral streptococci. Proc. Natl. Acad. Sci. U.S.A. 1978. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00034-10 LMI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Mechanisms of Histamine Release

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hook, William A.	Research Microbiologist	NIDR LMI
COPI:	Siraganian, Reuben	Chief, Clinical Immunology	NIDR LMI
	Barsumian, Edward	Visiting Fellow	NIDR LMI
	Morita, Yutaka	Visiting Fellow	NIDR LMI
OTHER:	Vlagopoulos, Triphon	Guest Worker	NIDR LMI
	Chan, Suzanne	Microbiologist	NIDR LMI

COOPERATING UNITS (if any)
Arthritis & Rheumatism Branch, NIAMDD
Laboratory of Cell Biology, NCI
Laboratory of Theoretical Biology, NCI

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Clinical Immunology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4 3/4	3 3/4	1

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the release of histamine from blood basophils and mast cells as one of the immunological mechanisms implicated in inflammation. Histamine-releasing agents studied included IgE antibody, formyl methionine-containing peptides and a complement factor (C5A) derived from human serum. The influence of anions and cations on the mechanisms of histamine release was studied. Although antigen-induced release requires only Ca^{++}, certain other ions were found which enhanced or inhibited the reaction. The desensitization of human basophils by environmental antigens was characterized as to the requirements for antigen dose, temperature and time. The release of inflammatory mediators from cultured mastocytoma cells is being compared with the mechanisms of release from basophils and mast cells collected from humans and laboratory animals. The research is being expanded to determine the role of IgE-mediated mechanisms in apthous ulcers.

NIDR CLASSIFICATIO: 20312
20314

I. Project Description:

A. Influence of anions and cations on IgE-mediated histamine release. Antigen-activated human basophils require calcium for the in vitro release of histamine. The role of certain other ions was studied to determine their influence on the IgE-mediated reaction. Sodium or potassium acetates were more active in supporting histamine release than the corresponding chlorides, iodides, or bromides. This enhancement occurred primarily at the initial antigen-dependent stage of basophil activation. There was no absolute requirement demonstrable for Na^+, K^+ nor Cl^-. Sugar solutions also supported release. Several ionic media interfered with histamine liberation. Reactions carried out in K_2SO_4, Na_2SO_4, LiCl, or the Na salt of isethionic acid markedly inhibited the activation stage but had less effect on the Ca^{++}-dependent, histamine-releasing second stage. The influence of ions other than calcium is complex and cannot be explained by a requirement for a specific exchangeable ion during the secretory phase. Experiments are in progress to learn the effect of these ions on histamine release induced by fMet peptides and C5a.

B. Mechanism of antigen-desensitization of basophils. Human basophils can be made immunologically unresponsive to histamine release by incubation with antigen in the absence of calcium. The mechanism and specificity of this desensitization reaction is being studied, including the influence of antigen dose, temperature, time, ions, and pharmacologic agents. The results show that leukocytes from individuals with immediate hypersensitivity reactions rarely demonstrate in vitro specificity of desensitization with different environmental antigens. Inactivation of cells is maximal with excess antigen at 37°C and requires 45-60 minutes. Cells desensitized with antigen also fail to release histamine when challenged with antibody to IgE. It can be concluded that immunologic deactivation of basophils from naturally sensitized humans is predominantly non-specific and involves IgE on the surface of these cells.

C. Association of hypersensitivity to food antigens with apthous ulcers. Leukocytes from subjects exhibiting recurrent apthous ulcers were tested in vitro for histamine release by common food antigens including buckwheat, chocolate, coffee, corn, cottonseed, egg, fish, shellfish, meat, milk, cereals, soybeans, strawberry, tomatoes and whole wheat. The results obtained indicated that leukocytes from apthous patients may exhibit histamine release with one or more of the above antigens. This significance of this finding in comparison to control groups is being evaluated.

D. Five mastocytomas were found amount 99 mice of different strains, all of which have been treated with a single intraperitoneal injection of 0.5 ml of 2,6,10,14 tetramethylpentadecane and infected 20 to 60 days later with Abelson murine leukemia virus (MuLV-A). Abelson virus was

recovered from three of the tumors tested. The rarity of these tumors in mice, their apparent high incidence in the small number tested and the presence of the virus suggest that the mastocytomas were induced by MuLV-A. Four of the tumors were frozen in liquid nitrogen in early transplant generations and successfully recovered. The mastocytomas were shown to contain granules and to produce serotonin and histamine. These tumors are potentially useful in defining defects associated with neoplastic transformation and in studies of histamine secretion. Presently tumors are maintained by passage in mice and are being adapted to tissue culture growth.

E. Cultured mastocytoma cells were stimulated to release histamine and serotonin by an IgE-mediated mechanism without loss of viability. Stimulation was achieved by incubation of the cells with rat IgE - anti-IgE, rat IgE - anti-light chain, fluoresceinated rat IgE - antifluorescein, IgE enriched mouse antiovalbumin - ovalbumin, or covalently linked dimers of rat IgE, at doses similar to those optimal for normal peritoneal mast cells. Active cell metabolism and Ca^{++} were required to obtain release. Despite the latter, no dose of the calcium ionophore, A23187, could be found which caused degranulation without concomitant cytotoxicity. Phosphatidylserine did not enhance degranulation.

F. Histamine release from mastocytomas. The histamine releasing mastocytoma has been characterized further by chromosome counts, karyotyping, LDH enzyme isotypes and injection into rats and mice. The cells were found to be of rat origin and therefore can be assumed to have arisen as a mutation from the RBL (rat basophil leukemia) cell line.

This cell line has now been cloned and both histamine releasing and non-releasing clones have been obtained. Studies are underway to characterize the different non-histamine releasing clones and determine whether their defects are identical. The effect of metabolic inhibitors on histamine release are being investigated to compare the function of the cells with normal mast cells.

G. Mathematical models for analysis of histamine release. In collaboration with the Laboratory of Theoretical Biology, NCI, mathematical models for analyzing the histamine release from basophils are being developed. The model relating the number of antigen sites bound to the cell surface at low doses indicates that the number of active signals required to initiate release is small. For example, the average number of concanavalin A or ragweed antigen E sites required is about 5, whereas for anti-IgE it is 10. This type of analysis is being extended to studies on the activation - desensitization of cells.

II. Significance to Biomedical Research and the Program of NIDR.

Immunologic release of mediators such as histamine is being studied as one of the effector mechanisms by which microbial cells, allergens

and other stimulatory agents may interact with human serum and leukocytes to cause inflammation. The biologic response of basophils to C5a, formyl-methionine-containing peptides or to environmental antigens could be part of a defense mechanism against microbial infection.

III. Proposed Course.

Experiments will further pursue the mechanisms for the different pathways and regulators of histamine release from mast cells and basophils.

IV. Publications:

1. Siraganian, R.P. and Hook, W.A.: Mechanism of histamine release by formyl-methionine-containing peptides. J. Immunol. 119: 2078-2083, 1977.

2. Taurog, J. Mendoza, G.R., Hook, W.A., Siraganian, R.P. and Metzger, H.: Noncytotoxic IgE-mediated release of histamine and serotonin from murine mastocytoma cells. J. Immunol. 119:1757-1761. 1977.

3. Wennstrom A.O., Wennstrom, J.L., Mergenhagen, S.E., and Siraganian, R.P. The Mechanism of basophil histamine release in patients with periodontal disease. Clin. Exp. Immunol. In press.

4. DeLisi, C., R.P. Siraganian. Receptor crosslinking and histamine release. I. The quantitative dependence of basophil degranulation on the number of receptor doublets. J. Immunol. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 DE 00084-05 LMI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Pathogenesis of Autoimmunity in New Zealand Mice

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Chused, Thomas M.	Medical Officer (Internal Med.)	NIDR LMI
OTHER:	Davidson, Wendy	Visiting Fellow	NIDR LMI

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Lab. of Microbiol Immunity, NIAID
Arthritis and Rheumatism Branch, NIAMDD
Biology Branch, NCI
Cellular Immunology Sec., LMI, NIDR
Lab. of Oral Medicine, NIDR

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Clinical Immunology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/2	1 1/2	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to understand the pathogenesis of autoimmune disease in mammalian systems by studying the model of New Zealand Mice. The present areas of investigation are the early activation of the immune system, manifested by macrophage activation and immunoglobulin production; B and T cell membrane receptors and differentiation antigen abnormalities; and the regulation of xenotropic murine leukemia virus production and virus coded cell surface antigens. The genetic analysis of these parameters has identified several separate genes which presumably contribute to the development of autoimmune disease.

NIDR CLASSIFICATION: 34200
34300

PHS-6040
(Rev. 10-76)

Project Description

I. Objectives:

The purpose of the project is to identify and map the several genes which produce autoimmune disease in New Zealand mice, to determine their function, to understand how they contribute to the development of disease, and to attempt to design a specific therapeutic intervention at one or more loci. Because of the similarity of this model system to several human disorders it may be possible to extrapolate from the animal studies to the human diseases.

II. Methods:

The following metheods were used: 1. Fluoresence activated cell sorter (FACS), a rapid and highly accurate method of quantifying cell surface antigens and receptors; 2) Xenotropic murine leukemia virus focus formation assay on $S^{+}L^{-}$ mink cells-, a one-step, 5 day, highly reproducible assay performed by Dr. Janet Hartley, LVD, NIAID; 3) Two site immunoradiometric assay for mouse IgM-, an ultrasensitive (10-20 picogram), technically straightforward, labelled antibody method. 4) Detection of macrophages activation by LAF production; and 5) Detection of antibodies to DNA, thymocytes and leukemia virus by standard techniques.

III. Major Findings:

Much evidence has been presented in recent years to document the fact that the immune system of New Zealand mice functions aberrantly at a fairly young age (6-8 weeks). This includes the inability to develop tolerance, hyperresponsiveness to certain antigens in vivo, and the inability to respond to sheep erythrocytes in vitro. This had led to the concept that there is a primary abnormality of the immune system of New Zealand mice which causes their autoimmune disease. Virologists, on the other hand, have demonstrated high levels of an unusual murine leukemia virus (MULV) in NZB mice which is termed xenotropic (X-MULV) because it replicates in a wide range of mammalian cells but not in cells of the species which produces it, i.e. the mouse. Excessive X-MULV production could also be the primary genetic lesion of NZB mice. Whether the virus or the immune system or both is "abnormal" is currently the most pressing question in the investigation of this strain.

With regard to the immune system abnormalities mentioned above one must ask the crucial question of whether they represent a primary, genetic, "misprogramming" or could be secondary to premature activation of the immune system to, say, a unique MULV. To this end we have carefully examined the immune function of New Zealand mice in the critical period from birth to 8 weeks of age.

Two lines of investigation have provided direct evidence of early immunologic activation in NZB mice. We have developed an ultrasensitive two site immunoradiometric assay for mouse IgM. Rabbit anti-IgM is covalently attached to Sepharose beads. The standards or samples are added and the IgM they contain is bound to the beads. The beads are washed and affinity purified iodinated rabbit anti-mouse IgM is added. It is bound to the IgM held by the beads. The sensitivity is between 10 and 20 picograms per tube, nearly 100 fold more sensitive than the usual labelled antigen, double antibody method. This technique is sensitive enough to detect IgM released by spleen cells in very short term (4 hour) cultures. Our results show that NZB mice and their Fc hybrids release from 40 to 100 times as much IgM as control mice from birth on. Thus their B cells are "activated" long before any T cell abnormalities have been noted. We have developed a method of enumerating plasma cells with the FACS and found that NZB spleen contains significantly more IgM containing plasma cell than normal mice by one week of age. Furthermore, spontaneous B-cell activation occurs to the same extent in thymectomized irradiated NZB mice regardless of whether Tcells are added or not, but not in normal strains. Moreover, NZB nu/nu mice manifest spontaneous B-cell activation. Thus we conclude NZB mice have a primary B-cell abnormality.

In collaboration with Drs. Monte Meltzer (NCI) and Joost Oppenheim (NIDR) we have examined macrophages from NZB and normal mice. Peritoneal macrophages from BCG infected or LPS infected normal 8 to 10 week old mice show increased chemotactic responsiveness and increased lymphocyte activating factor (LAF) production. LAF is assayed by its ability to stimulate thymidine incorporation by mouse thymocytes. Macrophages from 6 week NZB mice are "activated" by these criteria. Interestingly the level of activation decreases as the animal ages. We have not yet defined the age when activated macrophages appear.

These three studies support the same conclusion: NZB mice activate their immune system at birth. We believe many of the immunologic "abnormalities" may be the consequence of an activated immune system rather than a primary genetic defect.

It was reported several years ago that NZB spleens contained "null" cells, i.e. lymphocytes without T or B-cell markers. We have reexamined this question with the aid of the FACS. All of the reagents used were fluoresceinated Fab'_2 immunoglobulin fragments. Most were affinity-column purified. The findings are: the ontogeny of B cells, followed by the appearance of surface IgM and IgD (operationally determined with anti-mouse Fab which detects much more surface antigen than anti-IgM) in NZB mice is the same as in BALB/c mice, but the adult pattern, normally attained by 5-6 weeks, does not develop in NZB mice. Instead, there is an excessive number of "dull" staining B cells with small, but definite, amounts of surface Ig. We suspect this is because

the B cells begin an immune response as they become immunocompetent and that splenic B cells in NZB mice are an example of activated B cells. We have combined the cell sorting ability of the FACS with the IgM assay to show that surface Ig density does not correlate with IgM production. We have developed a method to measure the Fc receptor of B cells on the FACS and found that it is normal in NZB mice.

The main thrust of the laboratory and its collaborators is to examine the interaction of the X-MULV of NZB mice with their immune system. While a great deal is known about the genetic control of ecotropic (replicating on mouse cells) MULV, data on X-MULV are scanty.

X-MULV can be detected in two ways: 1) by its ability to infect and transform xenogeneic cells, in this case mink cells carrying the murine sarcoma virus genome (focus -formation assay) and 2) by pelleting particles from cell supernatants and measuring either the core protein p30 and/or reverse transcriptase. The second method is not specific for X-MULV but NZB carries only xenotropic MULV. By the focus formation assay large amounts of X-MULV are produced by NZB spleen cells, but not by thymocytes. With the p30 method we have found both NZB and B/W produce about 20 times as much virus as normal mice and NZW 4 times as much. NZW also carries X-MULV antigen (see below) but is free of autoimmune disease. While the viral burden of NZW is lower than NZB this is probably not an adequeate explanation for their normal clinical state.

We have identified three independently segregating X-MULV related genes in NZB mice. One produces high titers of X-MULV and surface gp70; the second produces low titers of X-MULV with high gp70 and the third produces only gp70. We have put these genes on the NFS background and found that the viral genes alone do not produce autoimmune disease. We are also transferring autoimmune disease from NZB to NFS to determine if the viral genes are an obligate part of the immunologic disorder.

Two crosses have been tested for control of X-MULV production. In the first NZB x NZW F_1 (X-MULV +/-) were crossed with NZW (-/-). In the second NZB x NZW F_1 were crossed with NFS (-/-). In both 50% of the progeny produced X-MULV at a level about 1/2 that of NZB and 50% were essentially negative. Thus X-MULV production is controlled by a single codominant gene.

We then asked what effect the above X-MULV gene and H-2 type had on disease production. NZB x NZW F_1 (X-MULV +/-, H-2 d/w) were crossed to NZW (X-MULV -/-, H-2 w/w). Suprisingly, neither H-2 nor X-MULV correlated with the development of autoimmune disease as manifest by the development of immune complex glomerulonephritis and proteinuria. This implies that there is another gene which influences the development of autoimmune disease.

In collaboration with Dr. Herbert Morse, LMI, NIAID we have developed specific antisera to the gp70 surface protein of X-MULV. We are able to detect this antigen on NZB and NZW lymphocytes with the FACS. We have found that thymus and spleen cells of NZB, NZW and DBA/2 mice bear considerable amounts of X-MULV gp70 on their surface. Interestingly in all 3 strains splenic B lymphocytes have more X-MULV gp70 than the T lymphocytes. This distribution is not seen with antisera to other MULV. In our examination of DBA/2 crosses with C57BL/6J and C57L/J we have mapped a gene on chromosome 4 near Fv-1 which controls the expression of X-MULV gp70. We are developing a congenic NZB mouse bearing the suppressing allele of this gene. In addition we have surveyed over 60 strains of mice for lymphocyte expression of X-MULV gp70.

IV. Significance:

The above results represent a good beginning in the attempt to unravel the complex interaction of the immune system of NZB mice with their excessive X-tropic MULV production.

At the present it is clear that these animals are making an immune response at birth. There are genetic factors other than X-MULV and H-2 involved in the development of autoimmune glomerulonephritis.

Identifying the other factor(s) will lead to a complete understanding of New Zealand mouse disease and hopefully contribute to our understanding of human Sjogren's syndrome (see separate Project Report), lupus erythematosus, and, perhaps, other diseases.

V. Proposed Course:

The only definitive method of assessing the functions of various genes and their contribution to autoimmune disease is by genetic analysis. The finding described above now makes this endeavor possible. In particular the presence of activated macrophages and elevated IgM production can probably be considered markers for the future development of autoimmune disease. This will allow us to bypass the one year waiting period for overt clinical disease and breed our animals while they are still fertile. In the various crosses X-MULV production, X-MULV gp70 expression, pelletable p30 production, H2, IgM production and macrophage status will be followed, as required. We are breeding a strain congenic to NZB but without X-MULV production or antigen expression. We wish to know whether their immune system becomes normal, i.e. deactivated. This will be done by selecting the repeated backcross of NZB x FVD or NZC for one half the NZB virus production (i.e. X-MULV +/- and low cell surface gp70). After 8 or 9 generations a brother-sister mating will give two very closely related strains, one +/+, which should be vitually identical to NZB and one -/- which should be congenic NZB without X-MULV. Similarly we are introducing the X-MULV gene into NFS. By following X-MULV antigen

expression and immunologic status we hope to isolate the additional gene described above which influences the development of autoimmune disease.

We also are investigating several other strains of mice which develop autoimmune disease: motheaten, MRL/1 and BXSB. Preliminary results indicate that these strains are different from NZB and the last two may, infact, have a primary T-cell abnormality.

VI. Publications:

1. Moutsopoulos, H., Boehm-Truitt, M., Kassan, S.S., and Chused, T.M. Demonstration of activation of B lymphocytes in New Zealand Black mice at birth by an immunoradiometric assay for murine IgM. J. Immunol. 119: 1639-1644, 1977.

2. Chused, T., Moutsopoulos, H., Sharrow, S., Hansen, C., and Morse, H.C.: Mechanism of autoimmune disease in New Zealand Black mice, Genetic Control of Autoimmune Disease. Elsevier, Amsterdam, 1978. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00085-05 LMI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies in Sjogren's Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Chused, Thomas M.	Medical Officer (Internal Med.)	NIDR LMI
OTHER:	Brown, Elinor M.	Biologist	NIDR LMI

COOPERATING UNITS (if any)
T. Lawley, Dermatology Branch, NIC
D. Mann, Immunology Branch, NCI
Arthritis and Rheumatism Branch, NIAMDD

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Clinical Immunology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/4	1/4	1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is the purpose of this project to determine the etiology of the autoimmune disease Sjogren's Syndrome. Genetic factors are of particular interest, such as HLA-A, -B and -D types and B cell alloantigens.

NIDR CLASSIFICATION: 34000

Project Description

1. Objectives:

To determine the etiology of Sjogren's syndrome (SS) and develop a treatment for it.

II. Methods:

1. B-cell typing, a cytoxicity test against purified B cells with antisera that detect antigens which correlated with HL-D type rather than HL-A or HL-B type, was done by Dr. D. Mann. The HL-D and B-cell antigens appear to correspond to the Ia antigens of mice which, in turn, are very closely related to immune response genes. Thus, although immune response genes have not yet been demonstrated in humans, it is quite possible that when detected, they will correlate with HL-D and B-cell typing.

2. A fragment of the first component of complement, Clq, binds to immune complexes. In collaboration with Dr. T. Lawley we have utilized this to determine the level of circulating immune complexes in patients with SS and controls.

III. Major Findings:

It has been known for years that there is a greater familial incidence of autoimmune disease (Sjogren's syndrome, thyroiditis, etc.) than expected by chance. Whether this is due to genetic or environmental factors was not known. This fact and the report of an abnormal distribution of HL-A types in Grave's disease led us to HLA type 24 SS patients. The diagnostic criteria for SS are 1) positive Schirmer's test for decreased tear production and positive Rose Bengal staining of the cornea, and/or 2) decreased parotid flow and abnormal parotid scintigraphy and 3) a positive lip biopsy showing focal lymphoid infiltrates around the ducts. As in all previously reported series 95% were female and 90% had a positive test for rheumatoid factor.

The result was that 50% of SS patients carried HL-A8 compared to 21% of the control population ($p < .001$). There is now a sizeable group of organ specific autoimmune disesase in which HL-A8 is strikingly increased. In addition to SS, they include gluten sensitive enteropathy, myasthenia gravis, Addison's disease, juvenile diabetes mellitus, Grave's disease, and chronic active hepatitis.

Twenty-four patients with Sjogren's syndrome have been typed for HLA-DW3. Eighty four percent of patients with SS without rheumatoid arthritis (RA) carried this antigen compared to 24% of controls, a greater proportion than HLA-B8. Statistical analysis revealed that the

primary association of SS is with DW3 rather than B8. This result suggested that an immune response gene linked to HLA-DW3 may be involved in the pathogenesis of SS.

Then B-cell typing was carried out on 24 SS patients using antisera which recognize the human homologue of Ia antigens. Two such antisera (Ia-172 and Ia-AGS) reacted with 100% of SS patients compared to 25 and 61% of normals respectively. These two antigens are not associated in the normal population. Thus there are two Ia-like B-cell alloantigens which are prerequisites for the development of SS. Family studies have indicated that patients can be heterogenous for the Ia antigens.

A second area of activity is the presence of antibodies to various extractable nuclear antigens (ENA) in autoimmune disease. Several years ago an entity was defined by the correlation of high titers of antibody to ribonucleoprotein and a distinctive clinical presentation and response to therapy. Very recently it was reported that many SS patients had antibody to a different ENA termed Ha. In collaboration with Drs. Masashi Akazuki and Stuart Kassan we have tested many SS sera for antibody to Ha. By a relatively insensitive immunodiffusion test 50% of SS and 50% SS plus systemic lupus erythematosus (SLE) were positive. 16% of SLE were positive and on clinical basis these probably have SS. Only 5% of SS + RA were positive and no alone or normals were positive.

By the use of affinity chromotography we have purified the Ha antigen to molecular homogeneity and developed a Farr assay for antibody to it. Elevated Ha binding was observed in 73% of patients with SS alone and 73% of patients with SS and lupus erythematosus but only 6% of SS with RA, 3% with lupus erythematosus and 0% in normal individuals. There are two exciting implications of these results. The first is that the two groups of SS patients, with and without RA, are serologically quite different. The second is that antibody to Ha may be a specific serologic test for SS particularly in the absence of RA which often presents a clinical challenge.

Several SS patients have developed immune complex glomerulonephritis. We have demonstrated a high frequency of circulating immune complexes in our patients.

IV. Significance:

The correlation of two specific Ia antigens with SS provides an explanation for the familial occurrence of the disease and suggests the involvement of the major histocompatibility complex of man in the pathogenesis of the disease. The requirements for two distinct antigens is a novel finding in the genetics of autoimmune disease.

The high incidence of anti SS-B may lead to a specific diagnostic test for the disease.

The observation that both HL-A typing and Ha antibody distinguish the SS patients from those with SS and RA suggests that these are distinct populations, perhaps with a different genetic substrate. This is currently being evaluated

V. Proposed Course:

1. There is great similarity between human autoimmune disease and the animal model, New Zealand mice. Because murine leukemia viruses are strongly implicated in New Zealand mouse disease (see separate Project Report) we are looking for evidence of leukemia virus antigens in human SS patients.

2. We will try to identify the antigens circulating in immune complexes.

VI. Publications:

1. Kassan S.S., M. Akizuki, A.D. Steinberg, R.L. Reddick, and T.M. Chused, Antibody to soluble acidic nuclear antigen in Sjogren's Syndrome. Am. J. Med. 63: 328-335, 1977.

2. Akizuki, M., M.J. BoehmTruitt, S.S., Kassan, A.D. Steinberg, and T.M. Chused. Purification of an acidic nuclear protein antigen and demonstration of its antibodies in subsets of patients with sicca syndrome. J. Immunol. 119:932-938, 1977.

3. Gratwhol, A.A., Moutsopoulos, H.M., Chused, T.M. Akizuki, M., Wolf, R.O. Sweet, J.B., and Deisseroth, A. Sjogren-type syndrome after allogeneic bonw-marrow transplantation. Ann. of Internal Med. 87:703-706, 1977.

4. Moutsopoulos, H.M., Chused, T.M., Johnson, A.H., Knudsen, B., and Mann, D.L. B lymphocyte antigens in sicca syndrome. Science 199: 1441-1442, 1978.

5. Kassan, S.S., Thomas, T.L., Moutsopoulos, H.M., Hoover, R., Kimberly, R.P., Budman, D.R., Costa, J., Decker, J.L., and Chused, T.M. Increased risk of lymphoma in sicca syndrome. Ann. Int. Med. In Press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 DE 00114-05 LMI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (60 characters or less)

Control Mechanisms of IgE Production

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Siraganian, Reuben	Chief, Clinical Immunology	NIDR	LMI
COPI:	Fox, Philip C.	Clinical Associate	NIDR	LMI
	Chused, Thomas M.	Medical Officer (Int. Med.)	NIDR	LMI
OTHER:	Fischler, Cynthia	Medical Technician Micro.	NIDR	LMI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Clinical Immunology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2 1/4	1 1/4	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The control mechanisms of IgE production are under study utilizing a mouse model system. Currently the emphasis is on two types of approaches: first, genetic experiments to better understand the control of specific IgE production. Several mouse strains have been found that lack the ability to produce IgE after an antigenic challenge; breeding experiments are attempting to determine whether the genetic defect in these negative strains is identical. These strains are also being crossed to "normal" IgE producing strains to better understand the genetic controls. The second approach is an attempt to purify murine IgE and develop a radio-immunoassay capable of quantitating the small amounts of IgE present in mouse serum (estimated to be less then 1.0 µg/ml).

NIDR CLASSIFICATION: 20314

PROJECT DESCRIPTION

I. Objective:

The aim of this project is to better define the control mechanisms involved in the production of IgE. A model system in the mouse is utilized for this purpose and the approach has focused on strains genetically defective for IgE production to understand the control mechanisms. Another facet in this study is the purification of murine IgE for further studies utilizing sensitive radio-immunoassays, fluorescent techniques and tissue culture studies.

II. Method and Description:

A. Genetic Control Mechanisms of IgE Production. The immunization of mice with small amounts of protein antigen (~ 1.0 µg) in alum induces the production of both IgG_1, and IgE antibodies. This response is boosted by repeating the same antigenic challenge 21-28 days later. In most strains of mice this immunization schedule results in the production of both IgG_1, and IgE and the relative titers of both specific antibodies are equal (ratio of IgG_1 titer to IgE titer less than 5). In contrast studies have shown that in SJL, SWR, BUB/Bn and STB strains of mice this ratio is greater than 20 i.e. these mice produce normal levels of IgG_1, but fail to make IgE.

Breeding experiments between these 4 negative strains show a lack of complementation in the genetic defect of these mice. In contrast the F_1 mice of the cross between a negative mouse and a normal strain show normal IgE production. There is no sex-linked differences in IgE production in these mice. Studies with recombinant strains should further characterize the genetic locus for this defect.

B. Purification of Mouse IgE. The purification of mouse IgE would make available a useful reagent for studies of the cellular control of IgE production. This purification is being attempted through the use of mouse ascitic fluid and classical techniques (ammonium sulfate precipitation between 40 and 50%, DEAE separation follwed by gel filtration). These steps result in ~ 100 x purification. Removal of other contaminants is achieved through the use of solid phase immunoabsorbants prepared with antibodies to the contaminants. The method seems to be capable of purifying IgE even though it is present in serum in very small amounts. The recent development of hybridizing normal spleen cells with myloma cells to produce "hybridomas" producing monclonal antibodies is being utilized to obtained an IgE producing cell line.

A rabbit antibody to mouse IgE has been prepared and it will neutralize the mouse 72 hr PCA activity at a concentration of 2 µg of protein. Further use is being made of histamine release from mouse mast

cells to quantitate and follow the purification of both the anti-IgE and antibodies being prepared to impurities in the IgE.

C. IgE Suppression Due to Exposure to Nebulized Antigen. Mice exposed to different nebulized antigens are incapable of an IgE response when later immunized with the same antigen. The suppression is antigenically specific and is observed even after a short exposure of mice to a nebulized antigen. The suppression is observed only for the IgE class of antibodies, the mice mount a normal IgG_1 response. At present experiments are underway to determine whether the suppression is at the level of T or B cells and whether it is effective in turning off an ongoing immune response. Preliminary experiments suggest that this phenomenon is not due to suppressor T cells.

D. Presence of IgE receptors on murine B lymphocytes. Utilizing spleen cells from parasitized mice, antibody to mouse IgE and fluorescence activated cell sorter IgE receptors were found on mouse B cells. The IgE bound to the B cells is in lose associaton and therefore different than the mast cell-IgE interaction. Other classes of immunoglobulins do not compete with IgE for binding to this receptor. The physiological significances of this receptor is currently under investigation.

III. Significance:

Mediator release from mast cells and basophils is an important amplification system in immunological injury. IgE - antigen interaction is the specific trigger for the release of histamine. The present studies are an attempt to better understand the mechanisms which control the induction and synthesis of IgE. The mouse model is ideal for this type of study because of the extensive previous studies on cellular interactions in this animal and the availability of multiple inbred strains.

IV. Proposed Course:

1. Studies will determine the characteristic of the phenomenon of of IgE suppression induced by exposure to nebulized antigen. The cellular basis of this suppression will be investigated.

2. The breeding studies will be extended to backcrosses of the F_1 mice with the negative strains. This should help clarify whether the lack of IgE production is due to multiple genes.

3. Purified mouse IgE will be used in studies of the immunobiology of the IgE system; e.g. studies on the interaction of IgE with mast cell.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZOI DE 00205-02

PERIOD COVERED
October 1, 1977 to September 1978

TITLE OF PROJECT (80 characters or less)
Investigation of Cellular Immunological Mechanisms in Periodontal Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Fox, Philip C.	Clinical Associate	NIDR LMI
OTHER:	Wahl, Sharon M.	Research Microbiologist	NIDR LMI
	Oppenheim, J.	Medical Officer,	NIDR LMI
	Hook, William A.	Research Microbiologist	NIDR LMI
	Siraganian, Reuben	Chief, Clinical Immunology	NIDR LMI

COOPERATING UNITS (if any)

NONE

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Clinical Immunology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 3/4	1 3/4	0

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A group of patients with periodontosis is being studied to identify cellular immunologic deficiencies. They are being compared to patients with juvenile and adult periodontitis and individuals without periodontal pathology. A familial pattern of this disease is being investigated.

NIDR CLASSIFICATION: 20314

PROJECT DESCRIPTION

I. Objective:

The study group includes young people with periodontosis, a severe localized juvenile form of periodontitis. The aim of this project is four-fold:

1) To determine the immunologic status of patients with periodontosis.

2) To search for abnormalities in the immune system of these patients.

3) To attempt to differentiate periodontosis from other periodontal diseases (periodontitis and juvenile periodontitis) in a systematic manner.

4) To investigate familial patterns of periodontosis.

II. Method and Description:

All patients are screened for underlying medical conditions or vitamin deficiencies. A full medical and oral exam is carried out. Immune function is studied utilizing several assay systems including lymphocyte transformation, lymphokine production, monocyte and neutrophil chemotaxis and histamine release. Gingival fluid is analysed for cellular components and HL-A typing of lymphocytes is done. Dental plaque is evaluated for its' antigenic potential in these patients.

Control groups being studied include individuals with adult and juvenile periodontitis and normals. Chemotactic activity was studied in response to: 1) partially purified C5a derived from zymosan activated serum; 2) crude bacterial factor derived from (Coli; and 3) fMet-Leu-Phe. Results of the chemotactis assay demonstrate that patient and control neutrophil functin was the smae. Monocyte chemotactic activity was noted to be deficient in some cases studied. There was a great deal of variability noted in responses of normal and patient cells on repeated testing. Several factors were identifed which appeared to effect chemotactic responsiveness. These included: antibiotics, smoking, asperin, antihistamines and other drugs. There was a difference noted in chemotactic response of monocytes to self or foreign dental plaque. Plaque itself was not a chemoattractant. Lymphocyte transformation and histamine release studies showed a correlation between cell responsiveness and degree of gingival inflammation, with inflammation serving as a better predictor of response than severity of bone loss. HL-A frequencies were unremarkable.

III. Significance:

Periodontosis is a unique form of periodontal disease in several respects; 1) its occurs early in life (from puberty to age 30); 2) it is extremely localized in its' expression in the mouth, and 3) it is characterized by extensive bone loss without gingival inflammation. It has been reported that patients may have a systemic cellular defect. The absence or presence of this defect is important to document as it may contribute to an understanding of the mechanisms of bone loss in periodontal disease. The local expression of such a systemic defect would provide a valuable model for the study of cellular immune interactions.

IV. Proposed Course:

1) Additional patients are being recruited to extend these studies.

2) Any differences noted in our study group will be intensively investigated for expression in other than oral environment.

3) If a defect can be defined, efforts will be made to treat these patients by overcoming this defect with local or systemic therapy.

4) Several new assays will be utilized to better differentiate subtle differences between control and study groups. Additional chemotactic techniques are being investigated to resolve the question of the presence of a chemotactic defect in periodontosis patients.

ANNUAL REPORT OF THE LABORATORY OF ORAL MEDICINE
NATIONAL INSTITUTE OF DENTAL RESEARCH

The Laboratory of Oral Medicine is concerned with the etiology and pathogenesis of diseases of the soft tissue of the oral cavity with emphasis on: 1) viral infections such as herpes simplex virus; 2) aphthous ulcers and other dermatologic disorders; 3) exocrine and endocrine diseases with special attention to diseases of the salivary glands and pancreas (diabetes mellitus); and 4) malignant and pre-malignant lesions of the oral cavity. The program is disease oriented and highly interdisciplinary.

Over the last year in depth studies have continued on the projects discussed in the 197677 annual report. In addition, one new project was initiated and one old project was terminated. The new project is concerned with the "targeting" or "homing" of biologically active mediators (e.g., chemotactic factor) to specific sites (e.g., tumors, viral lesions) by the use of antibody molecules. The terminated project was concerned with the development and diagnostic use of a radioimmuno-assay for measuring salivary and pancreatic amylase. This project was discontinued because of the departure of the principal investigator.

The project on tumors of the oral cavity is now in its second year. Attempts are being made to determine whether the virus(es) which causes the common wart may also be the etiologic agent(s) in papillomas of the oral cavity (e.g., focal epithelial hyperplasia, laryngeal papillomas). A number of new approaches and techniques have been set up in the Laboratory (e.g., molecular hybridization, endonuclease restriction enzymes, isolation of supercoiled double-stranded DNA). These techniques are being used to identify and distinguish the various papilloma viruses.

As indicated last year, a major shift in emphasis from animal models to a greatly expanded use of clinical materials took place. This emphasis on clinical materials, in almost every phase of our work, continues and has resulted in even closer ties between the clinical and basic research aspects of the various projects. Since last year, a young investigator from Japan with a D.D.S. and Ph.D. joined our staff as a Visiting Fellow. Efforts are being made to strengthened the program even further by the addition of another clinically oriented investigator.

Members of the Laboratory continue to collaborate fruitfully with investigators from other laboratories within NIDR and several new collaborative projects have been initiated with investigators at universities in the United States and Europe.

The various service components of the Laboratory are functioning very well. Excellent support is provided by the Tissue Culture Unit and the Pathology-EM Unit. On the personnel side, no major new recruitment has taken place.

Since last year's annual report, substantial progress has been made in several areas. The potential significance and implications of some of our findings are briefly summarized below:

1. Inflammation is an important host response to a variety of diseases including viruses and tumors. Experiments were initiated to see if it might be possible to increase the inflammatory response at the site of lesions. It was reasoned that if fMet-leu-phe (a powerful chemotactic agent) was coupled to antibody directed against a specific virus or tumor, the resulting molecule might be capable of binding to the target tissue where it also might attract inflammatory cells. In a collaborative study with members of the Laboratory of Microbiology and Immunology and the Laboratory of Developmental Biology and Anomalies, it was shown that fMet-leu-phe could be coupled to anti-HSV antibody and that the resulting molecule retained its capacity to bind to antigen and was chemotactic. The effectiveness of chemotactic antibody in vivo is presently under investigation. If chemotactic antibody proves effective in vivo, it should be possible to couple a variety of other biologically active mediators to immunoglobulin molecules.

2. When human basophils are exposed to known allergens, such as ragweed antigen, histamine is released. Last year LOM investigators showed that the release of histamine could be greatly enhanced by interferon and suggested that the induction of interferon by viruses might be one of the cofactors responsible for potentiating attacks of bronchial asthma during viral infections.

Concentrating at the molecular level, LOM investigators now have found that the enhancement of histamine release by interferon requires an induction period of 6 to 9 hours and new RNA synthesis. In this connection, it has been known for some time that the antiviral effects of interferon also require an induction period and new RNA synthesis. These findings suggest that the histamine-enhancing properties and the antiviral properties of interferon may operate through a common pathway. If this turns out to be the case, then the mechanism by which interferon enhances histamine release may provide new insight into how interferon exerts its antiviral effect.

The fact that interferon enhances histamine release may have broader implications than just in viral infections. It is known that when leukocytes from individuals immune to a specific antigen (e.g., BCG) are challenged with the same antigen a variety of soluble mediators, including interferon, are released. During the last 12 months, investigators from LOM have shown that under conditions in which immunologically-induced interferon is released there is a marked enhancement of histamine

release. These studies suggest that the enhancement of histamine release (immediate hypersensitivity reactions) may be a common sequela secondary to a variety of immunological responses. This possibility is presently under investigation.

3. Several important new findings have been made in the area of virus-induced diabetes. First, past studies from the Laboratory have shown that only certain inbred strains of mice develop diabetes when infected with encephalomyocarditis (EMC) virus and that susceptibility is inherited as an autosomal recessive trait. The precise number of loci involved was not known. Studies completed during the last year have provided data consistent with a mendelian mode of inheritance, principally controlled by a single locus involving two or more alleles. The gene product controlling the development of viral-induced diabetes is still not known, but preliminary experiments suggest that there might be more receptors for EMC virus on the surface of beta cells from the strains of mice that developed diabetes than from strains of mice that did not develop diabetes. If this is found to be the case, then it is possible that the locus controlling susceptibility might actually operate by modulating the expression of viral receptors.

Second, using the beta cell cultivation technique previously developed in the Laboratory, LOM investigators now have succeeded in obtaining variants of two common human viruses which when injected into mice attack pancreatic beta cells and produce a diabetes-like syndrome. One of these viruses is a member of the Coxsackievirus B group and the other is a member of the reovirus group. These observations greatly strengthen the possibility that viruses might play a role in the etiology of some cases of juvenile-onset diabetes in humans.

4. New understanding about the role of the host's immune response in controlling the acute and latent phases of herpes simplex virus (HSV) infection in ganglia has been obtained by use of *in vitro* and *in vivo* systems. LOM investigators showed that antiviral antibody markedly reduced the amount of infectious virus in ganglia but did not eliminate the acute phase of the infection. From these and other studies it is concluded that both humoral and cellular components of the immune system are needed to eliminate the acute phase of the ganglionic infection.

Another major finding has been the development of an animal model for studying viral reactivation. By use of agents such as cyclophosphamide and/or x-irradiation, it has been possible to reactivate HSV in ganglia of latently infected animals. This model should prove useful in obtaining new information about the factors that control HSV latency.

On the molecular level, LOM investigators have continued their studies on the state of the viral genome in ganglia of chronically infected animals. There are two current theories. One states that

there may be a low level of viral replication in ganglia which cannot be detected by simply assaying ganglionic homogenates for infectious virus. The other states that the virus exists in a non-replicating, truly "latent" state. If the former is true, mRNA transcripts of HSV should be present in ganglia during the chronic phase of the infection. By hybridizing I^{125} HSV DNA with ganglionic mRNA, LOM investigators found less than one viral mRNA molecule per 2000 ganglionic cells or an equivalent of less than 400 mRNA molecules per trigeminal ganglion. This indicates that transcription of at least the vast majority of the viral genome is severely depressed and possibly blocked altogether. Since expression of genetic information is needed for viral replication, these results suggest that during the chronic stage of the infection the viral genome is likely to exist as a non-replicating, latent entity. Experiments are now under way to determine whether the viral genome is integrated into the DNA of the host cell. These molecular techniques should provide powerful tools for studying HSV latency in humans.

A detailed description of these and other projects follows.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER [illegible]

PERIOD COVERED

October 1, 1977 - September 30, 1978

TITLE OF PROJECT (80 characters or less)

Diseases of the Salivary Glands and Pancreas: Virus-Induced Diabetes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Notkins, Abner L.	Medical Director	LOM	NIDR
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	Jenson, Alfred B.	Surgeon	LOM	NIDR
	Onodera, Takashi	Visiting Associate	LOM	NIDR
	Dobersen, Michael J.	Staff Fellow	LOM	NIDR
	McClintock, Patrick R.	Staff Fellow	LOM	NIDR

COOPERATING UNITS (if any)

Laboratory of Developmental Biology & Anomalies, NIDR

LAB/BRANCH

Laboratory of Oral Medicine

SECTION

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.30	4.55	3.75

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two viruses of human origin, Coxsackie B4 and reovirus type 3, were shown to be capable of producing a diabetes mellitus-like syndrome in mice. Two other human viruses, mumps and Coxsackie B3, were capable of infecting and damaging human pancreatic beta cells in culture. Susceptibility of inbred strains of mice to virus-induced diabetes was studied.

DISEASES OF THE SALIVARY GLANDS AND PANCREAS: VIRUS-INDUCED DIABETES

Background

Diabetes mellitus is the third leading cause of death in the United States. Over five million Americans are diabetics. Ninety percent have maturity-onset diabetes (MOD) and ten percent have juvenile-onset diabetes (JOD). Current evidence suggests that diabetes is a heterogenous syndrome. MOD has a familial tendency and the secretory capacity of insulin remains relatively unchanged throughout the disease. JOD occurs in a significantly higher than normal incidence in persons with certain HLA types and there is a relatively abrupt loss of the capacity to secrete insulin. The development of JOD is thought to be dependent on the interaction of an environmental agent(s) with the beta cells of a genetically susceptible host. Clinically, JOD affects a younger population than MOD and causes a greater morbidity and more rapid mortality because of its more severe complications. Prevention of development of JOD may be dependent on identification and eradication of the agent(s) that trigger this insulin-dependent type of diabetes.

Seroepidemiologic studies and many case reports of human diabetes mellitus, particularly the juvenile-onset type, have yielded a large volume of circumstantial evidence suggesting that viruses may be involved as etiologic agents in some cases of diabetes mellitus. There has been no direct proof, however, that viruses actually cause human diabetes mellitus. During the past year, investigators from the Laboratory of Oral Medicine produced experimental evidence that viruses of human origin may have the potential to cause diabetes mellitus. Two of the most frequently mentioned candidates for human diabetogenic viruses, mumps and Coxsackievirus B3, were shown to be capable of infecting and destroying human pancreatic beta cells in culture. Two other widely disseminated human viruses, reovirus type 3 and Coxsackie B4, were shown to be capable of causing a diabetes-like syndrome in mice.

Population genetic studies of JOD have demonstrated the association between different alleles of the HLA-B-D locus and the development of diabetes. If viruses are involved in human JOD, they may interact in some way with the gene products of the HLA locus or a closely associated locus in linkage disequilibrium with the HLA locus. During the past year, investigators from this Laboratory have undertaken extensive genetic studies of inbred strains of mice and have shown that susceptibility to the murine diabetogenic virus, M-variant of encephalomyocarditis (EMC) virus, is transmitted as a recessive trait with a single gene locus controlling susceptibility. Studies of the interaction of EMC with murine beta cells in culture suggest that the gene products of the locus controlling susceptibility to EMC may be modulating a receptor for EMC on the beta cell surface.

We have developed two reliable techniques which are enabling us to study in depth the effect of viruses on beta cells. The in vitro isolation and enrichment of pancreatic beta cells from mice, rats and human and non-human primates have provided us with a technique for determining if prototype viruses have a tropism for beta cells of certain strains or species in culture. By consecutive passage of the virus through beta cell cultures, we can then select out a variant or mutant which has an increased tropism for beta cells in vitro and the capacity to cause diabetes mellitus in vivo. The double-label antibody technique has enabled us to determine if insulin-containing beta cells in vitro and in vivo are infected by specific viruses.

Objectives

The objective of this research project is to determine if viruses and/or autoimmunity have a role in the development and pathogenesis of JOD.

Major Findings

VIRUS INFECTION OF HUMAN PANCREATIC BETA CELLS

Viruses, particularly mumps and the Coxsackievirus B group, have been proposed as candidate diabetogenic agents for JOD since the turn of the century. A true causal relationship between viral infection and diabetes, however, has only been proven for the murine animal model system. Investigators in this Laboratory have shown that the susceptibility of murine beta cells to infection by diabetogenic viruses is operative both in beta cell cultures (in vitro) and in the intact host (in vivo). The application of these animal studies to human disease is dependent on showing whether or not human diabetogenic candidate viruses are capable of infecting beta cells in man. Since this type of study could not be carried out in vivo, it had to be done in vitro. Our goal has been to establish and characterize pancreatic beta cultures from both human and non-human primates and determine the capacity of human diabetogenic candidate viruses to infect and damage these beta cells.

Last year, we developed a technique for growing human and non-human primate beta cells in culture. The percentage of beta cells in each culture was quantitated by the fluorescent antibody technique. By a double-label antibody technique, primate (human and non-human) beta cells were shown to be susceptible to mumps virus infection. With the double-label antibody technique, we identified three populations of cells: uninfected insulin-containing beta cells which stained only with rhodamine-labeled anti-insulin antibody; mumps virus-infected (non-insulin-containing) cells which stained only with fluorescein-labeled anti-mumps antibody; and virus-infected insulin-containing beta cells which stained with both antibodies.

This year, we examined the capacity of Coxsackievirus B3 to infect insulin-containing beta cells in human pancreatic cell cultures. By using the double-label antibody technique, we were able to show that insulin-containing beta cells were susceptible to infection by Coxsackievirus B3. Virus assays of the infected cultures showed that virus titers were highest at 72 hours after infection. Examination of infected beta cell cultures revealed marked cytopathology at 4 days. Intracellular immunoreactive insulin levels decreased rapidly between 24 and 72 hours after infection and then leveled off at approximately 25% of the levels obtained from the control cultures. Extracellular immunoreactive insulin levels increased during the first 48 hours after infection and then decreased markedly over the next 48 hours, leveling off at 20% levels seen in the control cultures. These results suggest that replication of Coxsackievirus B3 in human pancreatic beta cells induce beta cell damage and degranulation. Release of immunoreactive insulin into the culture media was followed by death of affected beta cells.

In our experiments, beta cell cultures were highly susceptible to infection by either mumps or Coxsackievirus B3 regardless of the age, sex or HLA type of the donor of the pancreas. Neither the present study with Coxsackie nor the earlier study with mumps proves that these viruses infect and destroy beta cells in vivo. It is known that some viruses will grow in cultured cells derived from organs that, when in the host, are resistant to the virus. Experiments are underway to determine if there is a quantitative difference in susceptibility of human beta cells to infection by different prototype viruses or their variants based on the HLA type or genetic background of the donor of the pancreas.

MURINE VIRAL-INDUCED DIABETES

EMC Virus: We have previously shown that the M-variant of EMC virus has the capacity to infect and destroy pancreatic beta cells in mice. Because of this beta-cell damage, infection with EMC virus causes decreased insulin in the pancreas and serum, increased blood glucose levels, and impaired glucose tolerance. The extent of beta cell damage, when graded histologically correlates well with the hyperglycemia observed.

Single Locus Controlling Susceptibility: Earlier studies from our laboratory revealed that the development of EMC viral induced diabetes is genetically determined, only certain inbred strains of mice (e.g., SWR/J, SJL/J, CD-1, Swiss NIH, DBA/1, and DBA/2) develop hyperglycemia and/or abnormal glucose tolerance tests following infection with EMC virus. Other strains (e.g., C57BL/6J, CBA/J, A/J, AKR, C3H/He) do not develop overt diabetes or abnormal glucose tolerance tests under the same condition. Matings between susceptible (SWR/J or SJL/J) and resistant strains of mice (C57BL/6J) showed that in the F-1 generation,

the development of EMC-induced diabetes is inherited as an autosomal recessive trait. When the F-1 hybrids were compared with the resistant parental strains (e.g., C57BL/6J), the hybrids were found to be essentially resistant to the development of EMC-induced diabetes. When the F-1 hybrids were crossed with susceptible parents (e.g., SJL/J or SWR/J), 46% of the backcross mice were found to be susceptible to the development of EMC-induced diabetes. By Chi square analysis of the observed frequency of diabetic animals in the (F-1 x C57BL/6J and F-1 x SWR/J) backcross, the hypothesis that susceptibility to the diabetogenic effect of EMC virus is controlled at a single locus was not rejected ($p<0.05$).

When susceptible SWR/J mice are mated with the less susceptible DBA/1 mice, the susceptibility to EMC virus-induced diabetes is similar to the less susceptible DBA/1 parents. When two highly susceptible strains (e.g., SWR/J and SJL/J) are crossed, the F-1 hybrids develop diabetes. Since susceptibility is recessive, the development of viral-induced diabetes in the F-1 hybrids supports the argument that the same locus is of major importance in controlling susceptibility in both parental strains. The F-1 offspring of these susceptible strains, however, developed less severe diabetes than either parental strain. The dominance of the less susceptible DBA/1 genotype over the more susceptible SJL genotype, the resistance produced by the C57BL/6J genotype, and the differences among inbred strains of mice in the severity and percentage of animals developing hyperglycemia suggest that there may be more than one allele controlling the degree of susceptibility. In addition, by determining the number of infected beta cells with fluorescein-labeled anti-EMC antibody we found that some beta cells are infected even in resistant strains and that quantitative differences exist among the various strains. There appears to be a spectrum of susceptibility ranging from strains of mice showing only a few infected beta cells and no hyperglycemia to strains showing many infected beta cells and hyperglycemia. Although a variety of complex genetic interactions might account for these variable findings, the simplest explanation is a single locus involving two or more alleles.

Viral Receptors: Pancreatic beta cells from strains of mice that develop EMC-induced diabetes (susceptible), and pancreatic beta cells from strains of mice that do not develop EMC-induced diabetes (resistant) were grown in cultures and infected with EMC virus. Examination of these cultures revealed that beta cells from susceptible mice produced up to 50 times more virus than did beta cells from resistant mice. To see whether the higher viral titers yield in cultures from susceptible mice was due to more virus being produced per cell or more cells being infected, infectious center assays were performed by inoculating monolayers of beta cells with EMC virus. Ten to thirty times more SJL/J than C57BL/6J cells contained infectious virus. These results were confirmed

in an immunofluorescent assay for EMC virus antigens. The beta cells from the susceptible mice (e.g., SJL/J) contained 10 times as many fluorescent cells as the beta cell cultures from resistant mice. In a more direct assay, the ability of susceptible (SJL/J) and resistant (C57BL/6J) cells to bind virus was measured. The susceptible beta cells (SJL/J) bound twice as much virus as did the resistant beta cells (C57BL/6J). In contrast, other cell types from these two strains (e.g., pancreatic fibroblasts, primary kidney cells) bound equal amounts of virus. From this data, we concluded that genetically determined differences in viral receptors on surface of beta cells may be one of the factors controlling susceptibility to EMC-induced diabetes mellitus.

These preliminary results supported the hypothesis that the observed differences in susceptibility to EMC were related to events at the cell receptor level. However, due to the difficulty in obtaining large numbers of beta cells and the technical difficulties inherent in using monolayer cell cultures for the binding assays, a definitive answer could not be obtained. It became apparent that several key technical problems needed to be resolved. One of these was the necessity of plaque-purifying the virus in order to obtain a genetically uniform stock. When this was done, two interesting variants were obtained which differed in their ability to induce hyperglycemia in susceptible mice. When these variants were tested in SJL/J mice, one of them (108-D-I) produced hyperglycemia in 95-100% of the animals. In contrast, one of the other isolates (16-B-II) did not induce diabetes under the same conditions. These variants have proved to be genetically stable and comparative studies of their receptor binding characteristics and their in vivo behavior are planned.

Using the plaque-purified viruses, we have established an assay for receptor activity which is applicable to both suspension and monolayer cultures. The assay is based upon recently developed methods for measuring the binding kinetics of polio virus and rhino-viruses with human cell lines. However, unlike the above viruses, the binding of EMC to its receptor on mouse cells appears to be extremely rapid and extremely reversible. A well known characteristic of picorna-viruses is that after the initial contact with the receptor the virion may elute or dissociate from the receptor with the loss of infectivity. In the case of EMC, it appears that the elution process may be favored under physiological conditions making it difficult to estimate the true binding capacity of the host cell. Work is continuing on this problem, and by suitable modifications of the assay conditions, we expect to be able to obtain good kinetic measurements of the virus receptor interaction.

Coxsackievirus: Previous studies from our Laboratory with EMC and Coxsackieviruses revealed a remarkable tropism of viruses for specific cells of the pancreas: the M-variant of EMC virus attacks beta cells

but not acinar cells; whereas, the Coxsackieviruses attack acinar cells but not beta cells. To see whether the tropism of Coxsackievirus B4 for beta cells could be increased, virus that had been grown in secondary mouse embryo cells was serially passaged in pancreatic beta cell cultures. Coxsackievirus B4 from selected passages was inoculated into SJL/J mice and then glucose in the blood from the infected animals was measured. The virus, which was not passaged in beta cell cultures, failed to produce diabetes. However, beginning with the second passage, a number of animals showed elevated blood glucose levels. By the fifth passage, over 35% of the inoculated animals became diabetic and by the 14th passage, close to 80% of the inoculated animals became diabetic.

In the majority of the animals, the hyperglycemia induced by passaged Coxsackievirus B4 was transient. At 13 days after infection, about 86% of the animals were hyperglycemic. The severity of the hyperglycemia and the percentage of hyperglycemic animals declined rapidly. At 30 days after infection, only 43% of the animals were hyperglycemic. At 80 days, only 2% of the animals still were diabetic. This may well be due to the fact that a sufficient number of beta cells are left intact after the infection so that proliferation and/or hypertrophy of these cells results in metabolic compensation. When the non-fasting blood glucose of individual animals was examined over a period of 80 days, three general patterns were observed. The most common pattern, found in up to 80% of the animals, was a transient hyperglycemia lasting from two to eight weeks. The second pattern, occurring in less than 5% of the animals, was a severe and persistent hyperglycemia lasting for at least 12 weeks. The third pattern, found in 15-30% of the animals, was characterized by a failure to develop hyperglycemia. Despite the failure of these latter animals to develop hyperglycemia, many had distinctly abnormal glucose tolerance tests.

To study the relationship between virus-induced hyperglycemia and immunoreactive insulin (IRI), SJL/J male mice were infected with Coxsackievirus B4 and, at different times thereafter, the concentration of IRI in the pancreas was determined and compared to the concentration of non-fasting glucose in the blood. Within 2 days after infection, a number of animals showed signs of hyperinsulinemia, probably secondary to the release of insulin from damaged beta cells. This was followed, beginning on day 4, by hypoinsulinemia and hyperglycemia. As shown in the relationship between pancreatic IRI and blood glucose, the infected animals could be segregated into two groups: one with normal plasma IRI and normal glucose levels and the other with depressed plasma IRI and elevated glucose levels.

To determine whether the alterations in insulin might be secondary to Coxsackievirus-induced beta cell damage, sections of pancreas from infected and uninfected mice were examined microscopically. Islets from animals infected with Coxsackievirus B4 revealed infiltration of

mononuclear cells and disruption of the architecture of the islets of Langerhans. Mild inflammatory changes were seen within 3 to 4 days after infection and the inflammation was maximal at about 5 days. The severity of the inflammatory changes varied considerably among animals and within a single pancreas with some islets showing little, if any, change while others showed moderate to extensive infiltration of cells. Normal appearing islets often were seen adjacent to islets showing extensive infiltrations. In some cases, only a portion of the islet was involved. In the more severe cases, beta cell destruction and coagulation necrosis were observed. At two weeks after infection, atrophic islets were sometimes seen.

To show that virus was actually replicating in beta cells, sections from the pancreas of infected mice were stained with FITC-labeled antibody to Coxsackievirus B4. Within 3 days after infection, viral antigens were seen in the cytoplasma of beta cells and maximal involvement occurred at about 4 days. After 7 days, the viral antigens were faint and difficult to detect. The degree of beta cell involvement varied considerably with some islets showing only a few cells and others showing almost all the cells containing viral antigens. Occasional acinar and ductal cells also contained viral antigens.

To see whether the induction of diabetes by Coxsackievirus B4 was influenced by the host, several different inbred strains of mice were infected and glucose indexes determined. The male SWR/J, SJL/J and NIH Swiss mice readily developed diabetes, whereas C57BL/6J, CBA/J, AKR, Balb/c, C3H/J, DBA/1J and DBA/2J mice failed to develop diabetes. A similar pattern was observed in females of these strains, except the severity of the hyperglycemia and the percentage of diabetic animals were lower. These studies suggest that the genetic background of the host influences the development of diabetes.

Precisely how passage of Coxsackievirus B4 in cultures enriched for beta cells increases its diabetogenic capacity is not known, but alterations in the tropism of viruses after serial passage in animals or tissue culture is a widely recognized phenomenon. In the case of Coxsackievirus B4, there are at least several ways in which this could have occurred. First, the original stock virus pool may have contained two populations of virus; one tropic for beta cells and the other not tropic for beta cells. Growing the stock virus in cultures enriched for beta cells may have favored the replication of the beta-tropic virus. Second, mutation or recombination may have taken place during serial passage of the virus in culture and the presence of beta cells may have favored the selection of the beta-tropic virus. Third, non-genetic adaptation (e.g., host-controlled alterations in viral antigens or coat) may have occurred, thereby increasing the capacity of the virus to bind to beta cells. By plaque-purifying the virus and by passaging the virus

in beta cell cultures from different inbred strains of mice, it might be possible to distinguish between a stable mutant and non-genetic adaptation. These experiments are now underway.

Reovirus: Attempts to produce diabetes in animals with other than EMC virus have been largely unsuccessful. During the past year, we have found that a virus which is widely disseminated in the human population, reovirus type 3, can infect mouse beta cells and alter the host's capacity to handle glucose.

It has been previously reported that reoviruses produce a variety of lesions in newborn mice, including encephalitis, hepatitis, myocarditis, adrenalitis, and acinar pancreatitis. However, involvement of the islets of Langerhans has not been reported. To see whether the tropism of reovirus for beta cells could be increased, the virus was passaged at least 7 times in SJL mouse pancreatic beta cell cultures and then SJL suckling mice were infected. At different times after infection, the concentration of insulin in the pancreas and glucose in the blood were determined. Within 3 days after infection, the concentration of IRI in the pancreas had declined and was reduced by approximately 30% at day 5. Immunoreactive insulin remained depressed over the next 10 days and then began to return towards normal levels. Blood glucose levels fell in parallel with the levels of pancreatic insulin, presumably due to the acute release of insulin into the blood from damaged beta cells. No evidence of overt hyperglycemia was found. However, a number of infected animals showed a distinctly abnormal response to a glucose load. Abnormal glucose tolerance test values were first detected at 7 days after infection. Some of these animals had abnormal GTT at 17 and 21 days after infection.

Evidence that metabolic alterations were secondary to reovirus induced beta cell damage was obtained by light microscopy, immunofluorescence, and electron microscopy. As early as 3 days after infection, interstitial edema was seen in areas surrounding the islets. Shortly thereafter, pyknotic nuclei and degenerative cytoplasmic changes were observed within islet cells. Within 7 days after infection, many islets showed focal necrosis and some showed extensive coagulation necrosis with loss of islet architecture.

To see whether the pathologic changes found in the islets were due to replication of the virus in beta cells, a double-labeled antibody technique was used. Frozen sections from the pancreas of infected mice were stained with FITC-labeled anti-reovirus type 3 antibody and TRITC-labeled anti-insulin antibody. By double exposure photography, insulin-containing beta cells infected with reovirus were readily identified by their orange, green and yellow color. In addition, the double exposure showed that some non-insulin containing cells (e.g., acinar cells, ductal cells, and degranulated beta cells) also had become infected.

Further proof that reovirus type 3 replicated in beta cells came from electron microscopic examination of the islets. A reticulo-granular viral matrix, characteristic of active reovirus replication, was sometimes seen among insulin granules in beta cells. Focal cytoplasmic degradation, encapsulating large numbers of reovirus particles, also was found in the cytoplasm of many infected beta cells. In the cytoplasm of a damaged beta cell, numerous reovirus particles, 70nm in diameter, were interspersed among insulin granules and in crystalline array. Glucagon-containing alpha cells also were readily identified in many of the islets, but thus far, viral particles have not been found in these cells. Moreover, we recently found that unpassaged reovirus infects beta cells, but that passage of the virus in beta cell cultures markedly enhances this capacity.

The precise explanation for this enhancement is still not clear, but passage of the virus in beta cell cultures might have resulted in adaptation and/or selection of virus with increased tropism for beta cells. Moreover, the finding by electron microscopy of virus particles in beta cells, but not adjacent alpha cells, suggests that alpha cells may not be susceptible to reovirus and points to the possibility that there might be specific receptors for this virus on beta cells. Recently, it has been suggested that the δ-1 outer capsid polypeptide may be the component of the reovirion that binds to cell receptors and that differences in δ-1 may be the basis for differences in cell tropism and neuro-virulence among reovirus strains. Whether the tropism of reovirus for beta cells also is due to the δ-1 polypeptide is now being investigated.

Microassay and Immunological Studies: Several lines of evidence suggest an involvement of the immune system in the etiology of juvenile-onset diabetes mellitus. One of these has been the demonstration of humoral immunity against islet cells (islet cell antibody) in juvenile-onset diabetics.

The presence of islet cell antibody suggests the possibility of a convenient marker for identifying autoimmune diabetes. The indirect fluorescent antibody technique has been exclusively used for islet cell antibody detection.

During the past year, we have tested the incidence of islet cell antibody in the sera from newly diagnosed juvenile-onset diabetes by the indirect fluorescent antibody technique. About 41% of the tested samples turned out to be positive. However, the incidence was seen to fall significantly in sera from cases greater than six months onset, when only 14% of the tested samples were positive. Sera from families which have one or more diabetics and where the complete family haplotypes are known are being tested for presence of islet cell antibody.

Although the fluorescent antibody technique is highly specific due to the nature of antigen-antibody interactions, quantitation is based on visual interpretation, and the reproducibility and sensitivity are inherently limited. Furthermore, this technique gives little information on the biological significance of islet cell antibody.

In recent years, microcytotoxicity testing has proved to be a useful tool in the study of immune phenomena. The objective of this research is to investigate the feasibility of using a microcytotoxicity system as a quantitative, sensitive, and reliable tool in studying the immunology of juvenile-onset diabetes. There are two important features of this system. First, the number of cells required in the assay and second, the marker system. One of the major drawbacks in islet cell culture has been the relatively low number of cells that are obtainable. This is especially apparent in work with human and non-human primate tissue. The microcytotoxicity system allows for the maximum use of a minimum number of cells by making it technically possible to do a relatively large number of replicates. Secondly, the present state of islet cell culture yields only enriched beta cell populations which contain the other islet cells, fibroblasts and ductal cells. Measuring the release of insulin by radioimmunoassay therefore, as an indicator of beta cell lysis allows an unambiguous interpretation of the cytotoxic reaction. Further, the radioimmunoassay for insulin shows great sensitivity to the proposed system.

The development of the microcytotoxicity system involves three steps: (1) the establishment of an islet culture system; (2) determination of the functional integrity of the culture system; and (3) application of the system to the problem defined above.

During the past year, a method for islet enrichment and culture has been developed through modification of various standard techniques. Briefly, the technique involves collagenase digestion of newborn rat pancreas followed by purification of the islets by Ficoll density gradient centrifugation. Following enzymatic dissociation of the islets and removal of fibroblasts by selective adherence, the cells are plated in flat-bottom microwell trays. This method routinely yields approximately 40-60 islets/pancreas (10^5 cells/pancreas) with the cultures being comprised of 40-60% beta cells as determined by immunofluorescent staining for insulin. The newborn rat has been chosen to establish the feasibility of this approach. Adaptation to human or non-human primate culture systems will eventually be undertaken.

A functional characterization of the culture system has recently been completed. Time course experiments have indicated that insulin release and content of the cultures remain relatively unchanged over a 14-day period. Stimulation of insulin release (3-fold) by glucose has also been demonstrated. This response was found to be enhanced approximately 2-fold by theophylline and depressed approximately 2-fold by epinephrine. Insulin release increased in a sigmoid fashion in response to increasing glucose concentration. Finally, amino acids such as arginine, leucine

and isoleucine stimulated insulin release 3-fold, 2-fold and 4.5-fold, respectively.

Determinations of the intracellular insulin levels suggest that meaningful data can be obtained using as few as 10^3 to 10^4 cells, thus greatly maximizing the use of a small number of cells. This system is capable of yielding reproducible and quantitative results. The adaptation of this system to the study of islet cell antibody offers a meaningful approach to the quantitation and determination of the biological significance of this phenomenon.

Implications for Biomedical Research

There are over one million insulin-dependent diabetics in the United States. This form of diabetes is the most difficult to control and the short and long term side effects are enormous with respect to the patient's health and economics. Circumstantial evidence suggests that viruses play some role in the initiation of this form of diabetes. Identification of diabetogenic viruses and preparation of a vaccine might offer a form of preventive medicine that is not otherwise available for genetically predisposed individuals.

Future Plans

Our future plans for research on viral-induced diabetes are aimed at: (1) identifying viruses which produce diabetes in mice and rats with the long-term goal of testing those viruses in non-human primates; (2) identifying diabetogenic mutants of prototype viruses which can ultimately be used to study the molecular basis of virus-induced diabetes; and (3) identifying inbred strains of mice and rats that are susceptible and resistant to diabetes induced by these viruses with the goal of evaluating the genetic and non-genetic factors controlling susceptibility.

The in vitro isolation and enrichment of beta cells from different species will be aimed at: (1) studying the effects of various hormones, secretagogues and drugs on insulin regulation in beta cells, and (2) studying the effects of autoimmune antibody (ICA) on beta cells to provide information on the possible role of autoimmunity in the development of JOD.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00094-05 LOM CT 0600114

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Ulcerative Lesions and Tumors: Aphthous Ulcers

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Graykowski, Edward A.	Medical Director	LOM NIDR
COPI:	Hooks, John J.	Research Microbiologist	LOM NIDR
	Archard, Howell O.	Dental Director	LOM NIDR
	Notkins, Abner L.	Medical Director	LOM NIDR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Oral Medicine

SECTION

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.35	2.30	1.05

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The object of this project is to study the etiology and pathogenesis of mucosal diseases of the oral cavity, with the ultimate goal of treatment and prevention. At the present time, emphasis is on recurrent aphthous ulcers, Behcet's disease, and Sjogren's syndrome. Efforts are being made, by a variety of techniques, to demonstrate a viral etiology and to look for immunological abnormalities. Attempts also are being made to transmit the disease to rhesus monkeys. Experiments were begun to determine if injury to tissue plays a role in initiating oral ulcers in patients known to have recurrent disease, as compared to matched controls. Double-blind clinical trials are being carried out to determine the efficacy of various drugs.

ULCERATIVE LESIONS AND TUMORS: APHTHOUS ULCERS

Background and Objectives

This area of research is concerned with investigations in patients with recurrent aphthous ulceration (RAU) and Behcet's syndrome. Aphthous ulcers are characterized by recurrent painful single or multiple ulcers on the moist, movable oral mucosa. Behcet's syndrome may be the most severe form of RAU with the signs and symptoms of immune reactions in the eye, joints, and central nervous system, in addition to oral and genital aphthous ulcers. The etiology of both diseases is unknown, however, there is a strong familial tendency and the oral aphthous ulcers often appear following injury to the mucosa. Physical and emotional stress, decreased serum levels of folic acid, iron, and vitamin B12, as well as auto-immune reactions, have been implicated. Microbiological studies have failed thus far to identify any virus or unusual bacterial flora associated with the lesions. Except for the specific replacement therapy in folic acid, iron, and vitamin B12 deficiency, therapy to date has been directed toward palliation of the discomfort caused by the oral ulcers.

Major Findings and Implications

Studies on the Etiology of Aphthous Ulcers and Behcet's Syndrome. The most recent advances in viral isolation attempts were used to isolate or detect the presence of persistent or latent viruses from patients with recurrent oral ulcers and Behcet's Syndrome. Biopsy specimens of ulcerative lesions from these patients were grown in *in vitro* culture for up to 300 days. These cell cultures, along with leukocytes and body fluids, were examined by a variety of techniques for the presence of virus or viral antigens. Except for the presence of a transient multinucleation found in two of the RAU cell cultures, no evidence of virus was detected. Using cell cultures and leukocytes as the antigen source, there was no evidence of auto-antibody or auto-cell mediated immune reactions.

Although we have not implicated a persistent or latent virus with the diseases under investigation, the possibility of an infectious etiology still remains reasonable. First, the infectious virus may initiate events which lead to ulceration but may no longer be detectable when clinical lesions appear. Second, the virus may become integrated into the host cell genome at an earlier date. External stimuli might trigger the cell to produce viral antigens and the host immune responses might act against these antigens. The lesion would then appear to be induced by an autoimmune state. Finally, the virus may not replicate *in vitro* or in the limited animal host studied.

During the past year, we have expanded our studies to try to determine if the proposed etiologic agents replicate in cell cultures or in the limited animal host studied. We have obtained leukocytes from two patients with Behcet's syndrome and incubated these cells by multiple routes into two Rhesus monkeys. In addition, leukocytes from four patients with Sjogren's Syndrome have been incubated by multiple routes into Rhesus monkeys.

The animals are being observed daily for clinical signs of disease. Also, at four month intervals, the following assays are being performed: ophthalmologic examination with Schirmer's test, anti-salivary duct antibody, Coomb's test, anti-nuclear antibody, cold agglutinins assay, rheumatoid factor test, and routine clinical chemical assays and diagnostic immunology assays.

During this first year following incubation, these monkeys have remained healthy. Since the causative agents may induce a slow infection, the monkeys will be kept under observation for up to three years.

Salivary lysozyme in aphthous patients. Lysozymes present in saliva are important factors in the resistance of the oral mucosa to infection. A recent study has shown that the lysozyme content of saliva in patients with oral ulcerations was directly correlated with the severity of the disease. During the past year, a study has been initiated to determine if there is a significant change in the amount of lysozyme present in parotid saliva in patients with acute aphthous oral ulcers and during remission. The results in five patients show that the lysozyme content of saliva decreases an average of 29% when acute oral ulcers are present in the oral mucosa and returns to normal during remission. The study is being expanded to include more frequent salivary samples during the development of ulcers to determine at what stage the salivary lysozyme level falls and its possible relationship to the etiology of these ulcers.

Morphologic Studies on Oral Lesions. Ultrastructural studies utilizing electron microscopy techniques are being done on oral mucosal lesions in man and animals. Ultrastructural identification of the morphologic changes in oral mucosal cells in human and experimental animal disease states is desirable and necessary for the proper diagnostic evaluation of human oral mucosal diseases.

Nutritional alterations may act as pathogenic factors in the development of oral mucosal disorders. Ultrastructural studies done on the oral mucosal and skin epithelium of zinc deficient guinea pigs revealed an increase in the orthokeratinized cells of the epithelium of the buccal mucosa, dorsum of the tongue and the skin. Both control and zinc deficient guinea pigs possessed large numbers of gap junctions. In addition, annular gap junctions develop in great numbers, particularly

in the prickle cell layers of oral epithelium. They have been traced through the maturing epithelial layers as budding, looping, and ultimately as intracellular annular gap junctions. Their possible relationship to zinc deficiency is of considerable interest since zinc appears to be involved in nearly all aspects of cellular metabolism and has been implicated in the pathogenesis of human, as well as experimental, animal keratoses.

Treatment of Aphthous Oral Ulcers. During the past year the double-blind trial of a tetracycline suspension as a treatment for recurrent aphthous ulcerations (RAU) was completed and the results reported at the NIDR Aphthous-Behcet's Syndrome Workshop which was held September 14-15, 1977, at the National Institutes of Health in Bethesda, MD. In brief, the average duration of the oral ulcers, the maximum ulcer size, and the maximum ulcer pain were all significantly reduced in the aphthous patients who received tetracycline during the treatment period. The ulcer incidence, however, was not reduced. In addition, it was noted that the most frequent sites for oral ulcers were the lower labial mucosa, tongue, buccal mucosa, upper labial mucosa, and soft palate in order of decreasing frequency. The results of this study may provide a basis for further investigations into the etiology and treatment of this disease.

Relationship of Injury and Tissue Repair to Aphthous Ulcers. Localized mechanical trauma has been implicated in the etiology of oral aphthous ulcers in several reports in the literature. Investigators in a study comparing the effects of mechanical trauma on the mucosa of aphthous patients with individuals without oral mucosal disease produced oral ulcers in aphthous patients but not the controls. It has been postulated that recurrent oral aphthous ulcerations are an end result common to many etiological factors, provided they are capable of initiating sufficient tissue reaction to produce ulceration and that they are applied periodically to the oral mucous membrane. It also seems probable that aphthous ulcers may occur as the result of one or more precipitating factors superimposed upon a mucosa of limited recuperative or defensive potential - the latter aspect attributed to as yet unknown local or systemic factors.

A protocol entitled "The Relationship of Mechanical Injury to Oral Aphthous Ulcers" was initiated during the past year. The purpose of this study is to ascertain if mechanical injury to the oral mucosa is capable of inducing aphthous ulcers in patients known to have recurrent aphthous ulcers. The buccal mucosal injury is produced by inserting a surgical suture in the mucosa or placing a small portion of the mucosa in a surgical clamp for one second. These procedures are done after local anesthesia has been obtained in the operative area. The results will be compared with the findings in a matched control group of individuals free of oral ulcerative disease. The study is ongoing at the present time with a total of 10 patients; 7 aphthous and 3 controls

having been completed. A total of 13 ulcers occurred as the result of mucosal injury in the aphthous patients; no ulcers were produced in the control group. After the 10 aphthous and control patients have completed the study, the results will be evaluated. If significant differences are found between the two groups, the role of mucosal injury in the etiology of aphthous oral ulcers will be investigated further.

Future Plans

Etiology of Aphthous Oral Ulcers. Although a persistent or latent virus has not been isolated from patients with aphthous oral ulcers, Behcet's syndrome, or Sjogren's syndrome, an infectious etiology is still a possibility. Long-term studies on the inoculation of patient materials into subhuman primates are in progress and will be expanded during the next year. These animals will continue to be evaluated for the appearance of disease.

The etiology of recurrent aphthous ulcerations is unknown. The recent discovery of antigen-antibody complexes in the serum of patients with recurrent aphthous ulcerations (RAU) and the previous demonstration of a mononuclear cell tissue infiltrate early in the development of these ulcers suggest that a cell-mediated immune response may be a factor in the etiology of aphthous ulcers. A relationship between ulcer duration, pain, and mast cell content in RAU has been reported in the literature. The mast cell count, initially high, dropped sharply within the first two days of ulcer life, prior to the onset of severe pain, in the majority of the patients.

In order to determine whether mast cells in the oral mucosa play a part in the formation of aphthous ulcers two experiments have been designed: 1) the number of mast cells in tissue sections prepared from ulcer biopsy tissue will be recorded at the sites of epithelial mononuclear cell infiltration; and 2) the amount of histamine released from mast cells in similar tissue specimens will be determined. The tissue sections used in these experiments will be obtained from excisional biopsies of aphthous oral ulcers performed during the first two days after onset of the ulcer. If oral mucosal mast cells release the mediators of the immediate type hypersensitivity reactions, it would be an important finding relative to the pathogenesis.

Future plans also include a collaborative study with the NIDR Clinical Immunology Branch to investigate whether patients with oral mucosal ulcerative diseases are allergic to certain foods. A serum food allergy test utilizing the percent of histamine release from the circulating basophils will be used. Sixteen food allergens will be tested in patients with recurrent aphthous ulcers, other oral ulcerative diseases, and controls.

Treatment of Aphthous Ulcers. Excellent control of recurrent aphthous ulcerations (RAU) with sodium cromoglycate in the form of Intal, 2.5%, in a toothpaste, was reported recently. Approximately 60% of the patients had either full remission of oral aphthae or substantial improvement. The disodium salt of cromoglycic acid acts on the immediate type immune reactions to inhibit release of histamine and other pharmacological mediators. A random double-blind crossover trial with sodium cromoglycate in a toothpaste and a mouthwash will be done if the oral mucosal mast cells can be implicated in the pathogenesis of aphthous oral ulcers.

Morphologic Studies on Oral Lesions. Further ultrastructural examination of control and zinc deficient guinea pig epithelia will be carried out, particularly using perfusion-fixation techniques and appropriate tracer procedures. In addition, epithelia in other nutritional deficiency states (e.g., scorbutic guinea pig) will be studied, particularly with regard to alteration in cell contacts and annular gap junctions. Human oral epithelium will be investigated in various disease states in order to identify significant intracellular ultrastructural alterations such as those not visible by routine light microscopy.

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U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
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Herpes Simplex Virus: Latency

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The object of this project is to study the pathogenesis and treatment of herpes simplex ganglionic infection. Biological and molecular studies are used to determine the factors necessary for the development and maintenance of latency, the state and degree of expression of the genome during latency, and the mechanism of viral reactivation.

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(Rev. 10-76)

HERPES SIMPLEX VIRUS: LATENCY

Background

Herpes simplex virus (HSV) produces a wide spectrum of clinical manifestations in man ranging from the life threatening infection of herpes encephalitis to the common affliction of herpes labialis. Clinical episodes can be divided into primary or recurrent infections. After a primary infection, it has been shown that HSV can remain latent for months or years in sensory and autonomic ganglia. Following diverse stimuli, it is generally thought that new virus is synthesized in the ganglia and transported by axoplasmic flow to epithelial surfaces (e.g., lip, eye, genitalia) where subsequent replication produces the recurrent lesion.

Much of the information on the pathogenesis of HSV has been obtained from animal models. Inoculation of mice with HSV by various epithelial routes results in the centripetal neural spread of virus and ultimately an infection of local ganglia. During the first two weeks of the infection (acute phase), infectious virus can be recovered from ganglia by homogenization. After two weeks (latent phase), infectious virus can no longer be recovered from cell-free homogenates but can be recovered by explantation and cocultivation of the ganglia.

Past annual reports of the Laboratory of Oral Medicine have summarized the effect of immunization and anti-viral drugs on this system. It has become increasingly apparent, however, that in order to hope to eliminate recurrent HSV diseases, a different approach to anti-HSV therapy probably would be needed. The development of any new approach clearly would be greatly aided by a more basic understanding of latency and viral reactivation. To this end, the experimental studies in the last year have focused on three general aspects in this system: (1) the factors necessary for the elimination of the acute phase of the ganglionic infection and the development of latency, (2) the state and activity of the viral genome during latency, and (3) the mechanism of viral reactivation. The present report will review the results of studies under these three headings.

Major Findings

Acute ganglionic infection and the development of latency: In the past year, experiments were designed to study the mechanism by which HSV reaches the ganglia after epithelial inoculation. It was shown that when the sciatic nerve was sectioned just prior to footpad HSV inoculation of the mouse, the ganglionic infection was prevented. This result supports the view that virus reaches the ganglia by the neural route as

opposed to lymphatic, hematogenous, or direct tissue extension. The precise mechanism of neural spread, however, remained to be elucidated. The formal possibilities included spread by contiguous Schwann cell infection, via the peri-axonal space, or within axons. The third possibility was supported by an additional experiment which showed that ganglia did not become infected 72 hours after footpad HSV inoculation when the nerve was left intact but treated by a method that has been shown to stop axoplasmic flow locally (by application of a colchicine impregnated silastic cuff to the sciatic nerve). This study, therefore, strengthens the view that HSV reaches the ganglia by retrograde axoplasmic flow.

Once the virus reaches the ganglia (about 24 hours after skin inoculation), the acute or productive viral infection ensues. The annual report last year described an in vitro organ culture model of the acute ganglionic infection and the effect of anti-viral antibody on the infection. Although the in vitro organ culture system provided useful information, it had several deficiencies. First, ganglia removed from animals and placed in culture consist of nerve bodies in which most of the axon has been cut off. The nerve endings are absent and the neuronal membranes may be altered. Thus, in ganglionic cultures, the virus may enter the cut end of axons and spread to other nerve bodies in a different way than occurs in vivo. Second, other components of the host's immune system (e.g., complement, killer cells, and phagocytic cells) are not present in the organ cultures.

Because of these limitations, the continued study of the immune control of the acute ganglionic infection was carried out in an in vivo model. Immunocompetent and immunodeficient (nude or athymic) Balb/c mice were inoculated in both corneas and at certain times thereafter, ganglia were excised and assayed for infectious virus. In the immunocompetent mice, the mean ganglionic viral titer peaked at 4 days post inoculation and was negative at 12 days, the beginning of the latent phase of the infection. In contrast, the titer in the immunodeficient mice increased after day four post inoculation and all of the animals were dead by day 12. In addition to the deficiency in T-cell immunity, the nude mice do not make a neutralizing antibody response to HSV, presumably because of lack of helper T-cell function. These results, therefore, show that antibody plays an important role in the control of the acute infection. In fact, the ganglionic viral titer in the immunocompetent animals during the acute infection varied inversely with the serum neutralizing antibody titer.

However, additional experiments showed that antibody by itself was not sufficient to eliminate the acute ganglionic infection. This result was obtained from passive immunization studies of HSV eye inoculated nude mice. Despite high serum antibody titers, these mice, in contrast to passively immunized immunocompetent mice, still had a low grade

productive ganglionic infection as revealed by the assay of infectious virus from cell-free ganglion homogenates. These results, therefore, indirectly suggest that a T-cell mediated response may be necessary for the elimination of the acute phase. Preliminary immune spleen cell transfer studies in HSV inoculated nude mice support this view by showing that a latent infection was obtained in nude mice given cells and antibody, whereas the acute infection was not eliminated in the nude mice given antibody alone.

What role, if any, the immune response plays in establishing latency is not known. There are two formal possibilities. First, during the acute phase of the infection, neurons become productively infected. The productive infection is then converted into a latent nonproductive infection. According to this hypothesis, the host's immune response modulates the infection and brings about the conversion, possibly in a way similar to the modulation of measles virus infection by antibody. Second, the host's immune response may have nothing to do with establishing the latent infection. Instead, there may be two populations of infected cells in the ganglion: one is productively infected and the other is latently infected. In this situation, the host's immune response acts to eliminate the productively infected cells (i.e., the acute phase of the infection) and thereby allows recognition of the latently infected cells by explantation of the ganglia. At the present time, these two possibilities cannot be distinguished.

State of the viral genome during latency: During the latent stage of the infection, HSV cannot be demonstrated by infectivity assays of the cell-free ganglionic homogenate and viral antigens cannot be detected by immunofluorescent techniques. However, HSV can be reactivated in ganglia by explantation of the tissue in vitro. In order to study the state of the viral genome and the degree of viral transcription during latency, nucleic acid hybridization techniques were used. These studies, initiated in 1976, were continued and expanded during the present year.

DNA and RNA extracted from trigeminal ganglia of HSV-infected mice at the acute and latent stages were hybridized in vast excess to a trace amount of ^{125}I-labeled HSV DNA, and the hybridization kinetics was followed by fractionation of the hybrids in hydroxyapatite columns. These experiments were repeated three times, using animals from separate inoculation protocols as well as different preparations of iodinated DNA. The results of these experiments agree rather well and indicate that at the acute stage there are 1.2-2.0 viral genome equivalents per cell and 0.1-0.2 viral transcripts per cell on the average. In contrast, at the latent stage only 0.1±0.03 viral genome equivalents per cell are found, and the transcriptional level is less than one viral mRNA molecule per 2,000 cells or an equivalent of less than 400 mRNA molecules per trigeminal ganglion.

These results suggest that a transcriptional block occurs concomitantly with the transition of acute to latent stage, and that a low, undetectable level of viral multiplication is unlikely to be responsible for the persistence of HSV since, as indicated by our results, the viral genome appears to be silent at the latent stage.

As discussed in last years annual report, viral specific thymidine kinase (TK) was detected in pooled dorsal root ganglia taken from latently infected mice up to, but not beyond 60 days post epithelial HSV inoculation. At the present, we are unable to distinguish among three possible explanations for this result: persistence of viral TK synthesized at the acute stage, transcription of a minute fraction of the genome that happens to code for TK, or low level of complete transcription with <400 viral mRNA molecules per ganglion.

In an attempt to increase the sensitivity and to allow assay of TK on single ganglia, a different technique was introduced in the present year. In brief, this assay was based on the fact that the thymidine analogue, 5'-Iododeoxycytidine (Idc) is a good substrate for the viral specific thymidine (deoxypyrimidine) kinase, but a poor substrate for the cellular TK. Incorporation of Idc in uninfected cells occurs via deamination to give iododeoxyuridine (IdU) which is then phosphorylated to IDUMP by the cellular TK enzyme. In the presence of tetrahydrouridine, an inhibitor of the deamination reaction, incorporation of IdC is blocked in uninfected cells; whereas in HSV infected cells incorporation can occur via the viral TK. Anti-HSV IgG but not non-immune IgG blocks this incorporation in infected cells.

In a preliminary study using this method, viral TK was detected in extracts prepared from 6 out of 10 human trigeminal ganglia taken at autopsy. Thus, this assay provides a sensitive, rapid method which can be applied in screening studies of mouse and human tissue for the expression of HSV.

The viral DNA sequences coding for TK have recently been shown by others to be integrated in the chromosomal DNA of the biochemically transformed cell line carrying it. This observation prompted us to investigate the possibility that the viral genome might integrate into the ganglion cell DNA at some time during or after the acute infection. To answer this question, we designed a series of experiments in which ganglion DNA from infected animals was run in potassium iodide density equilibrium gradients. Each fraction of the gradient was hybridized to ^{125}I-HSV DNA and the extent of hybrid formation was determined in hydroxyapatite columns. Since the buoyant density of HSV DNA (1.725 g/cc) and mouse DNA (1.695 g/cc) differ, the presence of viral DNA at the buoyant density of cellular DNA should be an indication that the viral DNA is bound to cell DNA sequences that carry it along to the density position of the host cell DNA. It was found that at the acute stage,

less than 10% of the viral DNA was present at the cell DNA density, and this level could all be accounted for by overlapping between the two bands. In contrast, as much as 30% of the viral DNA present in latently infected ganglia banded at the position of cell DNA, forming a distinct peak that could not be ascribed to overlapping.

These results suggested that some viral DNA molecules may be integrated into ganglia DNA, but there are inherent problems with this experimental approach, such as aggregation, non-covalent interactions between molecules, and others, that need to be ruled out experimentally before a conclusion can be drawn from our results.

Ruling out these alternative explanations poses a major technical problem, since in all cases, recycling of the appropriate DNA fractions and purification through other types of gradients (alkaline potassium iodide, velocity sedimentation in sucrose) is required. However, due to the minute amount of viral DNA present in latently infected ganglia, an enormous number of mice (approximately 20,000) would be needed to do all the proper experiments.

We have decided to follow an alternative approach to determine whether the viral genome is truly integrated. This approach is based on the fact that if the viral genome is integrated, its termini would be covalently joined to cell DNA sequences. Thus, the viral termini isolated from latently infected ganglia should display biochemical and physical properties that would differ depending on whether the genome was integrated or not. Studies to evaluate this question are being carried out at the present time.

Viral Reactivation: As noted in last years annual report, viral reactivation could be detected after 48 hours of in vitro cultivation of ganglia from latently infected mice. Anti-viral drugs prevented reactivation, but continued drug presence in the culture was needed to maintain this in vitro latency. Anti-viral antibody and interferon did not prevent reactivation.

In contrast to this in vitro system, it has been very difficult to set up the more physiological in vivo model of reactivation in the mouse (without performing an axotomy). In the present year, however, there was some success in establishing this animal model by treating mice with cyclophosphamide or x-irradiation.

These studies used latently eye infected mice, 6-8 weeks after HSV inoculation. In over 100 control animals (latently infected, untreated), no infectious virus could be detected in cell-free ganglia homogenates. However, with cyclophosphamide treatment, viral reactivation could be detected in up to 70% of the animals by assaying ganglia homogenates in the second week of treatment (200mg/kg i.p. on days 1 and 3 of treatment, 15mg/kg i.p. daily beginning on day 5 until the end of the experiment).

Similarly, total body x-irradiation produced reactivation in up to 40% of animals in the second week after treatment (550 rads in a single dose). Eye homogenates were also positive for infectious virus in 10-20% of cyclophosphamide treated animals, but not in the controls. This last observation is consistent, but cannot provide definitive proof for the hypothesis that during a recurrence, HSV reactivates in ganglia and reaches the epithelial surfaces by the axonal route.

Although viral reactivation was seen in 7 out of 7 experiments, the percent reactivation was quite variable among experiments. Identification of viral antigens by immunofluorescent techniques verified the reactivation in a low percentage of treated mice and suggested that there was little or no spread from the reactivated cells. In order to try to increase the sensitivity for viral detection, a method of in vitro amplification was used. This technique was based on the idea that if the process of reactivation had been initiated by cyclophosphamide treatment in vivo, then when excised ganglia were cultured in vitro, infectious virus should be detected by homogenization and infectivity assay sooner than if ganglia were taken from untreated animals. This amplification method, in fact, did show that after 24 hours of in vitro cultivation, infectious virus could be detected in 70-90% of ganglia from 7-9 day cyclophosphamide treated mice as compared to 0-30% of saline treated (control) mice.

The mechanism of reactivation produced by cyclophosphamide and x-irradiation cannot be stated with certainty. A direct effect of these cytotoxic agents on ganglionic DNA is a possibility. If, on the other hand, the reactivation was secondary to immunosuppression, then it is more likely that cell mediated immunity rather than the humoral immune system is responsible for the maintenance of latency since reactivation occurred at a time when the PHA response was diminished but the serum antibody titer was still at high levels. Clearly, further studies are necessary to establish the mechanism of reactivation in these experiments, but this system does provide a badly needed, reproducible animal model of reactivation.

Future Studies

The studies reviewed in this report provide some tentative insight into the major areas of interest: the development of the latent infection, state and activity of the viral genome during latency, and the mechanism of viral reactivation. Over the next year, the studies in these areas will be continued and expanded. Greater emphasis will be placed on the detection of viral products during different stages of the infection and on the biology of the ganglion cell to determine ultimately what cell functions may be needed for the establishment and maintenance of latency. Finally, the molecular techniques developed in these animal studies will be further employed to study the natural infection in ganglia of man.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
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October 1, 1977 to September 0, 1978

TITLE OF PROJECT (80 characters or less)

Ulcerative Lesions and Tumors: Papillomas of Oral Cavity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Human cutaneous and oral cavity papillomas were examined in situ by electron microscopy and fluorescent antibody studies and in vitro by endonuclease restriction enzyme studies and molecular hybridization techniques for evidence of papilloma viruses. Human papilloma virus could be identified in approximately 55% of cutaneous papillomas but in only 5% of oral cavity papillomas. Molecular hybridization studies suggest that there is little if any DNA homology between the most common papilloma virus that causes cutaneous papillomas and those variants that probably cause the oral papillomas.

ULCERATIVE LESIONS AND TUMORS: PAPILLOMAS OF THE ORAL CAVITY

Introduction

Focal epithelial hyperplasias are benign lesions of the oral cavity seen primarily in native North American children. Papillomas are the most common benign epithelial neoplasms of the oro-pharyngeal and pharyngo-laryngeal cavities. Although the etiologies of these hyperplasias and papillomas are unknown, viruses of the papilloma virus subgroup of Papova viruses have been implicated. The oral cavity hyperplasias and papillomas have the same morphologic appearance as the human wart, the only human neoplasm known to be caused by a virus, the papilloma virus. Papilloma virus particles have readily been seen in human warts and focal epithelial hyperplasias but less frequently in oral and laryngeal papillomas. Clinical studies indicate that children with papillomas, particularly laryngeal papillomas, frequently have mothers with venereal warts. Finally, filtrates from oral and laryngeal papillomas, venereal warts and skin warts have reportedly resulted in skin warts after inoculation into the skin of volunteers.

All papilloma viruses have the same size (55nm in diameter) and substructure when examined by the electron microscope. However, papilloma viruses differ biologically by being highly species specific. Moreover, there are at least five variants of the papilloma virus(es) that cause the common wart in humans. These variants can be distinguished both immunologically and by different endonuclease cleavage sites. These variants can also be distinguished by molecular hybridization techniques. This technique is powerful enough to determine if papilloma virus is present in a cell in any one of several forms: 1) encapsidated in the form of a virion; 2) integrated into the cellular DNA; and 3) being actively expressed (transcribed) in the cells as a biochemical event. Identification of a papilloma virus, other than nonspecifically by the use of the electron microscope, is dependent on having previously isolated enough of that particular virus to either immunize an animal with it and obtain specific antibodies against the virus or label the viral DNA with a radioactive substance for use in the molecular hybridization technique.

Objectives

The objective of this research project is to determine if the hyperplasias and papillomas have a viral etiology and, if they do, to identify the etiologic agent(s).

Identification of papilloma viruses is being accomplished both *in situ* and *in vitro*. *In situ* identification is being done by (1) thin section electron microscopy, and (2) the fluorescent antibody technique, using antibodies produced against known papilloma viruses. *In vitro* identification is being done by (1) endonuclease restriction enzyme studies, and (2) molecular hybridization techniques.

Major Findings

Electron Microscopic and Immunologic Identification of Papilloma viruses. Approximately 100 cutaneous papillomas (warts), 55 laryngeal papillomas, 3 oral papillomas, 1 lip papilloma (wart) and 7 focal epithelial hyperplasias have been collected and examined by light and/or electron microscopy (EM). Approximately 55% of all cutaneous papillomas (foot and hand), but only one of 20 papillomas from the oral cavity and lip papilloma, were EM-positive for papilloma viruses. Antibodies prepared against purified virus extracted from individual foot warts (the only papillomas that yielded enough virus to use for immunization or molecular hybridization techniques) were FA-positive in 100% of EM-positive foot warts but only 25% of EM-positive hand warts. All oral cavity hyperplasias and/or neoplasias, including the EM-positive lip papilloma, were FA- negative. It appears that at least 50% of the EM-positive cutaneous warts in our study are caused by one variant of the human papilloma virus (the human foot wart virus). The other cutaneous papillomas and the oral cavity papillomas are apparently caused by other less common variants of the human papilloma virus.

In several of the plantar wart specimens, we found electron microscopic evidence of two different types of viruses. Crystalline arrays of a small virus, 25-30nm in diameter, were found in the same nuclei that contained the larger papilloma virus, 50-55nm in diameter. The role of this small parvo-like virus in the induction of cutaneous and, possibly, oral cavity neoplasia remains to be determined.

Molecular Hybridization

The studies initiated last year were extended to a total of thirteen individual laryngeal papillomas. Human papilloma virus DNA, labeled in vitro by nick translation was hybridized to an excess of laryngeal papilloma DNA and hybrid formation was determined by hydroxyapatite chromatography. As described in last year's progress report, one preparation (5 pooled papillomata) was positive for HPV sequences at a level of 10 copies/cell. Of the remaining twelve, two were borderline positive, at a level of 0.1-0.05 copies/cell and ten were negative. These results were reproducible and were confirmed by using different HPV DNA preparations obtained from different warts.

HPV DNA preparations derived from eight individual warts were compared by restriction enzyme analysis, and two of the eight were compared as well by crisscross hybridization. All eight isolates were identical, indicating to us that we had only one HPV variant of the four described in the literature. Our failure to demonstrate viral DNA sequences in a majority of the papillomata tested could be the result of not having the right representative of the HPV.

Since the three other HPV variants are infrequently found in common warts, we are following an alternative approach to the study of the viral etiology of oral neoplasia. We are attempting to purify DNA with properties of papilloma virus DNA (supercoiled double-stranded DNA of molecular weight of $5x10^6$ daltons) from oral papillomas and focal epithelial hyperplasia. The method of choice has recently been published by Dr. Felsenseld and colleagues, who have demonstrated that extraction of DNA with phenol at pH 4.0 in low ionic strength buffers leaves only supercoiled molecules in the aqueous phase. The purity of the preparations is comparable to that obtained by other lengthier, cumbersome techniques.

We have successfully applied this method in a trial with a single wart containing very few viral particles, as previously determined by electron microscopy. No cellular DNA sequences could be detected in the preparation of supercoiled DNA from this wart, which yielded 5µg of viral DNA out of a total of 75µg of nucleic acids (cellular and viral). Surprisingly, we found three different species of DNA, with molecular weights of $5x10^6$, $4x10^6$, and $2x10^6$ daltons. We have reexamined preparations of supercoiled viral DNA purified by classical methods (Ethidium bromide-CsCl gradients) and have found the same three species of DNA in them. It may be possible that there is more than one virus associated with the common wart.

Significance for Biomedical Research

A papova virus, the human wart virus, is the only virus that unequivocally causes a human tumor and is the leading candidate virus to cause other tumors such as the oral and laryngeal papillomas. Identification of the etiologic agent(s) of the hyperplasias and papillomas is necessary before any progress in rational treatment of these tumors is possible. The techniques and biological information generated by this study should not only provide valuable insight into the etiology of the oral and laryngeal hyperplasias and papillomas but could prove useful in attempting to establish a possible relationship between the papilloma viruses and more aggressive lesions of the oral cavity, such as inverted papillomas and squamous cell carcinomas of the larynx. In addition, our techniques could be useful for investigating papillary tumors in other parts of the body such as brain and urinary tract which are thought to harbor papova viruses.

Future Plans

In situ identification of papilloma viruses. We plan to: (1) continue to collect human cutaneous and oral cavity papillomas for EM and FA studies; (2) determine if there is a viral antigen common to all papilloma viruses such as exists in the polyoma SV-40 virus subgroup of papova viruses; and (3) study the development of canine oral papillomas after infection by the canine oral papilloma virus as an animal model system for human oral papillomas.

In vitro identification of papilloma viruses. We plan to: (1) utilize a new purification method to extract supercoiled DNA from oral cavity papillomas; and (2) test the specificity of the supercoiled DNA for the particular papilloma by molecular hybridization to determine its relationship to the papilloma virus DNA found in the cutaneous papillomas.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 DE 00219-02 LOM

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Herpes Simplex Virus: Cell-Mediated Immune Mechanisms and Interferon

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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3.45	2.10	1.35

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this research project is to determine the role of viruses and interferon in immunologic and immunopathologic processes.

The release of histamine from leukocytes (basophils) of allergic individual following stimulation with ragweed antigen (Antigen E) or anti-IgE is a reliable in vitro model for immediate hypersensitivity reactions (allergic reactions). Using this in vitro model, we have shown that viruses and interferon can enhance the release of histamine from leukocytes stimulated with antigen E. Further experiments demonstrated that an induction period and RNA synthesis are required for interferon to act.

More recently, our studies have shown that the interaction of leukocytes with a specific antigen results in the release of soluble factors which can enhance the IgE-mediated release of histamine. Whether or not interferon is acting in conjunction with another lymphokine is under investigation.

HERPES SIMPLEX VIRUS: CELL MEDIATED IMMUNE MECHANISMS AND INTERFERON

Background and Objectives:

Interferon has long been known as a soluble substance induced by viruses and nucleic acids which can interfere with virus replication. During the past few years, studies from several laboratories, including our own, have indicated that interferon induction and the effects of interferon are not limited to viruses. First, interferon can be induced by antigens or antigen-antibody complexes in specifically sensitized lymphocytes (T cells). Therefore, interferon is one of a variety of lymphokines. Second, interferon can have varied biologic effects including the modulation of the host's immune responses.

The objective of this research project is to determine the role of viruses and interferon in immunologic and immunopathologic processes. In our last annual report, we described our initial experimental data on the role of viruses and interferon in immediate hypersensitivity (allergic) reactions.

Immediate hypersensitivity responses can symptomatically result in asthma, hay fever, urticaria, and possibly atopic dermatitis. The target cells for anaphylaxis are tissue-fixed mast cells or blood basophils. The IgE antibody molecules fixed to the target cells form a complex with specific antigen. This antigen-antibody reaction on the surface of basophils initiates an enzyme cascade resulting in the release of pharmacologic mediators. These mediators are histamine, SRS-A, eosinophil chemotactic factor of anaphylaxis (ECF-A) and bradykinin.

Much of the information on the mechanism of IgE-mediated release of pharmacological mediators has been obtained from an in vitro model. The release of histamine from leukocytes (basophils) of allergic individuals following stimulation with ragweed antigen (antigen E) or anti-IgE is a reliable in vitro model for immediate hypersensitivity reactions (allergic reactions). Using this model, we found that incubation of human leukocytes with certain viruses before challenge with ragweed antigen or anti-IgE enhanced the release of histamine. Further studies showed that interferon induced by these viruses was responsible for this effect. These data suggest that interferon may be one cofactor in the potentiation of asthmatic attacks during viral infections.

Experimental studies in the last year have focused on two general aspects of this system: 1) To investigate the kinetics of interferon induced enhancement and to determine whether RNA synthesis is required; and 2) to determine if there is an enhancement of IgE-mediated histamine release following immune stimulation.

Major Findings

Interferon-induced enhancement of IgE-mediated histamine release. During the past year, we have expanded our initial studies to investigate the kinetics of the interferon-induced enhancement of IgE-mediated histamine release and to determine whether RNA synthesis is required for this enhancement. Human peripheral blood leukocytes (PBL) were incubated with interferon or medium for 3 to 24 hrs and then challenged with different concentrations of anti-IgE. Cells that had been treated with interferon for 3 to 6 hrs prior to challenge with anti-IgE failed to show any enhancement of histamine release. However, incubation with interferon for 9 hrs resulted in a significant enhancement of histamine release ($p<0.001$). Maximum enhancement was observed when cells were exposed to interferon for 12 to 24 hrs. These studies showed that an induction period was necessary and suggested that an intermediate product might be involved. To determine if RNA synthesis was needed for the enhancement of IgE-mediated histamine release by interferon, an inhibitor of RNA synthesis, actinomycin-D, was used. Actinomycin-D completely inhibited the interferon-induced enhancement of histamine release.

It is known that an induction period and RNA synthesis are required for interferon to exert its antiviral action. These data suggest that similar steps are involved in the interferon-induced enhancement of histamine release.

Enhancement of IgE-mediated histamine release by an immune-specific lymphokine. It is well established that if leukocytes from an immune host are stimulated with a specific antigen a variety of biological mediators or lymphokines are released. One of these mediators is interferon. Our second approach was to see if the IgE-mediated release of histamine would be affected by specific immune stimulation.

The initial experiments were designed to determine if there was an enhancement of the IgE-mediated release of histamine in PPD-treated leukocytes from BCG sensitive individuals in comparison to PPD-treated leukocytes from BCG non-sensitive individuals. Peripheral blood leukocytes obtained from 12 healthy donors were exposed to PPD for 2 hrs at 37°C, washed three times with RPMI media, and further incubated for an additional 22 hrs. After 24 hrs of incubation, the cells were challenged with either ragweed antigen E or anti-IgE for 45 min and the amount of histamine released into the supernatant fluids was determined.

Preincubation of leukocytes from 5 BCG-negative blood donors with PPD did not alter the amount of histamine released when the cells were challenged with either antigen E or anti-IgE ($p>0.01$). In contrast, when leukocytes from 7 BCG-positive blood donors were preincubated with PPD and then challenged with stimulant, there was a significant

enhancement in the amount of histamine released ($p<0.001$). Our preliminary studies revealed that the incubation of leukocytes obtained from either BCG-positive or BCG-negative individuals with PPD did not cause the release of histamine. These studies suggest that the enhancement of histamine release by PPD after challenge with antigen E or anti-IgE may be an immunologically specific phenomenon.

In order to determine if a soluble factor was involved, the supernatant fluids were collected from the PPD-stimulated leukocytes from a BCG-positive donor and were then added to leukocytes from a BCG-negative donor. These cells were incubated and then challenged with anti-IgE and the amount of histamine released was determined. The supernatant fluids had the capacity to enhance histamine release. These studies suggest that the IgE-mediated release of histamine can be enhanced on an immune basis and this effect is mediated by a soluble factor.

We next wanted to see if interferon was present in the supernatant fluids and thus may be the soluble factor enhancing histamine release. Indeed, the supernatant fluid contained a factor which can inhibit VSV plaque production on human cells, but not heterologus cells. Since this antiviral activity is stable after ultracentrifugation, but labile to trypsin, heat and pH 2.0, it is classified as type 2 or immune interferon. Our studies show a good correlation between the development of antiviral activity and the ability of supernatant fluids from PPD-stimulated leukocytes to enhance the release of histamine.

We next wanted to see if we could dissociate the antiviral activity of the supernatant fluids from their histamine enhancing capacity. Consistent with the idea that interferon is operative in this system is the fact that treatment with trypsin completely destroys both the antiviral and the histamine enhancing activity of the samples. Treatment with heat does not completely destroy either activity. In contrast, pH 2.0 treatment of samples can dissociate the antiviral activity and their histamine enhancing capacity. Treatment of the samples at pH 2.0 slightly decreased, but did not eliminate, the histamine enhancing capacity. This treatment completely eliminated the antiviral activity of the interferon containing samples.

These studies demonstrate that the interaction of leukocytes with a specific antigen results in the release of soluble factors which can enhance the IgE-mediated release of histamine. Whether or not interferon is acting in conjunction with another lymphokine remains to be determined.

Significance

There are millions of humans suffering from asthma and other forms of immediate hypersensitivity. Clinical evidence has suggested that

upper respiratory tract infections can potentiate or augment asthmatic attacks. Our studies have shown that viruses and other antigens (bacterial) can induce interferon and possibly other lymphokines. This enhances the release of histamine and other pharmacologic mediators of anaphylaxis and thereby augments immediate hypersensitivity reactions.

Interferon is generally known as an antiviral substance which can play a role in recovery from viral infections. Recent evidence indicates that interferon can interact in other systems such as suppression of tumor cell growth and regulation of immune responses. Our studies demonstrating that interferon can enhance the release of histamine represents a new biological role for interferon. The in vitro model of IgE-mediated release of histamine has been instrumental in elucidating the role of receptors, cyclic nucleotides, microtubules and microfilaments in the release of pharmacologic mediators from cells. Using this model, we may be able to determine if interferon acts on a common metabolic pathway and how it modulates a variety of other biological processes. Proposed Cours

Proposed Course

1. Studies on the mechanism of action of interferon in the enhancement of histamine release are continuing. These studies will attempt to determine if interferon can alter cyclic nucleotides, disrupt microfilaments, or microtubules, or stabilize membrane receptors.

2. We are attempting to establish a workable in vivo model of viral or interferon induced enhancement of immediate hypersensitivity reactions. Once such a model is developed, we can determine under what conditions viruses or interferon can intensify the reactions.

3. Since interferon can be induced in man by both viruses and specific antigens and antigen-antibody complexes and since interferon can modulate the immune responses, we plan to study the role of interferon and other lymphokines in autoimmune diseases such as Sjogren's syndrome, lupus erythematosus, and rheumatoid arthritis.

4. Certain types of delayed cutaneous hypersensitivity reactions are characterized by a large local accumulation of basophils. These reactions are called "cutaneous basophil hypersensitivity" (CBH). Normal human basophils are attracted to C5a and to culture fluids from human lymphocytes stimulated by mitogens or antigens. Studies are underway to determine if interferon can alter the response of basophils in this reaction. The role of basophils in various diseases of the oral cavity (e.g., herpes simplex infections, aphthous ulcers, periodontal disease) will be investigated.

Publications

1. Asher, D.M., Hooks, J.J., Amyz, H., Luber, N.P., Asher, L.V., Gibbs, C.J. Jr., and Gajdusek, D.C.: Persistent Shedding of A New Adenovirus from Chimpanzee Urine. Infect. & Immun. 21:129-134, 1978.

2. DiGiacomo, R.F., Hooks, J.J., Gibbs, C.J. Jr., and Gajdusek, D.C.: Pelvic Endometriosis and Simian Foamy Virus Infection in A Pigtailed Macaque. J. Am. Vet. Med. Assn. 171:859-861, 1977.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00232-01 LOM

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Chemotactic Antibody

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibody of the immunoglobulin G class to herpes simplex virus and antibody of the immunoglobulin M class to sheep red blood cells were coupled to the synthetic peptide formylmethionylleucylphenylalanine (fMet-Leu-Phe), which is chemotactic for both mononuclear and polymorphonuclear leukocytes. The resulting molecules were chemotactic and retained their antigen-binding activity. When antibodies coupled to fMet-Leu-Phe were incubated with antigen, the resulting immune complexes were also chemotactic. Chemotactic antibody may provide a potent means of enhancing the migration of inflammatory cells to specific sites.

CHEMOTACTIC ANTIBODY

Background

The inflammatory response plays a significant role in the host defense against virus, bacteria, or parasites. The resulting accumulation of leukocytes at the foci of infection is carried out by chemoattractant substances formed in the organism. However, in certain infections or tumors, the inflammatory responses are poor. The possibility exists that chemoattractant substances generated at the site of infection are not sufficient to attract leukocytes to that site.

Recently, it has been found that synthetic formylated peptides are chemoattractants at concentrations of 10^{-6} to 10^{-11} M. The most active of these peptides is formyl-methionyl-leucyl-phenylalanine (fMLP). The possibility of enhancement in the inflammatory response in specific areas would be a way of improving the host defense. Using an antibody as a carrier, we coupled the chemotactic peptide fMLP to antibodies of both IgG and IgM classes.

Major Findings

Chromatographically purified IgG anti-herpes simplex virus (IgG a-HSV) was coupled to fMLP in the presence of minute amounts of tritiated fMLP using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene-sulfonate (CDI) as a coupling agent. The sample was dialyzed and chromatographed on a Sephacryl S-200 column (molecular sieve chromatography). The ^{3}H-fMLP appeared in the same peak as the native IgG. This demonstrates that fMLP was coupled to the IgG molecule because of the great differences between the exclusion peaks of the IgG and fMLP on the Sephacryl S-200 column.

With the same procedure, IgG a-HSV and IgM anti-sheep red blood cells (IgM a-SRBC) were coupled to the chemotactic peptide in the presence of CDI. The samples were dialyzed and chromatographed on a Sephacryl S-200 (IgG a-HSV) and Sepharose 4B-Cl (IgG a-SRBC), and the peaks of native immunoglobulins were assayed for chemotactic activity with peritoneal guinea pig macrophages in a modified Boyden's chamber.

Both the IgG a-HSV and IgM a-SRBC have high chemotactic activity at 50 and 100µg of protein. The controls, the native immunoglobulins, and the immunoglobulins treated with CDI were devoid of chemotactic activity.

It was important to evaluate the antibody activity after the coupling procedure with CDI. The antigen-binding site was assayed by the viral neutralization test for IgG a-HSV, and hemagglutination for

IgM a-SRBC. Both the IgG a-HSV and IgM a-SRBC antigen-binding sites remained intact after treatment. The ability of these immunoglobulins to activate complement was slightly decreased with IgM a-SRBC and greatly decreased with IgG a-HSV.

It is known that the chemotactic peptide binds to neutrophil receptors. $_{3}$IgG a-HSV and IgM a-SRBC coupled to fMLP inhibited the binding of ^{3}H-Nor-leu-Phe. This demonstrates that the coupled fMLP is still capable of binding to specific receptors. In vivo, antibodies will not exist unbound, but rather exist bound as immune complexes. Our studies showed that immune complexes formed by IgM a-SRBC coupled to fMLP and bound to SRBC had high chemotactic activity at 50 and 100µg of antibody protein.

Significance to Biomedical Research

With this approach, it may be possible to couple a variety of other biologically active mediators to antibodies. The use of antibodies as a carrier may be useful to increase the pharmacological activity of different mediators at specific areas and to reduce systemic toxicity provoked by mediators or drugs.

Course of Further Studies

The biological activity of chemotactic antibody is under investigation. This will provide the answer to whether or not antibodies coupled to fMLP are active in vivo. Further investigation has begun in an attempt to couple antibodies to epidermal growth factor (EGF), a potent mitogenic factor of epithelial cells.

Publications

Isturiz, M.A., Sandberg, A.L., Schiffmann, E., Wahl, S.M. and Notkins, A.L.: Chemotactic Antibody. Science 200:554, 1978.

ANNUAL REPORT OF THE
NEUROBIOLOGY AND ANESTHESIOLOGY BRANCH
NATIONAL INSTITUTE OF DENTAL RESEARCH

The Neurobiology and Anesthesiology Branch is concerned with the elucidation of basic mechanisms of oral-facial sensation with particular emphasis on pain sensation, and with the assessment and measurement of experimental and clinical pain in humans. Fundamental research on peripheral and central nervous system mechanisms of oral-facial sensation is conducted by the Neural Mechanisms Section. The primary objective of this research group is to correlate behavioral responses to noxious and non-noxious stimuli with neuronal function at various levels of the trigeminal system. A multidisciplinary approach is employed and includes the following research activities at present: (1) electrophysiological studies of the response of trigeminal brain stem and spinal cord neurons to noxious and innocuous thermal and mechanical stimuli; (2) behavioral and pharmacological studies involving pain and temperature discrimination in monkeys trained to escape potentially-noxious or noxious stimuli and to detect non-noxious stimuli applied to the face; (3) correlative behavioral and neural studies to determine the role of different peripheral and central neural populations in pain and temperature discrimination; and (4) psychophysical studies on the measurement and assessment of experimental and clinical pain.

Fundamental studies on neuronal structure and function are carried out by the recently established Section on Neurocytology and Experimental Anatomy. This program is concerned primarily with neurocytological studies of the postnatal maturation and adult organization of the brain stem trigeminal nucleus in cat and monkey, utilizing light and electron microscopy and various tracer techniques. Correlative studies on neuronal structure and function are carried out in conjunction with investigators in the Neural Mechanisms Section. The Anesthesiology Section conducts clinical research on the use of intravenous sedative and anesthetic techniques to control anxiety and apprehension associated with dental procedures. Present activities include physiological, pharmacological and psychological studies of the effects of intravenous sedative drugs during and after oral surgery procedures.

Although the programs of the Branch are divided into these three Sections, there is considerable interactions between research groups. Correlative studies on neuronal structure and function are being carried out to elucidate in detail the circuitry of the upper layers of the dorsal horn of the medulla and spinal cord and their relationship to pain mechanisms. Studies of chronic pain in humans involve the use of behavioral and psychophysical techniques that also have been employed in correlative animal behavioral and physiological studies. Our previous animal research also has provided the conceptual framework for the initiation of human studies on endogenous pain-suppressing mechanisms.

Anatomical Studies

These studies are aimed at delineating the synaptic circuitry of the caudal end of the trigeminal brain stem nuclear complex and its role in the processing of pain and temperature information. Thus the nucleus usually called nucleus caudalis, is laminated and consists of an outer marginal layer (layer I), a middle substantia gelatinosal layer (layers II and III), and an inner magnocellular layer (layer IV). Recently, we have shown that nucleus caudalis and the contiguous areas of the lateral reticular formation are organized similar to the gray matter of the spinal cord. A new lamination scheme has been proposed in which the gray matter of the caudal medulla has been divided into dorsal and ventral horns. Nucleus caudalis comprises the head of the dorsal horn and consists of four layers. The lateral reticular formation comprises the neck of the dorsal horn and is divided into layers V and VI. The medial reticular formation comprises the ventral horn and is divided into layers VII and VIII. The laminar boundaries of this scheme can be recognized in all major mammals currently used as experimental models and it should prove very useful in relating findings in the trigeminal system to spinal cord sensory mechanisms.

Other studies have assessed the effects of tooth pulpotomies on the neural circuitry of the dorsal horn of the medulla. The removal of tooth pulps results in the degeneration of the primary trigeminal neurons which innervate the affected teeth. In addition, this loss of input results in transsynaptic degenerative changes in neurons located in layers I to III of the medullary dorsal horn. It is possible that such disruption of synaptic circuitry is a factor in some types of oral-facial pain syndromes.

During the past year studies using the protein tracer horseradish peroxidase have identified several sources of input to the medullary dorsal horn which appear to play a role in modulation of sensory information. These include input from the cervical spinal cord, contralateral input from the opposite medullary dorsal horn, and descending input from the more rostral subdivisions of the trigeminal brain stem nuclear complex. In addition, neurons in the raphe nuclei, the locus coeruleus, and the periaqueductal gray matter also send projections to the medullary dorsal horn. These descending pathways appear to be critical to our understanding of mechanisms of analgesia produced by narcotic drugs and by peripheral and central nervous system electrical stimulation.

Both light and electron microscope autoradiographic techniques have been used to elucidate further the synaptic connections between a descending serotonergic inhibitory pathway from the raphe nuclei and cells in the medullary dorsal horn. Two major categories of morphologically distinguishable axonal endings were found to contain radioactive serotonin which was applied to nucleus caudalis and presumably was taken up by terminals which normally use serotonin as a neurotransmitter. One type,

called dome-shaped endings, forms single synapses on dendrites [illegible] other type, called scalloped endings, forms multiple synapses on dendritic shafts and spines. Three subclasses of labelled dome-shaped endings and four subclasses of labelled scalloped endings have been identified. Two subclasses of dome-shaped endings are found in layers I to III of the medullary dorsal horn. The different subclasses of scalloped endings are confined to single layers of the superficial portion of the dorsal horn. Since most of the dendrites in layer I are derived from layer I trigeminothalamic neurons, these experiments suggest a direct modulation of serotonergic input on to these projection neurons. In addition, serotonergic inputs to layers II and III synapse on dendrites of interneurons which modulate the transfer of input from primary afferents to the projection neurons in layer I. These findings are of particular significance since the activation of a descending serotonergic pathway has been implicated in mechanisms of pain suppression.

Other studies have examined postnatal neuronal maturation in the dorsal horn of the medulla in newborn kittens. During the past year Golgi studies have been directed at the two major interneurons of layers II and III. At birth these interneurons are present in forms ranging from very immature to nearly mature. The dendritic arbors of these two interneurons follow similar developmental sequences. They are laid down in a preliminary form prenatally and altered postnatally through the selective elongation of some dendrites and the retraction of others. In addition, the axons of these interneurons do not begin to develop until the dendritic arbors have taken on some of their adult characteristics. At birth, layers II and III already have reached their adult width and the neuropil is compact. Space for elongating neuronal and astrocytic processes is made available as a result of the disintegration of many dendrites and by an overall fourfold increase in the rostrocaudal length of the dorsal horn of the medulla during postnatal maturation.

Results of our electron microscopic analysis show that the postnatal elongation of layers II and III dendrites and axons and the retraction of other dendrites take place utilizing the same basic cellular mechanism, which we have termed, membrane addition. At many sites within dendrites and axons the agranular reticulum gives rise to numerous vesicles called addition vesicles. In elongating dendrites and axons addition vesicles migrate to the surface and fuse with the surface membrane. This sequence is responsible for the lengthening of the dendritic and axonal membranes. In retracting dendrites neurotubules fragment, and addition vesicles, instead of adding to the dendritic surface membrane, fuse with each other to form first small and subsequently large cavities within the dendrites. Eventually, the cavities become continuous with the intercellular space as their membranes fuse with the dendritic surface membrane. Ultimately, retracting dendrites, having been almost completely hollowed out by cavities, fragment and disintegrate.

Axonal endings of primary trigeminal neurons in the dorsal horn of the medulla are present at birth in layers II and III. They synapse on

the elongating dendrites of layers II and III interneurons but do not synapse on retracting dendrites. These synapses are considered to be an important determinant for continued dendritic elongation and maturation.

These studies indicate that although the dorsal horn of the medulla is one of the first nuclei in the brain to develop, local trigeminal pain pathways which mediate reflex movement of the head and neck in response to noxious stimuli may only be partially formed at birth.

Correlative Anatomical and Physiological Studies

Previous anatomical studies in our laboratory have provided a detailed analysis of the morphology of neurons in the upper layers of the dorsal horn that presumably participate in pain mechanisms. However, the function of the various types of projection neurons and interneurons identified in these anatomical studies is only poorly understood. In recent studies we have attempted to correlate structure and function using an intracellular labelling technique. Neurons in layers I, II and III have been functionally characterized and then intracellularly stained with horseradish peroxidase. The dendritic arbors and axonal extensions of these neurons were then examined at the light microscope level. Wide dynamic range neurons, responsive to innocuous stimuli but activated maximally by noxious stimuli, were found in layers I, II and III of the dorsal horn and had more extensive dendritic arbors than neurons in the same layers activated only by noxious stimuli (nociceptive-specific). One of the cell types found in layers II and III, called the stalked cell, had been described in detail in our previous studies. It occurs as either a wide dynamic range neuron or a nociceptive-specific neuron, and both send their axons into layer I. This evidence along with previous results showing similar properties of layer I projection neurons, support the hypothesis that the stalked cells are excitatory interneurons that synapse on layer I neurons. They are part of a synaptic pathway by which small fiber afferent input to layers II and III can ultimately reach the long spinothalamic projection pathways that carry nociceptive input to levels of conscious sensation.

This study should provide long needed knowledge on the role of the substantia gelatinosa (layers II and III of the dorsal horn) in pain mechanisms as well as adding to our understanding of the function of short interneuronal circuits in the brain.

Physiological and Behavioral Studies

In previous studies we determined the peripheral nerve fiber groups responsible for the ability of the monkey to detect innocuous thermal stimuli and to escape noxious thermal stimuli applied to the face. These correlated behavioral and neurophysiological studies formed the foundation for present and future studies of central nervous system coding of pain and temperature sensation.

Where do these different peripheral fiber populations project in the central nervous system and what are the properties of central neurons involved in the monkey's ability to detect non-noxious and noxious thermal stimuli? We began to answer these questions by examining the response properties of neurons in trigeminal nucleus caudalis in the brain stem in anesthetized monkeys. Many of these neurons are part of a pathway that projects directly to the thalamus and is exclusive of other components of the trigeminal brain stem nucleus. This analysis revealed two general classes of trigeminothalamic neurons that convey information related to pain. Studies are currently being conducted to extend these observations to the awake, behaving monkey. These studies allow detailed analyses of how the brain codes various properties of nociceptive input. In contrast to the relatively static and passive input-output relationships attending experiments on anesthetized preparations, studies of awake behaving monkeys provide the opportunity to examine variables such as attention, response appropriateness, expectation and stimulus salience, and the role they play in nociception.

In the behavioral task, monkeys are trained to discriminate the onset of innocuous thermal stimuli (less than 45°C) from noxious thermal stimuli (45°C or greater), and to detect the termination of innocuous thermal stimuli. Neuronal activity in the dorsal horn of the medulla has been correlated with a number of behavioral events such as panel press, temperature onset and termination, and panel release. One class of neuron studied provides information about the intensity of noxious heat stimuli applied to the monkey's face. A second class of neuron signals the presence of behaviorally significant stimuli. A third class of neuron appears to be associated with attentional or motor processes utilized during the execution of certain behaviors associated with the task. Each class of neuron studied in the awake monkey consisted of both wide dynamic range and nociceptive-specific types previously identified in the anesthetized animal. Thus, it appears that both nociceptive-specific and wide dynamic range neurons can provide information related to the discrimination of noxious thermal stimuli in awake behaving animals. However, the functional description based on experiments in anesthetized monkey is incomplete and does not take into account the wide response capabilities of neurons in the dorsal horn of the medulla. The activity of these neurons is influenced in a dynamic fashion by behavioral contingencies such as the relevance of the stimulus to the animal's goal-directed behavior. These functional properties demonstrate the importance of descending modulatory influences on medullary dorsal horn neurons and their role in the perception of relevant and salient stimuli in the awake animal. By increasing our knowledge of the circumstances which lead to the activation of "pain modulating" pathways in the brain, we may be able to utilize these endogenous "analgesic" mechanisms in the future to control chronic intractable pain problems in humans.

We have employed the same behavioral task in pharmacological studies in awake monkey to assess the relative effects of morphine on the sensory discriminative and affective dimensions of responses to noxious thermal stimuli. Our results indicate that morphine attenuates the monkey's ability to discriminate noxious and innocuous thermal stimuli independent of its effect on the aversive quality of the stimulus. This result parallels results in human experiments described below: narcotic analgesic drugs appear to alter the sensory-discriminative aspect of the pain experience apart from their effect on its affective component. The results in the animal studies also demonstrate that our behavioral model may be useful in the evaluation of new pain control agents.

Using the human model of first and second pain sensations, we now have correlated these sensations with activity in dorsal horn projection neurons in layers I, IV and IV and in interneurons located in the substantia gelatinosa (layers II and III). Heat pulse stimuli in the noxious range produce an initial pricking pain which is followed about 1 sec later by a burning pain sensation. These first and second pains previously had been correlated with activity in A delta heat nociceptive afferents and C polymodal nociceptive afferents. We now have found that wide dynamic range and nociceptive-specific neurons have responses to trains of noxious heat pulses that are analogous to human sensations evoked by identical stimuli. Several observations suggest that some substantia gelatinosa interneurons are part of an excitatory pathway to layer I projection neurons. Our previously discussed correlative anatomical and physiological studies indicate that the stalked cell likely is such an excitatory interneuron. Late neuronal responses of substantia gelatinosa neurons summate with repeated heat stimulation, similar to second pain sensations. Since these substantia gelatinosa neurons probably receive direct input from primary nociceptive afferents, they represent the critical stage at which mechanisms of central summation are first manifested.

These central summation mechanisms appear to play an important role in mechanisms of acute and chronic pain. The increase in the magnitude of second pain after repeated heat pulses occurs in the face of reduced activity in the unmyelinated afferents that initially evoke the response. This is a significant finding related to pathological pain conditions. Prolonged summation and pain that outlast the period of stimulation are characteristics of post-herpetic neuralgia, causalgia and other chronic pain syndromes. Second pain may serve as a useful model in the study of central neural mechanisms that contribute to the development and expression of such chronic pathological pain conditions.

Human Psychophysical and Clinical Studies

The clinical program of the Branch was established to take advantage of new knowledge gained in the laboratory and apply it to the clinical situation. This program has been expanded this year to include new

studies of chronic oral-facial pain conditions such as temporomandibular joint pain and trigeminal neuralgia.

A major area of study in this program involves the development of psychophysical measurement techniques for experimental and clinical pain, and the use of validated techniques to evaluate pharmacological and non-pharmacological pain control agents. Verbal descriptor scales in this project have been used to assess the influence of fentanyl, a short-acting narcotic analgesia, alone, or in combination with diazepam, a minor tranquilizer, on the sensory intensity, unpleasantness, and pain associated with electrical tooth pulp stimuli. The influence of nitrous oxide and oxygen on verbal reports of various dimensions of the pain experience also have been evaluated. The administration of fentanyl in a double-blind study resulted in a significant reduction in sensory intensity verbal descriptor responses to electrical tooth pulp stimuli. Unpleasantness verbal responses were not reduced after fentanyl. Since fentanyl and saline placebo produce distinctly different subjective effects, a second study used an active placebo, diazepam, to mask these different effects. The results were the same as in the first study, with sensory intensity responses reduced significantly after fentanyl and unpleasantness responses unchanged. An additional series of experiments determined the reliability and objectivity of verbal pain descriptors. Unlike sensory intensity or unpleasantness verbal descriptor responses, pain descriptor responses did not distinguish between a narcotic analgesic and an active placebo drug. These findings indicate that classical assumptions about narcotic action are incorrect, and that narcotic analgesic drugs can reduce the perceived sensory intensity of noxious stimuli without reducing unpleasantness associated with such stimuli. The different results with sensory intensity and unpleasantness descriptors indicate that the type of words used to measure pain significantly influence the outcome of a pain experiment. This was confirmed in the findings that general descriptors of "pain" were not able to separate the effects of an analgesic drug and a non-analgesic drug.

Other experiments continue to evaluate the quality of sensations elicited from the tooth pulp and the underlying neural mechanisms responsible for these sensations. All subjects report sensory detection thresholds that are significantly lower than pain thresholds at all frequencies of stimulation used. These findings suggest that non-nociceptive input may originate from the tooth pulp, previously presumed to be a pure pain source. This interpretation is supported by other findings: 1) subjects are able to scale the intensities of these non-pain sensations, and 2) the electromyographic silent period in masseter muscle produced by tooth pulp stimulation can be dissociated from the perceived pain sensations. It appears likely that reflex activity not associated with pain sensations may arise from tooth pulp and utilize distinct central neural pathways.

The electromyographic silent period in the masseter muscle produced by tooth pulp stimulation also has been used in the evaluation of

temporomandibular joint pain or myofascial pain-dysfunction (MPD) patients. Approximately one-half of the MPD patients exhibit a silent period at current levels below sensory detection threshold whereas this is the case for only 10% of normal subjects. These findings also support the conclusion that there are distinct central pathways related to reflex activity that are separate from sensory detection and pain sensation pathways activated by tooth pulp stimulation. Hyperactivity in this reflex pathway in MPD patients may be useful in diagnosis and treatment.

Studies on the effectiveness of intravenous sedation techniques in dental patients have concentrated on amnesia effects and recovery of sensory and motor functions. Recovery tests indicate that psychomotor performance recovers to pre-drug levels before higher order perceptual and integrative function. There is also a trend toward faster recovery when there is partial substitution of diazepam with fentanyl, a short-acting narcotic. Significantly more amnesia was noted for painful and auditory stimuli than for visual or tactile stimuli using either recall or identification questionnaires. Intravenous sedation is employed widely in dentistry to modify the dental pain--anxiety experience. This study provides baseline data on the extent and duration of sensory, psychomotor, and cognitive deficits after such procedures. Present studies also are assessing the patient's, the surgeon's and the anesthesiologist's evaluation of the efficacy of the procedure.

PROJECT NUMBER (DO NOT USE THIS SPACE) | HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT | Z01 DE 00132-04 NA

PERIOD COVERED
October 1, 1977 to September 30, 1978 | CT 0600102

TITLE OF PROJECT (80 characters or less)
Psychophysiological Evaluation of Intravenous Sedation - Ambulatory Oral Surgery

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gelfman, Stephen	Anesthesiologist	NIDR NA
	Driscoll, Edward J.	Chief, Anesthesiology Sec	NIDR NA
OTHER:	Wirdzek, Peggy R.	Clinical Nurse	NIDR NA
	Butler, Donald P.	Oral Surg-Anesthesiologist	NIDR NA
	Sweet, James B.	Sr Dental Surgeon	NIDR IR
	Clark, Barbara Ann	Clinical Nurse	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Anesthesiology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.90	2.00	1.90

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of the project is to test the effectiveness of two intravenous sedation techniques in an effort to approach an ideal psychophysiological environment for both the patient and the dentist. This ideal environment includes: (a) patient cooperation; (b) total amnesia; (c) minimal physiological challenge; (d) rapid complete recovery with minimal side effects; and (e) no loss of consciousness or protective reflexes. Technique "I" utilizes methohexital and diazepam. Technique "II" utilizes methohexital, diazepam and fentanyl. In addition, the effects of naloxone, a specific narcotic anatagonist are studied. The investigators wish to develop a combination of the aforementioned drugs which provides an ideal environment and the fastest most complete recovery to enable the patient to return to his daily activities as soon as possible as measured by various perceptual, psychomotor, integrative function and memory tests.

NIDR Classification: 60410--60%
60310--40%

1. Project Description

Objectives: The purpose of the project is to test the effectiveness of two intravenous sedation techniques in an effort to approach an ideal psychophysiological environment for both the patient and the dentist. This ideal environment includes: (a) patient cooperation; (b) total amnesia; (c) minimal physiological challenge; (d) rapid complete recovery with minimal side effects; and (e) no loss of consciousness or protective reflexes. In addition to recovery and amnesia studies, the surgeon and anesthesiologist will be rating the anesthetic experience to determine how closely these techniques approach an ideal psychophysiological environment for the patient. In addition, patient assessment of the anesthetic experience and recovery in the first 24 hours following surgery will afford data to determine how closely these techniques approach ideal conditions from the patients' point of view.

Methods Employed: Potential patients, 18 years or older of either sex, are screened by members of the NIDR Dental Clinic to verify the need for extraction of third molar teeth, the surgical procedure elected for use in this anesthesiology study. After validation of this surgical need, a complete history and physical examination (including appropriate laboratory studies) is performed by qualified NIDR staff.

There are three groups of patients selected randomly. Group I patients receive diazepam and methohexital. Group II patients receive diazepam, methohexital, and the narcotic, fentanyl. The patients in group II are further subdivided so that half receive naloxone at the end of the surgical procedure and the other half receives a normal saline placebo. Group III patients receive normal saline (placebo) injections only. All drugs are administered intravenously to achieve psychosedation only and local anesthesia is always employed. All patients have their surgery performed in two separate sessions. The patients are placed one time in Group I and the second time in Group II. The order of groupings is counterbalanced. This system allows for both between-patient and within-patient data analysis. The patients in group III have their second surgical procedure performed in a non-experimental session.

Emphasis in this study is being placed upon: 1) a comparison of recovery between the two sedation techniques; 2) study of the drug-induced amnesia which accompanies both of these techniques; 3) accumulation of physiological data to verify relative physiological safety; and 4) patient, surgeon, and anesthesiologist's evaluation of the overall anesthetic experience.

Recovery is measured by requiring the patients to complete a battery of psychomotor, perceptual, and integrative function tests designed for repeated measures testing. The patients generate a base-line or "non-drug condition" performance level on this battery of tests.

This baseline is compared to performance immediately postoperatively and 3 hours post-operatively.

Drug-induced amnesia is being studied by randomly presenting [illegible] types of stimuli to the patients at predetermined intervals after drug injection. The patients all must verbally identify these visual, auditory, tactile-cutaneous, and painful stimuli. Patients are [illegible] to retrieve this information post-operatively utilizing both recall and identification type memory questionnaires.

Basic physiological functions such as EKG, heart rate, arterial blood pressure, rate and depth of respiration, and oxygen saturation of blood are all being monitored non-invasively and recorded on a multi-channel polygraph.

Detailed data on the incidence of nausea, dizziness, etc. during the first 24 hours following surgery as well as the surgeon's and anesthesiologist's evaluation of the anesthetic experience will be compared to similar data on other anesthetic techniques.

Major Findings: No subjective differences in recovery were observed between Groups I and II. Placebo sedation effects including flaccid closure of eye lids, decreased rate and depth of respiration, and self reports of sedation were observed in Group III (normal saline controls). Significant deficits ($p<0.01$) were evidenced in psychomotor performance as measured by the Trieger Dot test in both drug groups I and II immediately and 1 hour post-operatively. Groups I and II demonstrated complete recovery to baseline levels on this test at the three-hour post-operative test session. The control patients (Group III) showed neither deficit nor practice effects on the Trieger Dot test. Central integrative ability as measured by critical flicker fusion frequency threshold was significantly depressed ($p<0.01$) both immediately and 3 hours post-operatively in Groups I and II. Group III showed no deficits or practice effects on this test in any of the post-operative test sessions. Perceptual ability as measured by Moran's Perceptual Speed test was significantly ($p<0.01$) depressed in Groups I and II. In addition, Group II (fentanyl-naloxone) performed significantly ($p=0.02$) better immediately post-operatively than group I, thus suggesting that reduction of diazepam and substitution of a reversible narcotic may hasten recovery. Group III showed no deficit or learning on this test.

These results indicate the following with respect to recovery as measured in this study from the two intravenous sedation drug combinations: 1) psychomotor performance recovers to pre-drug levels before higher-order perceptual and integrative function; 2) there is a trend toward faster recovery when there is partial substitution of diazepam with fentanyl-naloxone; 3) there is no measurable deficit in the normal saline (placebo) group on any of the tests employed, i.e., no "placebo effect" is noted on objective measures although by observation the patients displayed sedation-like effects.

The drug-induced amnesia produced by both drug combinations I and II did not differ significantly in any of the parameters tested. No amnesia was evidenced in the normal saline-placebo patients (Group III). No retrograde amnesia was produced in any of the patients on this study. Virtually complete amnesia (99%) for the local anesthetic injections was accomplished. Significantly more amnesia was noted for painful and auditory stimuli than for visual or cutaneous-tactile stimuli. Percent amnesia as measured by recall questionnaire was 89%, 89%, 81%, and 68% at 8, 13, 15½, and 18 minutes, respectively following drug administration. Corresponding percents of amnesia produced using identification questionnaires was 64%, 62%, 51%, and 46%, respectively.

Significance to Biomedical Research and Program of the Institute: Although it is widely accepted that patients undergoing ambulatory anesthesia are incapable of returning to normal daily activities ("street fitness"), the areas and duration of perceptual, psychomotor, and cognitive deficits are not well known. Intravenous sedation is widely employed in dentistry as an adjunct to local anesthesia to provide sedation, relaxation, amnesia, and to modify the dental-pain-anxiety experience. This study directed at modifying the psychological aspects of pain in the clinical dental situation will provide quantitative data on recovery after use of two commonly employed intravenous sedation techniques. Valuable data concerning amnesia and both surgeon and patient evaluation of the techniques are also being compiled. Evidence of "placebo sedation" without measureable central depression may have important implications in non-pharmacological manipulations of pre-operative anxiety in surgical patients.

Proposed Course: This project will form a model from which various pharmacological and non-pharmacological sedation techniques can be studied. In addition, future studies will attempt to verify the physiological safety of these techniques by emphasizing sophisticated non-invasive data collection. Future studies are planned which will attempt to speed recovery without decreasing amnesia levels by modifying the dosage schedule including more substantial reductions in diazepam and replacement with larger doses of fentanyl and naloxone.

2. Publications:

Gelfman, Stephen S., Gracely, Richard H., Wirdzek, Peggy R., Sweet, James B. and Butler, Donald P.: Conscious sedation with intravenous agents: an amnesic study. Journal of Oral Surgery, 36:191, 1978.

Gelfman, Stephen S., Gracely, Richard H., Wirdzek, Peggy R., Sweet, James B. and Butler, Donald P.: Tests of recovery from intravenous sedation with diazepam-methohexital and diazepam-methohexital and fentanyl. Journal of Oral and Maxillofacial Surgery, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 DE 00031-10 NA

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (30 characters or less)

Design and Computer Interfacing of Neurophysiologic Instrumentation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Brown, Frederick J. Electronic Engineer (Instru) NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neural Mechanisms Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This work involves the development of suitable electronic and electro-mechanical instrumentation to be used in neurophysiological, physiological and behavioral research. It involves the adaptation and interfacing of these and other instruments to a laboratory or multipurpose computer installation.

1. Project Description

a) A stimulator has been designed and built to facilitate pulsed constant current electrical stimulation of teeth. The stimulus pulse width and frequency are fixed at one millisecond and 100 Hz, respectively, and a train of 100 pulses is presented each time the START button is activated. Stimulus current is adjustable from 0.1 to 100 microamperes. The output polarity is negative, and voltage compliance extends to approximately -275 volts.

Because of changing priorities, this unit was built instead of a computer controlled stimulator which had been planned.

b) A cutaneous thermal stimulator has been interfaced to a DEC 11/40 computer to provide computer control of stimulus parameters. Subject response data such as stimulus-response latency and stimulus intensity judgements are entered into the computer through a special response panel.

c) A DEC 11/34 computer will be connected to equipment in two laboratories to allow interactive control of behavioral and neurophysiological experiments on monkeys. Each laboratory will be provided with on-line data collection and analysis, and a remote facility will be provided for processing data previously collected or for entering or re-entering additional neurophysiological data from analog tapes recorded during an experiment.

2. Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00081-01 NB

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Activity of Trigeminal Afferent Fibers in Response to Noxious and Innocuous Stimuli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Dubner, Ronald Chief, NAB NIDR NIH

COOPERATING UNITS (if any)

LAB/BRANCH

Neurobiology and Anesthesiology Branch

SECTION

Neural Mechanisms Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.45	0.10	0.35

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Under barbiturate anesthesia, microelectrode recordings from the trigeminal ganglion of monkey are performed and electrical activity is recorded and stored on magnetic tape. Thermal and noxious heat stimuli are delivered with a precisely-controlled thermode which has a temperature range of 20°C to 60°C and can produce a temperature change of 10°C at the thermode-skin junction within one second. Data is analyzed with the aid of an on-line, real-time computer system.

1. Project Description

Objectives: In this study we have examined the response properties of peripheral nerve fibers that innervate the monkey's face. We have attempted to characterize these neurons based on their response to innocuous and noxious levels of mechanical and thermal stimuli applied to facial receptive fields. One major aim is to correlate the activity of these peripheral neuronal populations with behavioral observations in monkeys trained to detect or escape thermal stimuli applied to the same region of their face. Another aim is to define the peripheral input systems that are capable of coding various aspects of sensation and to determine what transformations, if any, take place during central coding. A third aim is to correlate the physiologic properties of these fibers with anatomical receptor structures isolated from the skin.

Methods Employed: Under barbiturate or N_2O/O_2 anesthesia, microelectrode recordings are made from the trigeminal ganglion after hemispherectomy. With glass-coated tungsten microelectrodes, we are able to isolate single units including those innervated by unmyelinated (C) fibers. Thermal and noxious heat stimuli are delivered with a precisely-controlled thermode. Mechanical stimuli consist of hand-held probes and von Frey filaments. Data is recorded on magnetic tape and analyzed with the aid of an on-line real-time computer system.

Major Findings: Previously, we have reported on the properties of three peripheral nerve populations sensitive to temperature changes in the 47°C to 51°C range and capable of initiating central neuronal activity involved in the monkey's ability to escape from noxious heat stimuli. We concluded that activity in C polymodal nociceptors alone was insufficient to account for the monkey's escape behavior to such stimuli, and that A delta heat nociceptors probably are essential for such discriminations.

Recent experiments have been concerned with the responses of Aδ heat nociceptors and Aδ high-threshold mechanoreceptors to warming and noxious heat. A delta heat nociceptive afferents responded maximally to noxious heat (temperatures ≥45°C) and noxious mechanical (forces ≥25 g, measured with von Frey filaments) stimuli and had receptive fields (RF) limited to a single spot (1-2 mm diam). Conduction velocities averaged 15.0 m/sec (S.D.=9.9, n=15). Thermal and mechanical thresholds usually were below noxious levels and ranged from 37-47°C and 0.17-15.0 g, respectively. Repeated noxious heat stimuli produced a decrease in thermal thresholds and an increase in sensitivity to subsequent stimuli. Stimulus-response functions were monotonic from threshold to final temperatures of 51°C, with the steepest part of the curve in the 45-51°C range. On the other hand, A delta high-threshold mechanoreceptive afferents, responded maximally to noxious mechanical stimuli only.

conduction velocities averaged 10.2 m/sec (S.D.=7.3, n=54) and RFs were mostly single spots (1-2 mm diam). Mechanical thresholds ranged from 0.17 g to 30 g (median=1.2 g) but all exhibited monotonic stimulus-response functions into the noxious range. These afferents initially were not activated by noxious heat stimuli in the 45° to 51°C range. However, repeated exposure to noxious heat sensitized 24% of these afferents, so that subsequently they responded weakly to temperatures above 49°C. These data indicate that A delta heat nociceptive afferents are the only myelinated afferents that respond reliably to noxious heat. Correlation of their heat responses with escape latencies in monkey indicate that activity in this neural population is sufficient to account for fast escape latencies occurring at 49° and 51°C.

Significance to Biomedical Research and Program of the Institute: These studies shed light on the way in which the brain processes information related to the presence of tissue-threatening or tissue-damaging stimuli in the environment. By defining the parameters of stimulation necessary to activate specific classes of nociceptors, and comparing them with psychophysical measurements using similar parameters, we can ascertain their role in pain sensation. Transformation or processing of activity from each nociceptive class then can be evaluated at different levels of the neuraxis. In addition, the animal behavioral model which has been developed in this laboratory, in combination with electrophysiological studies, should be useful in the development and testing of new and improved methods of pain control.

Proposed Course: With completion of the above studies we will initiate studies which correlate functional properties of primary afferents with the morphology and location of their terminal axonal arbors in the brain stem, utilizing intracellular tracer techniques.

2. Publications

Beitel, R.E., Dubner, R., Harris, R., and Sumino, R.: Role of thermoreceptive afferents in behavioral reaction times to warming shifts applied to the monkey's face. Brain Research, 138:329-346, 1977.

Biemesderfer, D., Munger, B.L., Binck, J. and Dubner, R.: The Pilo-Ruffini complex: A non-sinus hair and associated slowly-adapting mechanoreceptor in primate facial skin. Brain Research, 142:197-222, 1978.

Dubner, R., Price, D.D., Beitel, R.E. and Hu, J.W.: Peripheral neural correlates of behavior in monkey and human related to sensory-discriminative aspects of pain. In: Anderson, D.J. and Matthews, B.M. (Eds.): Pain in the Trigeminal Region, Amsterdam, Elsevier, 1977, pp. 57-66.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 DE 00133-04 NA

PERIOD COVERED October 1, 1977 to September 30, 1978

CT 0600116

TITLE OF PROJECT (80 characters or less)

Assessment of experimental and clinical pain

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gracely, Richard H.	Research Psychologist	NIDR NA
OTHER:	Gelfman, Stephen	Anesthesiologist	NIDR NA
	Dubner, Ronald	Chief, NAB	NIDR NA
	Sweet, James B.	Sr Dental Surgeon	NIDR IR
	Heft, Marc W.	Clinical Assoc (Dentistry)	NIDR IR
	McGrath, Patricia A.	Biologist	NIDR NA
	Sharav, Yair	Visiting Scientist	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neural Mechanisms Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.90	1.30	0.60

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are (1) to assess psychophysical methods of experimental pain measurement, i.e., magnitude estimation, category scaling, and cross-modality matching. Pain will be experimentally induced by electrocutaneous, electric tooth pulp, and mechanical heat stimulation; (2) to assess clinical pain measures, such as pain questionnaires and sensory matching methods, in a dental setting; (3) to determine the validity of experimental pain models by comparison of experimental and clinical pain responses; and (4) to evaluate known pharmacological and non-pharmacological pain-control agents.

NIDR Classification: 60214--100%

1. Project Description

Objectives: The purpose of these studies is to develop psychophysical measurement techniques for experimental and clinical pain, and to use validated techniques to evaluate pharmacological and non-pharmacological pain control agents. Assessment techniques must attempt to independently measure the sensory and motivational dimensions of the pain experience. The sensory dimension is generally assumed to be associated with discriminatory aspects of pain. The motivational or unpleasantness dimension is generally assumed to be a complex perceptual cognitive component influenced by psychological factors. There is evidence that these two factors may be distinguished at a physiological level and evidence which suggests that pain control agents can act differentially on these two pain components.

Methods Employed: Experimental pain is produced by electrical stimulation of skin and teeth, by heat applied to skin and by noxious cold stimulation of exposed dentin. The sensory intensity and unpleasantness associated with these stimuli are assessed by verbal descriptor scales and cross-modality matching techniques. Clinical pain is being assessed by verbal scaling, by sensory matches to experimental pain stimuli, and by questionnaire. The verbal pain descriptors have been objectively and reliably quantified in previous studies.

Major Findings: The reliable, objective and valid verbal descriptor scales developed in this project have been used to assess the influence of nitrous oxide, oxygen, fentanyl and diazepam, on the sensory intensity, unpleasantness or pain associated with electrical tooth pulp stimuli. The experiments concerned with the quality of sensations elicited from the tooth pulp and the underlying neural mechanisms responsible for these sensations are described in a new NIRP, "Sensations produced by tooth pulp stimulation".

The pilot study that demonstrated that the narcotic fentanyl reduced the sensory intensity but not the unpleasantness of electrical tooth pulp stimuli was replicated with the addition of a saline placebo group to control for order and placebo effects. Subjects used verbal descriptors to rate the sensory intensity or unpleasantness of painful 100 Hz electrical tooth pulp stimuli before and after the random double blind intravenous administration of either fentanyl or saline. Sensory intensity responses were significantly reduced following fentanyl and unaltered following placebo. Unpleasantness responses were reduced significantly following placebo, but not following fentanyl, although the post-drug change in responses was not significantly different between the fentanyl and placebo groups.

Because fentanyl and saline produce distinctly different subjective effects, a second study used an active placebo, diazepam, to mask the

difference between the subjective effects of these drugs. Subjects used the verbal descriptor method to rate the sensory intensity or unpleasantness of painful 100 Hz electrical tooth pulp stimuli before and after the intravenous administration of diazepam followed by either fentanyl or saline. The results were the same as those found in the first study in which fentanyl or saline were administered without a pre-injection of diazepam. Sensory intensity responses were reduced significantly after fentanyl and unaltered after saline. Unpleasantness responses were reduced significantly after placebo but not after fentanyl, although the post-drug change in responses was not significantly different between the fentanyl and placebo groups.

An additional series of experiments determined the reliability and objectivity of verbal pain descriptors (e.g., slightly painful, moderately painful, very painful) and used these descriptors as responses to painful 100 Hz electrical tooth pulp stimuli. These stimuli were presented before and after the double blind administration of saline or fentanyl with and without a pre-injection of an active placebo (diazepam). Pain responses were reduced significantly following fentanyl, diazepam with fentanyl, and diazepam with saline, but not after saline alone. In terms of the analgesic action of fentanyl in comparison to a placebo control, pain responses were reduced significantly following fentanyl only when an active placebo was not used to mask the difference between the subjective effects of fentanyl and saline. Unlike sensory intensity or unpleasantness verbal descriptor responses, pain descriptor responses did not distinguish between a narcotic analgesic and an active placebo drug.

A final experiment used verbal descriptor scales to assess the sensory intensity or unpleasantness of 100 Hz electrical tooth pulp stimuli before and after the double blind administration of air, oxygen or 33% nitrous oxide. Nitrous oxide significantly reduced the sensory intensity and unpleasantness of the electrical tooth pulp stimuli in comparison to either air or oxygen. The effect of oxygen was not significantly different from that of air.

Significance to Biomedical Research and Program of the Institute: The present results confirm earlier findings that, in contrast to classical assumptions about narcotic action, the verbal descriptor method showed that a narcotic analgesic drug reduced the perceived sensory intensity of noxious tooth pulp stimuli without reducing unpleasantness. In addition, the different results with sensory intensity and unpleasantness descriptors after either saline or fentanyl indicate that the type of words used to measure pain may significantly influence the outcome of a pain experiment.

These results show also that verbal descriptors of sensory intensity and unpleasantness distinguish between a narcotic analgesic and an active placebo while general descriptors of "pain" may not separate the

effects of an analgesic drug and a nonanalgesic drug that produces a marked subjective effect. Finally, these studies provide experimental evidence that nitrous oxide produces analgesia both by reducing the intensity of pain sensations and by reducing the unpleasantness associated with those sensations.

Proposed Course: The validation of sensory and affective verbal pain descriptors will continue with a manipulation of sensory intensity by a local anesthetic. These descriptors will be used also to assess the effects of non-pharmacological pain control methods including transcutaneous electrical stimulation and verbal suggestion. Previous studies that compared natural dental pain and electrically evoked tooth pulp pain have been extended to include endodontic pain, postsurgical pain and chronic oral facial pain syndromes such as myofascial pain dysfunction and trigeminal neuralgia. Additional studies will examine the effects of surgical trauma, postsurgical pain, and experimentally evoked tooth pulp pain on the levels of endogenous opiate-like compounds (endorphins). Endorphin levels will be inferred from hypoanalgesia produced by injections of naloxone, a narcotic antagonist. A new series of studies will examine the psychophysical characteristics of contact thermal stimulation and the influence of pharmacological and non-pharmacological pain control methods on responses to this noxious natural stimulus. Both thermal and electrical stimuli will be used by normal subjects to assess the influence of narcotic analgesics on both subjective pain ratings and on neural mechanisms of suppression and summation. Parallel studies will assess cognitive, perceptual and psychomotor effects of narcotic analgesics. These studies and the subjective ratings of experimental pain stimuli and the analysis of neural mechanisms will also include patients suffering from chronic pain syndromes. These patients will also receive clinical pain questionnaires developed from the verbal assessment of postsurgical pain and chronic pain. These assessment procedures will in addition be applied to chronic pain patients scheduled to receive chronic stimulating electrodes implanted in central brain sites for pain relief. A comparison of the responses from these patients to those from non-surgical chronic pain patients and normal subjects will provide new information about the neural mechanisms, efficacy, tolerance and side effects of analgesia produced by central brain stimulation and by conventional narcotic analgesics.

2. Publications:

Gracely, R.H., McGrath, P. and Dubner, R.: Ratio scales of sensory and affective verbal pain descriptors. Pain 5:5-18, 1978.

Gracely, R.H., McGrath, P. and Dubner, R.: Validity and sensitivity of ratio scales of sensory and affective verbal pain descriptors: manipulation of affect by diazepam. Pain 5:19-29, 1978.

Gracely, R.H., Dubner, R., McGrath, P. and Heft, M.: New methods of pain measurement and their application to pain control. International Dental Journal, 28:52-65, 1978.

PROJECT NUMBER (Do NOT use this space)	HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00151-04 NA

PERIOD COVERED

October 1, 1977 to September 30 1978

TITLE OF PROJECT (80 characters or less)

Trigeminothalamic Neurons Responsive to Innocuous and Noxious Stimuli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Hayes, Ronald L.	Staff Fellow	NIDR NA
OTHER:	Dubner, Ronald	Chief, NAB	NIDR NA
	Price, Donald D.	Research Physiologist	NIDR NA
	Hoffman, Donna	Postdoctoral Fellow	NIDR NA
	Wolskee, Patricia J.	Psychologist	NIDR NA
	Ziriax, John M.	Psychologist	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH

Neurobiology and Anesthesiology Branch

SECTION

Neural Mechanisms Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.40	1.95	1.45

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project studied the responses of several classes of neurons located in trigeminal nucleus caudalis responding to noxious and innocuous mechanical and thermal stimuli in awake, behaving rhesus monkeys. Monkeys were trained to detect the termination of innocuous heat stimuli and the onset of noxious heat stimuli. Nociceptive neurons often had thermal thresholds below 45°C, but stimulus response functions were positively accelerating in the noxious heat range. The responses of phasic neurons could provide information about the presence of behaviorally significant stimuli. Tonic neurons exhibited complex response properties primarily characterized by tonic excitation or suppression throughout the execution of certain task related behaviors and/or the presentation of certain task related stimuli. The activity of such neurons may be associated with attentional or motor processes utilized during the execution of certain behaviors employed in this task.

1. Project Description

Objectives: Previous work in this project employed anesthetized preparations to examine the response properties of nucleus caudalis and subjacent reticular formation neurons that are part of the trigeminal nuclear system involved in nociceptive responses by rhesus monkeys. The present studies represent an effort to evaluate the contribution of general anesthesia to the results of earlier work. Moreover they allow detailed analyses of the dynamic properties of the encoding of nociceptive input. In contrast to the relatively static and passive input-output relationships attending experiments on anesthetized preparations, studies of behaving monkeys provide the opportunity to examine the interactive properties of somatosensory systems. Variables such as attention, response appropriateness, expectation and stimulus salience can be studied, and their role in nociception evaluated.

Methods Employed: In the physiological single unit recording experiments, a microelectrode is stereotaxically introduced into the brainstem trigeminal nucleus caudalis through a sealed chamber chronically fixed to the monkey's skull. During the experiment the monkeys are temporarily restrained. Otherwise the animals are kept unrestrained in their home cages.

In one behavioral task currently being used and reported on previously, monkeys are trained to detect the termination of innocuous heat pulses delivered via a feedback-controlled contact thermode (1 cm diam) applied to the upper hairy lip. Adapted skin temperature is 35°C. A panel is illuminated to signal the beginning of each trial. Monkeys depress the lighted panel to initiate innocuous heat pulses (39°, 41°, 43°C) of randomly varying durations (2-8 sec). Panel releases of less than 2 sec (reaction times or RTs) after the termination of these stimuli are rewarded by water (0.4 cc). Additional heat pulses of a constant duration (20 sec) are presented quasi-randomly in which the temperature increase reaches the noxious range (45°, 47°, 49°C). Release of the panel at the termination of noxious stimuli is not rewarded by water. However, early releases terminate heat pulses on these trials, thereby allowing the monkeys to escape noxious heat stimuli. Thus the task assesses RTs indicating the detection of the termination of innocuous heat stimuli or the discrimination of the onset of higher stimulus intensities shifting into the noxious range.

Major Findings: Neuronal activity has been correlated with the following behavioral events: 1) panel light onset indicating that a new trial could be initiated; 2) panel press initiating onset of innocuous (37°-43°C) and noxious (45°-49°C) thermal stimuli; 3) termination of thermal stimuli; and 4) panel release. Three classes of neurons have been studied. Receptive fields were almost always limited to the

ipsilateral face and often were confined to the trigeminal maxillary division. Nociceptive neurons exhibited responses correlated with onset and termination of thermal stimuli. Thresholds often were below 45°C, but stimulus-response functions were positively-accelerating in the noxious heat range. Behaviorally non-relevant thermal stimuli evoked longer latency responses which sometimes were reduced in magnitude, whereas responses to particularly relevant stimuli were potentiated. Phasic neurons had flatter stimulus-response functions than nociceptive neurons and had phasic response components which correlated with panel light onset and panel press. Behaviorally non-relevant thermal stimuli evoked weaker responses in the innocuous range. Responses in the noxious range were delayed in onset but of similar magnitude. One type of tonic neuron exhibited maintained discharges throughout the panel press period which ceased when the monkey escaped noxious thermal stimuli or detected the termination of innocuous warming stimuli. This maintained discharge was present although reduced when thermal stimuli were passively presented to the monkey. Response magnitude sometimes increased during panel press and often high-frequency discharges preceded panel release or followed temperature offset when stimuli were presented passively. Unit firing was enhanced by the appropriateness of the motor response. Response magnitude was independent of thermal stimulus intensity except for increased responses to 47° and 49°C noxious heat stimuli. Panel light onset produced either an increase or a decrease in activity. A second type of tonic neuron displayed maintained discharges to innocuous tactile stimuli. This response to innocuous tactile stimuli was tonically suppressed throughout the panel press period. Response suppression ceased when the monkey released the panel. The magnitude of suppression was independent of thermal stimulus intensity. Suppression was observed even when thermal stimuli were passively presented or the thermal probe placed outside of the receptive field of the neuron. The magnitude of responses for both types of tonic neurons could be reduced when reinforcement was withheld from the animal. These data suggest that (1) nociceptive neurons provide information about the intensity of noxious heat stimuli, (2) phasic neurons signal the presence of behaviorally significant stimuli, and (3) tonic neurons may be associated with attentional or motor processes utilized during the execution of certain behaviors during this task. For example, the high frequency discharge of some tonic neurons which attends panel release and/or temperature offset could be related to the monkey attending to the presentation of the task related stimulus, temperature offset. Alternatively the discharge could be related to lip movements involved in preparation to receive liquid reinforcement. Tonic neurons which are suppressed could be involved in labeling non-relevant tactile stimuli while the monkey is attending to task-related thermal stimuli.

In more recent studies, the thermal discrimination task outlined above has undergone three significant revisions. First, a signal light was added which predicted the onset of a 4.0 s noxious (47°-49°C) heat pulse. Signal light onset occurred simultaneously with temperature

onset. Second, a behaviorally non-relevant light was added. The light is illuminated only during inter-trial intervals. Neuronal responses can be correlated with the onset or termination of this non-relevant light. Finally, the presentation of the water reward can now be delayed until 2.0 s after execution of a correct response. This was done in an effort to dissociate events attending the reception of the liquid reward from those attending the presentation of certain stimuli or the execution of certain behaviors instrumental in securing the reward. The monkey can be tested with either the immediate or the delayed reinforcement contingency in effect. This manipulation is available in the thermal task as well as the light detection task described below.

A second behavioral paradigm has now been introduced to address the role of attentional processes as well as the effects of requiring the monkey to respond primarily to visual rather than thermal stimuli. In this task the water restricted monkey is trained to detect the onset of light stimuli. Correct detection of the onset of a light stimulus is rewarded by water (0.4 cc). Constant duration (4.0 s) heat pulses are presented at quasi-random intervals both during and between trials. Responses by the monkey do not have any effect on the presentation of thermal stimuli. The monkey is randomly exposed to the occurrence of both innocuous temperature (37°-43°C) and noxious (45°-49°C) temperature shifts. As in the thermal detection task, neuronal events can be correlated with: 1) panel light onset indicating the start of a trial; 2) panel press; 3) temperature onset; 4) temperature offset; 5) the onset of the light discriminandum; 6) panel release; 7) delivery of liquid reinforcement-optional in both tasks.

Preliminary results from these additions to the behavioral tasks used during single unit recordings from nucleus caudalis in awake, behaving monkeys are: (1) the maximum responses of some nociceptors to noxious thermal stimuli are reduced, as compared to unsignaled trials, when a light predicts the onset of noxious heat stimuli; (2) the threshold temperature at which a nociceptor responds is increased from 39° when the monkey is behaving in the thermal task to 43° when the monkey is detecting the onset of a light stimulus; (3) in the thermal task, the response of tonic neurons to trial light onset is absent or reduced when a light is not relevant to the task; (4) all tonic neurons studied to this date exhibit a maintained discharge throughout the panel press period in both the thermal and light tasks. Higher frequency discharges can also precede panel release in the light task. However, we have not excluded the presence of more subtle differences in the responses of tonic neurons during thermal and visual detection tasks; (5) tonic neurons can show high frequency discharges at panel release even when reward is delayed. The interpretation of these results awaits the collection of oral-facial EMG activity coincident with neuronal responses. Such data would elucidate the relationship of tonic neuron activity to oral-facial motor behavior.

These more recent data suggest that: (1) the activity of nociceptive neurons in nucleus caudalis can be modulated by behavioral contingencies; (2) relevant stimuli contributing to maintained discharge in some tonic neurons include visual as well as thermal stimuli; (3) increases in discharge rate of some tonic neurons previously seen at panel release or temperature offset can be temporally dissociated from the actual delivery of the liquid reinforcement and perhaps from the motor behavior in reward reception. However, the possible role of tonic neurons in oral-facial motor behavior related to reward consumption awaits EMG and neural data collected simultaneously during changes in delay of reinforcement.

Significance to Biomedical Research and Program of the Institute: These studies have successfully identified nociceptive neurons in trigeminal nucleus caudalis--that is, neurons which could transmit information about noxious stimuli and thus possibly participate in the perception of and responses to oral-facial pain. Since these data were collected in awake, behaving animals, our observations extend and modify previous work in acute, anesthetized preparations. Notably, certain classes of nociceptors described for acute preparations respond similarly when tested with stimuli passively administered to awake monkeys. However, when the monkey performs the task, neurons described as potential nociceptors in anesthetized preparations are classified as tonic or nociceptive neurons in awake behaving monkeys. Thus descriptions of the functional properties of potential nociceptive neurons in anesthetized preparations are incomplete. These studies have also shown that the responses of nociceptive neurons can be modified by environmental and behavioral contingencies. These data strongly suggest that the neural representation of oral-facial nociceptive input can be modified at the earliest stages of central nervous system integration. In addition, this work could suggest non-pharmacological approaches to the control of dental pain. Finally, this work has shown promise of providing valuable insights into the roles of such variables as attention, motivation and expectancy in the neural representation of somatosensory stimuli.

Proposed Course: Future experiments include the following: (1) antidromically activating neurons from chronically implanted thalamic stimulating electrodes; (2) requiring the animal to work to threshold intensities of light stimuli in the visual task. This should maximize the attentional variables mediating neural response differences seen in the visual versus the thermal task. (3) comparing signalled and unsignalled presentation of noxious heat stimuli presented as the monkey performs the light detection task; (4) shifting the temperature values which distinguish reinforced from non-reinforced trials. While presently established at a 43°C vs. 45°C comparison, movement in either direction could influence stimulus-response functions observed both for nociceptive and phasic neurons.

2. Publications:

Dubner, R. and Hayes, R.L.: Pain mechanisms in the trigeminal system. Proceedings of the 1978 Miles Symposium, New York, Raven Press, in press.

Hayes, R., Price, D.D. and Dubner, R.: Primate behavioral models in neurobiological studies of pain. Proceedings of the Second World Congress on Pain. New York, Raven Press, in press.

Dubner, R. and Price, D.D.: Mechanisms of oral-facial pain. Proceedings of the Bite Centennial, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00152-04 NA

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (30 characters or less)

Psychophysical and Electrophysiological Studies of First and Second Pain

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Price, Donald D.	Research Physiologist	NIDR NA
OTHER:	Dubner, Ronald	Chief, NAB	NIDR NA
	Ruda, Maryann T.	Staff Fellow	NIDR NA
	Hayashi, Haruhide	Visiting Fellow	NIDR NA

COOPERATING UNITS (if any)

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Neurobiology and Anesthesiology Branch

SECTION
Neural Mechanisms Section

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NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.80	1.45	0.35

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Brief noxious heat pulses (2.0 sec duration; peak temperature = 51.5°C) programmed by a computer and generated by a contact thermode have been used to study peripheral and central nervous system mechanisms of first and second pain. Previous research has demonstrated that heat-induced first and second pains are related to impulse conduction in Aδ heat nociceptive afferents and C polymodal afferents respectively. When 4 identical heat pulses are applied to the same location on the hand, first pain decreases in perceived intensity while second pain summates. Previous work in our laboratory has shown that the latter is related to CNS mechanisms in the spinal cord dorsal horn. Work in progress demonstrates that both lamina I spinothalamic neurons and lamina II-III substantia gelatinosa neurons of the dorsal horn respond to heat in a manner consistent with psychophysical responses, that is, with a brief latency (Aδ heat nociceptor mediated) response that shows suppression and a long latency response (C polymodal nociceptor mediated) that shows summation. Thus we have determined the neurons of origin of this summation phenomenon. Data are analyzed with the aid of a computer system.

I. Project Description

Objectives: Single unit recordings are made of spinothalamic tract neurons of lamina I and substantia gelatinosa (SG) neurons of laminae II-III to determine a) the physiological characteristics of dorsal horn neurons which convey information about first and second pain; b) the functional interrelation between SG neurons and lamina I spinothalamic neurons (especially with regard to their role in first and second pain); c) the neuronal mechanisms that are involved in first and second pain.

Methods employed: Independent variables in these experiments include constant waveform heat pulses (2.0 sec duration; 51°C peak temp) that reliably evoked first and second pain in human subjects as well as other forms of natural stimuli. The heat pulses, generated by a contact thermode (1 cm diameter), raise receptive field skin temperatures to noxious levels (45-51°C) for 0.7 sec. Responses of single monkey spinothalamic tract neurons and SG neurons to trains of heat pulses are then recorded, stored on magnetic tape, and analyzed with the aid of computer facilities. Lamina I spinothalamic neurons are identified by stimulation of their axons at thalamic sites and recording the antidromic action potential within the dorsal horn with conventional microelectrode techniques. SG neurons are identified just ventral to lamina I projection neurons. Thus "pairs" of neurons are recorded and their physiological properties determined.

Major Findings: Two major classes of neurons are found within lamina I (spinothalamic neurons) and in SG: (1) wide dynamic range (WDR) and (2) nociceptive-specific (NS). Their responses to trains of noxious heat pulses are analogous to human sensations evoked by identical stimuli. Each single heat pulse reliably evokes first and second pain in human subjects and short and long latency neuronal responses in NS and WDR neurons. The early neuronal response, like first pain, decrements in intensity with repeated heat pulses but not with repeated electrical stimuli. The second or late neuronal response, like second pain, outlasts the arrival of incoming C fiber impulses and summates in intensity with repeated heat pulses or electric shocks. A critical interstimulus interval is necessary for this summation (ca. 3 sec). WDR and NS neurons receive input from Aδ heat nociceptive (AHN) and C polymodal nociceptive (CPN) afferents whose activity is correlated with heat-induced first and second pain, respectively. The responses of AHN and CPN afferents decrease in intensity with repeated heat pulses but not with repeated electrical shocks. We conclude that heat-induced suppression of first pain and the early spinothalamic neuronal response are the direct result of suppression of AHN afferents. On the other hand, prolonged temporal summation of heat-induced second pain and of the late neuronal response are the result of spinal cord facilitatory mechanisms activated by CPN afferents.

Several observations strongly indicate that some SG neurons excite lamina I spinothalamic tract neurons. First, anatomical Golgi studies reveal a possible excitatory interneuron, the stalked cell in lamina II, whose axon projects to lamina I. Second, our physiological studies support this anatomical finding in showing that (1) the lamina I and lamina II neurons within a given microelectrode tract have overlapping receptive fields with the latter neuron having a smaller receptive field area; 2) the lamina II neuron usually has a shorter first spike latency to electrical stimulation of its receptive field. Both neurons within this functional "pair" respond similarly to repeated noxious heat pulses. Therefore, the stalked cell may represent the critical neuron at which mechanisms of central summation evoked by C polymodal nociceptor input is first manifested.

Significance to Biomedical Research and Program of the Institute: These results indicate that information about first and second pain is transmitted by two classes of neurons in lamina I and in SG. The decrement of first pain produced by successive heat pulses is related to suppression of Aδ heat nociceptor activity. In contrast, summation of second pain produced by successive heat pulses occurs in the face of partial suppression of C polymodal nociceptor activity. Central facilitatory mechanisms that could account for this summation exist within lamina I and SG of the dorsal horn and are reflected in the output of spinothalamic tract neurons. Thus prolonged temporal summation mechanisms, that occur with second pain and probably various forms of chronic pain, exist within the superficial layers of the dorsal horn. Understanding neural mechanisms of these superficial layers may be important in finding procedures that reduce chronic pain and pathological pain such as post-herpetic neuralgia.

Proposed Course: Future studies involve further analysis of the neuronal circuitry of layers I, II and III of the dorsal horn using intracellular recording and marking techniques.

2. Publications

Price, D.D. and Dubner, R.: Neurons that subserve the sensory discriminative aspects of pain. Pain, 3:303-338, 1977.

Price, D.D. and Dubner, R.: Peripheral and central mechanisms of first and second pain. J. of Investigative Dermatology, 69:167-171, 1977.

Price, D.D., Hayes, R.L., Ruda, M., and Dubner, R.: Spatial and Temporal Transformations of Input to Spinothalamic Tract Neurons and Their Relation to Somatic Sensations. J. Neurophysiol. 41:933-947, 1978.

Price, D.D., Hayes, R.L., Ruda, M., and Dubner, R.: Neural representation of cutaneous aftersensations by spinothalamic tract neurons. Fed. Proc. 37:2237-2239, 1978.

Price, D.D., Dubner, R., Hayes, R., Ruda, M., and Hu, J.W.: Trigeminothalamic and spinothalamic neurons that subserve sensory-discriminative aspects of pain. In Anderson, D.J. and Matthews, B.M. (Eds.): Pain in the Trigeminal Region. Amsterdam, Elsevier, 1977, pp. 225-232.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 DE 00165-03 NA

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Neuropharmacological Analysis of Supraspinal and Narcotic Modulation of Pain

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Harris, Robin D.	Research Psychologist	NIDR NA
OTHER:	Hayes, Ronald L.	Staff Fellow	NIDR NA
	Dubner, Ronald	Chief, NAB	NIDR NA
	Wolskee, Patricia J.	Psychologist	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH

Neurobiology and Anesthesiology Branch

SECTION

Neural Mechanisms Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.25	0.3	0.95

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major concern of this project was twofold: 1) to develop a primate, behavioral model of pain in which the animal's response to noxious stimuli is primarily, if not totally, dependent upon the sensory (intensity) dimension of pain while independent ot the affective dimension of pain; 2) to test the hypothesis that narcotic analgesics (viz, morphine) can suppress the sensory (intensity) dimension of noxious heat stimuli. A corollary of this hypothesis that altering the affective state by minor tranquilizers, like diazepam, is not sufficient to suppress the sensory (intensity) dimension of pain was also tested. Techniques employed in this study were application of thermal heat via a contact thermode, using latencies as a behavioral index of perceptual sensitivity, i.m. injections of drugs, and real-time computer control of the experimental sessions. The objectives of this study were to develop a more comprehensive behavioral model of pain in animals (primates) and to broaden our understanding of the analgesic action of narcotics.

1. Project Description:

Objectives: The general objective of this project was to extend the behavioral model of pain developed earlier by Dubner, Beitel, and Brown (1976) so that the sensory-discriminative (intensity) component and the motivational-affective (aversive) component of the pain experience could be separated from each other. The sensory-discriminative dimension defines such stimulus characteristics as intensity, duration, and location. The motivational-affective dimension delineates emotional qualities, such as anxiety, fear, aversiveness, or unpleasantness, associated with the stimulus. Initially, we developed a behavioral model in which the animal had to discriminate the magnitude of both innocuous and noxious final temperature: a behavioral task dependent upon stimulus intensity. In the second part of the project we addressed a more circumscribed objective concerning morphine analgesia. For many years many investigators have attributed the mechanism of morphine analgesia to morphine's attenuation of the aversiveness of noxious stimuli. An alternative hypothesis to explain the mechanism of morphine action in analgesia is that morphine attenuates the intensity component of the pain experience instead of or in addition to its attenuation of the aversive component. If this alternative hypothesis is correct, then, morphine should attenuate the discrimination of stimulus magnitude in our task. A corollary hypothesis that altering the affective state (anxiety) of the animal with minor tranquilizers (viz, diazepam) should not be sufficient to suppress the sensory (intensity) dimension of pain was also tested.

Methods Employed: The following two experiments evaluated the relative effects of morphine and diazepam on responses in a task requiring two male rhesus monkeys to attend to the sensory-intensity component of innocuous and noxious thermal stimuli.

In the experiments the animals had to concurrently perform two types of tasks: A detection task (reinforced response) and a discrimination task (nonreinforced response). First, the detection task will be explained. To signify a ready condition for a trial initiation, the panel button was illuminated. In response to the light the monkey initiated the trial by pushing and holding down the button. Simultaneous with the button press, the temperature of the contact thermode rose to an innocuous final temperature and remained elevated for a random period of time before returning to the baseline temperature (35°C). To receive a reward the animal had to release the button as soon as he detected the temperature turn-off. The resulting reaction time (RT) of the rewarded response served as an index of the animal's sensitivity to detect a decrease in temperature. Interspersed among the detection trials were the discrimination trials. These unreinforced trials also began with button illumination. Upon pressing the button the temperature of the contact thermode rose to a final temperature which was always greater

than those temperatures presented in the detection trials. The temperature remained elevated for 20 sec or until the animal released the button, whichever occurred first. Button releases, either before or after the temperature turn-off, were never rewarded. The time from the temperature onset until the button release was called the discrimination latency (DL). The DL served as an index of the animal's ability to discriminate the larger unreinforced final temperatures from the smaller reinforced temperatures, to which the animals would not release the button until after the temperature turn-off. The animals easily learned to discriminate the two types of final temperatures. From this paradigm, then, two different measures of sensitivity resulted: the detection RT, a measure of the detection of a temperature decrease; and the DL, a measure of discrimination of different stimulus magnitudes.

In Exp. I the reinforced final temperatures were 39°, 41°, and 43°C. The unreinforced final temperatures were 45°, 47°, 49°, and 51°C: all in the noxious range. In Exp. II the reinforced, detection trials had final innocuous temperatures of 37° and 39°C. The unreinforced, discrimination trials had final temperatures of 41°, 43°, 45°, and 49°C. Forty-one and 43°C are innocuous and 45° and 49°C are considered noxious temperatures. Thus, in the first experiment the monkey had to discriminate unreinforced noxious heat stimuli from reinforced innocuous heat stimuli; and in the second experiment the animal had to discriminate unreinforced noxious and innocuous heat stimuli from other reinforced innocuous heat stimuli.

Each day the animal was given an i.m. injection of either morphine, diazepam, or saline. Two saline days followed each morphine or diazepam day. Three doses of each drug were tested: 0.5, 0.75, and 1.0 mg/kg. The drugs were injected one hour before each day's data collection began.

All a priori statistical tests of significance were done using the Friedman's two-way analysis of variance (0.05 level of significance). The posteriori test used was the Wilcoxon matched-pairs signed-ranks test using Ryan's adjusted levels of significance.

Major Findings: In regard to the detection RT's, diazepam in both experiments slowed RT's significantly. The increase was dose dependent and animal dependent. Since the magnitude of RT increase was independent of temperature, the change in RT by diazepam probably is related to its effect on motor performance. Therefore, the magnitude of the RT increase was subtracted from the diazepam DL's to correct for this nonspecific effect. Morphine, on the other hand, did not consistantly increase RT.

In Exp. I morphine significantly increased the DL's at 47°, 49°, and 51°C. At 45°C morphine did not significantly change the DL's from the saline baseline. However, at 45°C the DL distribution exhibited excessive variability because it was the threshold discrimination

temperature. In neither monkey was the DL increase caused by morphine dose-dependent. Thus, in both monkeys morphine suppressed the discrimination of stimulus intensity.

Diazepam, in contrast to morphine, did not consistently suppress discrimination of stimulus intensity in both animals. In both monkeys the 1.0 mg/kg diazepam dose showed signs of general behavioral disruption.

From these results it was concluded that the morphine effect was probably related to morphine's attenuation of the perceived stimulus intensity apart from its effect on aversive aspects of the stimulus, since diazepam did not consistently suppress the discrimination of the noxious temperatures from the innocuous temperatures. To further eliminate the possibility that aversiveness was a factor in this discrimination task, the second experiment was performed. In it the animals had to discriminate both noxious and innocuous heat stimuli from other innocuous heat stimuli.

In Exp. II morphine again significantly increased the DL's in both monkeys. The increase in DL's was not only at the noxious temperatures of 45° and 49°C but also at the innocuous temperatures of 41° and 43°C. In one monkey a dose-response effect was evident from 43°-49°C. Thus, morphine suppressed the discrimination of stimulus intensity even when the discriminated intensity was in the innocuous range and not aversive to the monkey.

Unlike morphine, diazepam did not have a systematic influence on DL's in Exp. II.

Conclusion: Exp. I demonstrated that an alteration in the aversive quality of noxious stimuli was not sufficient to suppress the discrimination of noxious thermal stimuli from innocuous thermal stimuli, since diazepam failed to consistently increase the DL's to the noxious final temperatures. Furthermore, Exp. II showed that morphine suppresses the sensory-intensity dimension apart from its attenuation of aversiveness since morphine increased the DL's to 41° and 43°C, both innocuous, nonaversive temperatures. Also, it should be noted that the increase in the DL's caused by morphine administration occurred in the absence of any appreciable motor or general behavioral disruption. While discrimination of thermal intensity is not equivalent to analgesia, these results indicate that morphine analgesia may result at least in part from morphine's attenuation of the sensory-intensity component of the pain experience independent of its attenuation of aversiveness.

Significance to Biomedical Research and Program of the Institute: A long standing need in basic pain research has been an animal model of pain that can reflect the different attributes of pain perception. In the past most neurophysiological studies have been done in anesthetized,

acute preparations. However, it is clearly evident that recordings from such preparations do not yield an accurate picture of the neural activity of the normal, awake animal. Thus, it has become necessary to record from awake, behaving animals. In awake animals the behavioral model is important because it defines the situation and allows the investigator to relate the neural activity to a specific behavior. The development of animal behavioral models of pain also has importance independent of neurophysiological recording studies. They make it possible to study the psychological mechanisms of pain and its relief. This study, for example, particularly focused upon the mechanism of morphine analgesia. By showing that morphine can attenuate the sensory dimension it provides a rationale for developing new methods of analgesia which focus upon sensory attenuation rather than influencing the affective dimension of pain.

Proposed Course: Future plans for the use of this project include the following. First, the model will be used in a neurophysiological experiment recording from the trigeminal nucleus in awake, behaving monkeys. Second, the results will be published in an appropriate scientific journal. Third, a study needs to be done which tests the effects of morphine and diazepam upon an animal's ability to discriminate different noxious stimulus intensities in order to receive a reward.

2. Publications:

Hayes, R.L.: Experimental design and data analysis in studies of drug discrimination: some general considerations. In Ho, B.T., Richards, D.W. and Chute, D.L. (Eds.): Drug Discrimination and State Dependent Learning. New York, Academic Press, 1978, 173-201.

Hayes, R.L. and Mayer, D.J.: Discriminative control of behavior by electrical stimulation of the brain: a new neuropharmacological research strategy. In Ho, B.T., Richards, D.W. and Chute, D.L. (Eds.): Drug Discrimination and State Dependent Learning. New York, Academic Press, 1978, 249-260.

Hayes, R.L., Bennett, G.J., Newlon, P.G. and Mayer, D.J.: Behavioral and physiological studies of non-narcotic analgesia in the rat elicited by certain environmental stimuli. Brain Research, in press.

Hayes, R.L., Price, D.D., Bennett, G.J., Wilcox, G.L. and Mayer, D.J.: Differential effects of spinal cord lesions on narcotic and non-narcotic suppression of nociceptive reflexes: further evidence for the physiologic multiplicity of pain modulation. Brain Research, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00245-01 NA

PERIOD COVERED
October 1, 1977 to September 30, 1978 CT 0600117

TITLE OF PROJECT (80 characters or less)

Sensations Produced by Tooth Pulp Stimulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	McGrath, Patricia A.	Biologist	NIDR NA
OTHER:	Sharav, Yair	Visiting Scientist	NIDR NA
	Gracely, Richard H.	Research Psychologist	NIDR NA
	Heft, Marc W.	Clinical Assoc (Dentistry)	NIDR IR
	Dubner, Ronald	Chief, NAB	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neural Mechanisms Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.05	0.55	0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of this project is to determine the nature of sensations produced by tooth pulp stimulation. Non-pain, as well as pain sensations are evoked when low intensity electric current is applied to human teeth. In order to assess the role of non-pain sensations in the pulp--a traditionally exclusive pain system, these sensations were studied both psychologically and physiologically: 1) the minimum levels of current necessary to produce non-pain and pain sensations were determined for different frequencies of stimulating current; 2) the intensities of sensations from detection threshold to pain threshold were scaled by magnitude production and by verbal descriptors; 3) electromyographic (EMG) activity of the masseter reflex was recorded during tooth pulp stimulation at both non-pain and pain currents; and 4) the effect of a narcotic on sensations produced by tooth pulp stimulation and on the masseter reflex activity was evaluated.

NIDR Classification: 60214--100%

1. Project Description:

Objectives: The purpose of these studies is to investigate sensations evoked by electrical tooth pulp stimulation. The tooth has been assumed to be an exclusive pain or nociceptive system, and thereby a unique model for the study of pain, pain pathways, and pain control agents. However, a wide variety of non-pain sensations (such as warmth, tingling, and pressure) is experienced when low intensity electric current is applied to human teeth. The existence of non-pain sensations, in addition to pain sensations, may indicate the presence of a sensory system distinct from a pain system, or these non-pain sensations may simply be a parasthesia-and result from near threshold stimulation of an exclusive pain system. If there are 2 distinct sensory systems, there may be consistent differences between the levels of current sufficient to produce sensation and pain-assuming different thresholds for non-pain and pain nerve fibers. Or, the 2 sensory systems may differ in another neural property, temporal summation. Temporal summation can be studied by varying the frequency of stimulation (that is, the number of pulses within a stimulus train) and noting whether there is a uniform or differential effect on non-pain and pain sensations. If all sensations produced by tooth pulp stimulation have similar threshold and summation properties, it is probable that all sensations result from stimulation of one sensory system.

Another means of studying possible differences in the sensory innervation of the tooth pulp is monitoring the masseter reflex, the inhibition of masseter activity during sustained contraction that normally occurs when the tooth pulp is stimulated. This reflex may provide physiological correlates for the non-pain and pain sensations produced by tooth pulp stimulation. Reflexes produced by currents that produce definite non-pain sensations may differ from those caused by currents that produce pain sensations. In order to assess the value of these reflexes as physiological correlates of sensation: 1) the reliability and stability of EMG recordings of masseter activity during tooth pulp stimulation at non-pain and pain currents are determined; 2) the correlation between sensation experienced (non-pain or pain) and the reflex activity, as well as the correlation between stimulating current and the reflex activity are determined; 3) the effects of a narcotic (fentanyl) on sensations experienced and on the reflex are evaluated.

Methods Employed: Non-pain and pain sensations were produced by electrical stimulation (1 sec trains of monopolar, monophasic, cathodal, 1 msec duration constant current pulses) delivered to the labial and the incisal edge of upper central incisors. Frequency of stimulation ranged from 5 to 500 Hz.

Detection and pain thresholds were determined, and the intensities of sensations between these thresholds were scaled by magnitude production and by verbal descriptors.

For EMG monitoring, upper central incisors were stimulated by electrical pulses of 1 msec duration, at currents ranging from detection threshold to supra-pain threshold. Subjects maintained low or high muscle activity by audio-feedback from surface electrodes placed over the masseter muscles. Activity was monitored at the onset of each pulse in a 30 pulse series; recordings were rectified and averaged.

Major Findings: As reported previously in preliminary observations, all subjects reported detection thresholds that were significantly lower than pain thresholds at each of the frequencies studied. Sensory thresholds remained constant, regardless of the frequency of the stimulating current while pain thresholds varied monotonically with frequency with a maximum threshold at 5 Hz and a minimum at 100 Hz.

Temporal summation was evidenced at higher currents; a current that produced a non-pain sensation at a low frequency, 5 Hz, could produce a pain sensation when the frequency of stimulation was increased to 100 Hz. A more descriptive view of temporal summation was evidenced when the entire range of non-pain sensations was scaled. 1) Temporal summation occurred at a rate proportional to stimulus intensity; the lower the current, the less summation, the higher the current the more pronounced the summation. 2) The strength of sensation increased as current increased, and as frequency was increased at higher currents.

There were 3 types of reflex inhibition produced by tooth pulp stimulation: 1) a single depression or "silent period" in the normal activity of the masseter during sustained contraction; this silent period (SP) had a latency of 10-15 msec after pulse onset and a duration of 10-20 msec; 2) a double silent period, in which 2 depressions were evidenced separated by a short bursting of muscle activity; the first (SP1) had a latency and duration similar to those of SP, while the second depression (SP2) had a latency of 40-50 msec and a duration of 10-20 msec; and 3) an elongated silent period, in which SP1 and SP2 seemed to merge; the depression had a latency similar to SP and extended to approximately the end of SP2.

Previous investigators have hypothesized that SP1 was caused by low current stimulation, while SP2 was caused when high currents activated a nocioceptive or pain system. However, double silent periods were often evident at low currents that produced only non-pain sensations. No mention has been previously made about the merged silent period.

The reflex silent period first occurs at or near an individual's sensory threshold; it varies about 3 μA above and below sensory threshold. There is no unequivocal correlation between this masseter reflex and the sensation experienced when the tooth is stimulated. A merged silent period is seen more frequently during stimulation that produces pain, but not always. The reflex appears to be more related to the strength of the stimulating current; as current increases from sensory threshold to above pain threshold, there is a progressive decrease in the latency

of the reflex and a progressive increase in the duration. Merged silent periods usually occur at high-current stimulation, even if those currents do not produce pain. Single and double silent periods usually occur at lower currents.

The reflex inhibition is more pronounced at high levels of muscle activity.

Studies of the effects of the narcotic on both sensation and reflex inhibition are now in progress.

Significance to Biomedical Research and Program of the Institute: In recent years the tooth pulp has replaced the cornea (now known to have thermal and pressure innervation) as an exclusive nocioceptive system. Consequently much research has focused on producing pain in the tooth by mechanical, chemical, and electrical stimulation in order to investigate pure experimental pain and various methods of controlling it--drugs, hypnosis, electroanalgesia and acupuncture.

The present results show there is also a wide range of non-pain sensations produced when the tooth pulp is stimulated electrically. Subjects are able to scale the intensities of these non-pain sensations, in addition to the pain sensations usually associated with tooth pulp stimulation. Their scales or ratings may be used to assess the effects of various pain control methods and to determine if they act uniformly or differentially on non-pain and pain sensations.

Electromyographic monitoring of masseter reflex activity (following chin tap) has been used as a diagnostic tool for assessing temporo-mandibular joint dysfunction. TMJ). This reflex (following tooth pulp stimulation) has been used also as a physiological correlate for pain. However, the present results show that the reflex is not correlated with pain sensation but with any sensation produced from the tooth. The reflex is correlated more with the intensity of the stimulation, than with the felt sensation. A subject may feel a non-pain sensation, or in some cases feel no sensation and continue to have a reflex. The reflex may be used as an index of the current applied to an un-anesthesized tooth (in an anesthesized tooth, there is no sensation and no reflex), but not as a reliable index of pain.

Proposed Course: The investigation of non-pain sensations produced by tooth pulp stimulation will continue with experiments designed to differentiate further between non-pain and pain sensations. One such design involves the application of high currents to the tooth to note its effect on non-pain and pain sensations, since preliminary observations have shown that pulse trains at intense current levels may continuously increase pain threshold, while increasing sensory threshold by a finite amount.

Also, the effects of a narcotic both on sensation (non-pain and pain) and on reflex activity will be determined.

In addition to the temporal summation that was shown to be different at non-pain and pain sensations, future research will study spatial summation of the different sensations.

Inhibition of pain sensation in a tooth (produced either by a current applied to the tooth itself or to an adjacent tooth) will be evaluated. The success of some of the electroanalgesia currently used to relieve pain in dentistry is based on this inhibition principle. Yet, the specifics of the lateral sensory inhibition process--a generalized phenomenon common to other sensory systems--are not determined for the tooth. Future research will focus on elucidating this inhibition process in the tooth. By concurrent EMG monitoring of masseter reflex activity, it will be possible to assess the extent to which the sensory inhibition is peripheral or central.

In summary, research will continue on the nature of sensations produced by stimulating the tooth pulp (both non-pain and pain sensations) by studying: spatial summation properties, narcotic action, and inhibitory processes.

2. Publications:

None

PROJECT NUMBER (Do NOT use this space)

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00246-01 N.

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Masseteric exteroceptive reflex and sensory responses in MPD patients.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sharav, Yair	Visiting Scientist	NIDR NA
OTHER:	McGrath, Patricia A.	Biologist	NIDR NA
	Gracely, Richard H.	Research Psychologist	NIDR NA
	Dubner, Ronald	Chief, NAB	NIDR NA
	Heft, Marc W.	Clinical Assoc (Dentistry)	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH

Neurobiology and Anesthesiology Branch

SECTION

Neural Mechanisms Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.45	1.05	0.40

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objectives of this project are: (a) to establish sensory and pain thresholds to tooth pulp electrical stimulation in myofascial pain dysfunction (MPD) patients; (b) to relate sub-sensory, sensory and pain producing stimuli of the tooth pulp to the masseteric exteroceptive reflex in MPD patients, by means of electromyographic (EMG) recordings; (c) to compare the exteroceptive to the proprioceptive suppression of ongoing EMG activity in the masseters of these patients. In all these cases comparison between MPD patients to normal subjects will enable a better understanding of the MPD syndrome pain and dysfunction mechanisms, and the gain of insight into possible protective mechanisms of the exteroceptive reflex.

1. Project Description:

Objectives: The purpose of the present study is to measure and correlate the masseteric exteroceptive reflex at different biting forces to electric pulp stimulation of non-pain and pain producing intensities in patients with the myofascial pain dysfunction syndrome (MPD).

The ongoing electromyographic (EMG) activity of the human masseter during sustained contraction can be reduced or stopped by means of a proprioceptive (e.g. chin tap) or exteroceptive (e.g., electrical pulp stimulation) reflex.

MPD patients have demonstrated depressed EMG activity or a silent period (SP) after a chin tap that was of longer duration than in normal controls. This has usually been explained on the basis of muscle spasm and a peripheral mechanism of prolonged discharge from Golgi tendon organs and muscle spindles. Additional or different mechanisms of a central orgin were not excluded, however.

A chin tap in these patients also could have produced pain more often than in normal subjects. Thus, pain producing stimuli could have been the cause for the prolonged SP in MPD patients. The present study will examine: 1) the exteroceptive masseteric reflex to electric tooth pulp stimulation; thereby bypassing the proprioceptive input. This will enable the examination of mechanisms other than those associated with the muscle spindle or the Golgi organ; 2) the correlation between the reflex and different intensity stimuli at subsensation, non-pain and pain producing levels. This will enable us to relate the reflex to pain and non-pain exteroceptive reflexes in the MPD patient as compared to normal subjects; 3) the correlation between the EMG activity to different biting forces in order to examine the effect of biting force on the latency and duration of the silent period.

Methods Employed: Upper central incisors were stimulated by electric pulses of 1 msec duration at current range from detection threshold (sensory or reflex) to above pain threshold. Subjects maintained low or high muscular activity by auditory feedback during a train of 30 pulses. Muscle activity was monitored at the onset of each pulse, and EMG recordings were rectified and averaged.

Major Findings: Although final analysis of the data is not completed certain trends could be identified. (1) the exteroceptive suppression in MPD is not more prolonged that in the normals; (2) while 47% of MPD patients have a SP at sub-sensory thresholds this is true for only 9% of the normals; and (3) certain EMG configurations have been found to be more prevalent in MPD patients (e.g. cyclic activity). Comparison with normal subjects after the administration of a short-

acting narcotic (fentanyl) has demonstrated some common configurations with the EMG of MPD patients (i.e., cyclic activity). This is being further investigated.

Significance to Biomedical Research and Program of the Institute: The silent period has been found to be significantly longer in MPD patients. This has been attributed to muscle spasm and a peripheral mechanism of prolonged inhibitory discharge from Golgi tendon organs and muscle spindles. A central motorneuron pool mechanism could not, however, be excluded. The present study demonstrates a normal exteroceptive suppressive reflex in MPD patients which suggests that the disturbance is not in the central motorneuron pool. The etiological concept associated with a disturbed exteroceptive feedback due to a "pathogenic" occlusion seems unlikely in light of these new findings. This will have further implications in the treatment of the syndrome.

Proposed Course: Further analysis of the change in reflex threshold and EMG configurations (e.g., single SP, cyclic activity) in MPD patients will be carried on in order to better understand these changes. The low reflex threshold will be further investigated in connection with patient's sensory and pain thresholds. Comparison with normal subjects after the administration of a short activity narcotic (fentanyl) has been found of value, since certain changes in EMG configurations (i.e., cyclic activity) are common to both MPD and fentanyl effects.

2. Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00247-01 NA

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Cytomorphology of functionally characterized spinal cord dorsal horn interneurons

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bennett, Gary J.	Postdoctoral Fellow	NIDR NA
OTHERS:	Abdelmoumene, Mohammed	Visiting Fellow	NIDR NA
	Hayashi, Haruhide	Visiting Fellow	NIDR NA
	Dubner, Ronald	Chief, NAB	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neural Mechanisms Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.85	2.85	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Neurons in the spinal cord dorsal horn of monkeys and cats, especially neurons in lamina I and the substantia gelatinosa (SG), have been functionally characterized and intracellularly stained with horseradish peroxidase. The dendritic arbors and axonal extensions of these functionally identified neurons have been examined at the light microscope level. Wide dyramic range neurons, responsive to innocuous stimuli but activated maximally by noxious stimuli are found in layers I-V of the dorsal horn and have more extensive arbors than neurons in the same layers activated only by noxious stimuli (nociceptive-specific). The cytomorphological analysis of these functionally characterized neurons will soon be extended to the level of resolution of the electron microscope.

1. Project Description:

Objectives: The interneurons of the dorsal horn of the spinal cord and the homologous area of the trigeminal nucleus caudalis have received extensive scrutiny because of their participation in neural systems underlying sensations evoked from the skin. Neurophysiological analyses have made major progress in elucidating the responses of these neurons to various stimuli. Neuroanatomical analyses have provided a wealth of information about the cytomorphology of these neurons. Progress in unraveling the neural circuitry serving somatosensory phenomena has been hampered, however, by the difficulties encountered in bringing together the work of neurophysiologists with that of neuroanatomists. This project is a direct attempt to unambiguously correlate neuronal function and structure. Particular attention is paid to those interneurons that have been implicated in pain sensation. The project employs a recently introduced technique (described below) which permits one to study a single neuron with conventional electrophysiological techniques and then to histologically stain the very same neuron.

Methods Employed: The lumbosacral spinal cords of anesthetized monkeys and cats are prepared for single cell recordings in the conventional manner. Microelectrodes made from glass tubing drawn to very fine tips (c. 1μ) are filled with an electrolyte solution which contains the enzyme horseradish peroxidase (HRP). Isolated single neurons are 50functionally characterized with respect to the following stimuli: light touch (brushing the skin with an artist's brush or moving hair shafts); firm, but non-painful, squeezing of skin folds with blunt forceps; pinching folds of skin with small serrated forceps; noxious heat (delivered via a contact thermode); and percutaneous electric shock (2 msec. duration) of graded intensity. Following functional characterization an attempt is made to impale the neuron. After successful intracellular penetration small currents are passed through the electrode in order to drive the ionic HRP into the cell. Once introduced, the HRP invades the neuron's dendritic arbor and often the first few millimeters of its axon. When the post-mortem tissue is treated by certain histochemical procedures the HRP turns reddish brown to black. Optimally, the neuron's cytomorphology is revealed to the finest detail; a picture which rivals the best results obtained with anatomical silver techniques, with the crucial advantage that the neuron's function is known.

Major Findings: Interneurons in the most superficial portion of the dorsal horn and trigeminal nucleus caudalis (lamina I) are of two general functional types: wide dynamic range (WDR) neurons and nociceptive specific (NS) neurons. The former respond to innocuous stimuli and also to noxious stimuli; their responses to noxious stimulation consist of a higher discharge frequency than that evoked by innocuous stimulation. Several lines of evidence indicate that WDR neurons participate in pain mechanisms. NS neurons respond only to noxious or nearly noxious stimulation. It has been inferred from several lines of evidence that WDR neurons are significantly larger than NS neurons. The lamina I

neurons that we have characterized and stained clearly support this hypothesis. WDR and NS type neurons are also found in the deeper laminae (IV and V) and here also we have found WDR neurons to have larger cell bodies and more extensive dendritic arbors than NS neurons.

The small interneurons of the substantia gelatinosa (SG) have received extensive cytomorphological analyses. The physiological properties of these cells, however, have only recently been described. We have found that one of the cytomorphologically defined SG cell types, the stalked cell, occurs as either a WDR neuron or as a NS neuron. Both types of stalked cells send their axons into lamina I. This evidence supports the hypothesis that stalked cells are interneurons that synapse on lamina I neurons. The presently available evidence indicates that the dendritic arbors of NS stalked cells extend ventrally only into the superficial one-third of the SG. WDR stalked cells, on the other hand, emit dendrites which tranverse the entire thickness of the SG. This observation supports other data that suggest that primary afferent terminal arborizations of different functional types of neurons terminate in a laminated fashion within the dorsal horn.

Another cytomorphologically defined SG cell type, the islet cell, is of particular interest because of its presumed inhibitory function. The islet cells we have studied have been of two general types. The first type is essentially similar to a WDR neuron. It receives input from high threshold mechanoreceptors as well as from low threshold mechanoreceptors. The second type appears to receive input only from low threshold mechanoreceptive primary afferents.

Significance to Biomedical Research and the Program of the Institute: The neural mechanisms which generate pain sensation are poorly understood. The design of rational therapies for various forms of pain requires an accurate picture of the neural systems involved in signaling pain. This project is a contribution towards a better understanding of the neural circuitry of that part of the spinal cord involved in pain mechanisms.

Proposed Course: This project is continuing with the goal of enlarging our sample of the different cell types in lamina I and the substantia gelatinosa. Our cytomorphological analysis will be extended to the electron microscope level.

2. Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00020-13 NA

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Anatomical studies of the main sensory and spinal trigeminal nuclei

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gobel, Stephen	Chief, NEA Section	NIDR NA
Coinvestigator:	Falls, William	Staff Fellow	NIDR NA
Other:	Wharran, Gordon	Biological Lab Tech	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neurocytology and Experimental Anatomy Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.05	0.9	1.15

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is concerned with the synaptic connections of the sensory root of the trigeminal nerve and the morphology of the neurons which comprise the main sensory and spinal trigeminal nuclei. These studies employ electron microscopy and the Golgi method. The objectives of these studies are to delineate trigeminal pain-temperature pathways and to broaden our understanding of oral-facial sensation.

1. Project Description

Objectives: During the past year, our anatomical studies have had three objectives. First, to demonstrate that the lower end of the spinal trigeminal nucleus, i.e., nucleus caudalis, together with the rest of the gray matter of the caudal medulla is organized like the gray matter of the spinal cord. Second, to utilize the Golgi method to examine the laminar distribution of the terminal axonal arbors of primary trigeminal neurons in the substantia gelatinosa and to study those neurons in layer IV which send their dendrites into the substantia gelatinosa. Third, to examine in detail in electron miscroscopical studies the transsynaptic degenerative changes in the neurons of the substantia gelatinosa following primary deafferentation consequent to peripheral nerve injuries such as tooth pulpotomies.

Methods employed: The Golgi method is used to deposit silver salts inside of neurons in order to visualize their axonal and dendritic branches in terms of their laminar distribution, branching pattern, patterns of dendritic spine and axonal ending distribution. Electron microscopy is used to examine synaptic connections and the morphology of the axons and dendrites of neurons in normal and experimental situations.

Major Findings: The lower end of the spinal trigeminal nucleus has been traditionally viewed as a separate nucleus, i.e., nucleus caudalis, while the rest of the gray matter of the caudal medulla medial to it has been simply divided into the medial and lateral reticular formation. Several findings in our laboratory have suggested that this view of the caudal medulla is inadequate since it does not recognize several patterns of organization which parallel those in the spinal cord. The more important parallels include a similarity in the distribution of trigeminothalamic projection neurons in the medulla and spinothalamic neurons in the spinal cord, direct continuity between nucleus caudalis and the dorsal horn in the spinal cord, the presence of similar neuronal cell types in the substantia gelatinosa in both locations, and the similar morphological appearance of the primary endings in the substantia gelatinosa in both locations in terms of their size, shape, internal axoplasmic features and synaptic connections. In view of these similarities, a new lamination scheme has been proposed in which the gray matter of the caudal medulla has been divided into dorsal and ventral horns. Nucleus caudalis comprises the head of the dorsal horn and is made up of four layers. What has been called the lateral reticular formation comprises the neck of the dorsal horn and is divided into layers V and VI. The medial reticular formation comprises the ventral horn and is divided into layers VII and VIII. The laminar boundaries in this lamination scheme can be recognized in all of the major mammals currently used as experimental models including rodents, the opossum, the cat, the Rhesus monkey as well as in man. The laminar boundaries

and cytological characteristics of the eight layers in the dorsal and ventral horns of the medulla correspond closely to those of Rexed's first eight layers in the spinal cord and provide for the first time a sound anatomical basis for relating anatomical and physiological observations in the two locations.

During the past three years, three major kinds of neurons have been identified and the morphology and laminar distribution of their axons and dendrites characterized in Golgi studies of the substantia gelatinosa (layers I-III). These include the projection neurons of layer I and two kinds of interneurons in layers II and III. The other major contributor to the neuropil of the substantia gelatinosa are four kinds of neurons in layer IV which send some of their dendrites into the substantia gelatinosa. These include smooth and spiny varieties of pyramidal neurons, spindle-shaped neurons and neurons with beaded dendrites. These layer IV neurons may be important in trigeminal pain pathways because they may convey noxious inputs from primary axons in the substantia gelatinosa to neurons in layers V and VI which respond to noxious stimuli.

Two major findings have come from a series of experiments which assess the effects of tooth pulpotomies on the neural circuitry of trigeminal pain pathways. First, the removal of tooth pulps results in the degeneration of the primary trigeminal neurons which innervate the affected teeth. Second, the loss of input from these primary neurons results in transsynaptic degenerative changes in second order neurons in the substantia gelatinosa. Two kinds of transsynaptic changes have been identified. Layer I neurons lose their organelles and disintegrate 14 days after pulpotomy. By 30-60 days after pulpotomy, many of the dendrites of the interneurons in layers II and III have become eroded by numerous small cavities which have formed inside their dendritic shafts. The cavities ultimately open to the intercellular space and the dendrites disintegrate. These changes result in an extensive disruption of trigeminal pain pathways through the substantia gelatinosa.

Significance to Biomedical Research and the Program of the Institute: The rationale for utilizing orofacial pain in diagnosing dental pathology, for providing anesthesia for dental procedures and for understanding the role of pain in reflex movement of the musculature of the head and neck is based on our knowledge of orofacial pain pathways. Our knowledge of much of these neural pathways today is fragmentary. Our neuroanatomical studies are aimed at establishing a more definitive circuit diagram of trigeminal pain pathways.

The trigeminal nerve is involved in a host of chronic pain states which include trigeminal neuralgia (tic douloureux), glossodynia (burning tongue) and other facial neuralgias. Explanations of these pain states usually involve mechanisms related to pathology of the peripheral nerve. However many of the symptoms of tic douloureux, for example, cannot be

explained without considering synaptic circuitry in the central nervous system. For example, why are only the maxillary and mandibular divisions involved i.e., only those divisions supplying the teeth? Why do the most innocuous stimuli trigger the pain episode? Is the loss of teeth somehow involved in a disruption of synaptic connections in the spinal trigeminal nucleus?

Our studies on the synaptic circuitry of the dorsal horn of the medulla have provided us with a base of knowledge from which we can now design experiments to explore these questions. Our tooth pulp extirpation experiments have demonstrated that the loss of neural input from the teeth consequent to dental disease results not only in the degeneration of primary trigeminal neurons but in major disruptions of the neural circuitry of trigeminal pain pathways through the dorsal horn of the medulla since the neurons which receive primary input from the teeth also degenerate when this input is removed. These findings suggest that transsynaptic degenerative changes may be taking place at several additional sites in the brain e.g., the thalamus and cerebral cortex. It is quite possible that disruption in the synaptic circuitry of several sites in trigeminal pain pathways may be a factor in some orofacial neuralgias in which severely painful episodes are triggered by nonnoxious stimuli in the absence of obvious pathology.

Proposed Course:

a) Neurocytological Studies

The Golgi method will be used to characterize the remaining cell types of layer IV during the coming year. In a second series of experiments, the roots of the trigeminal nerve and cervical spinal nerve will be severed. The cut central ends of these nerves will then be soaked in horseradish peroxidase. This protein will be picked up by the cut axons and transported centrally where it will fill the axons, and in so doing, outline their terminal axonal arborizations. These experiments will allow us to examine the morphology and laminar distribution of axonal endings of all of the different kinds of primary neurons with much greater precision than has been possible previously.

b) Experimental Anatomical Studies

The tooth pulp extirpation studies will be continued and extended to longer survival times. In addition, experiments with postoperative survival times of 7, 21, 45 days are planned in order to examine in a more detailed way the transsynaptic changes in second order neurons as well as the time course of these changes. The trigeminal and facial motor nuclei will be examined in order to determine whether transsynaptic degenerative changes can be demonstrated in neurons which do not receive direct monosynaptic input from primary trigeminal neurons innervating tooth pulps.

2. Publications

Gobel, S. and Binck, J.M.: Degenerative changes in primary trigeminal axons and in neurons in nucleus caudalis following tooth pulp extirpations in the cat. Brain Res. 132:347-354, 1977.

Gobel, S., Falls, W.M. and Hockfield, S.: The division of the dorsal and ventral horns of the mammalian caudal medulla into eight layers using anatomical criteria. In Anderson, D.J. and Matthews, B.M. (Eds.): Pain in the Trigeminal Region. Amsterdam, Elsevier/North Holland Biomedical Press, 1977, pp. 443-453.

Gobel, S. and Hockfield, S.: An anatomical analysis of the synaptic circuitry of layers I, II and III of trigeminal nucleus caudalis in the cat. In Anderson, D.J. and Matthews, B.M. (Eds.): Pain in the Trigeminal Region. Amsterdam, Elsevier/North Holland Biomedical Press, 1977, pp. 203-211.

Gobel, S.: Golgi studies of the neurons in layer I of the dorsal horn of the medulla (trigeminal nucleus caudalis). J. Comp. Neur., 180:375-394, 1978.

Gobel, S.: Golgi studies of the neurons in layer II of the dorsal horn of the medulla (trigeminal nucleus caudalis). J. Comp. Neur., 180:395-414, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 DE 00166-03 NA

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Anatomical Studies of Postnatal Maturation in the Spinal Trigeminal Nucleus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Falls, William	Staff Fellow	NIDR NA
Other:	Gobel, Stephen	Chief, NEA Section	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neurocytology and Experimental Anatomy Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.15	0.85	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies examine the postnatal development of neurons in trigeminal pain pathways in the dorsal horn of the medulla (trigeminal nucleus caudalis) with Golgi and electron microscopic techniques. They compare in newborn kittens the maturity of the two major interneurons in the substantia gelatinosa (the stalked cell and the islet cell) with their adult counterparts. They follow the developmental sequences in the formation of the dendritic and axonal arbors of both stalked cells and the islet cells and examine how these interneurons eliminate excess dendrites as well as how they provide additional plasma membrane for their elongating dendrites and axons. These studies also determine which components of the synaptic circuitry in trigeminal pain pathways are present at birth and which develop postnatally.

1. Project Description

Objectives: The overall objective of these anatomical studies is to examine in newborn kittens the postnatal development of neurons in the substantia gelatinosa of the dorsal horn of the medulla. The objectives over the past three years have been fourfold. First, we wanted to assess the maturity of the two major interneurons in layers II and III (the stalked cell and the islet cell) by comparisons with their adult counterparts. Second, we wanted to follow the developmental sequences in the formation of the dendritic and axonal arbors of these interneurons. Third, we sought to uncover basic cellular mechanisms underlying how stalked cells and islet cells eliminate excess dendrites as well as how they provide additional plasma membrane for their elongating dendrites and axons. Fourth, we wanted to find out how tightly these interneurons are hooked into the neural circuitry of trigeminal pain pathways at birth.

Methods employed: These studies utilize the Golgi method and electron microscopy.

Major Findings: At birth the two major interneurons of the substantia gelatinosa can be found in three stages of dendritic development. These include immature forms, in which a large number of short dendrites radiate out in all directions from the cell body; intermediate forms, in which some dendrites have begun to elongate in specific directions while numerous other dendrites are in the process of beading up and disintegrating; and relatively mature forms, where the adult dendritic branching pattern is already established although the dendrites are only one fourth their adult length. Once the dendrites have assumed their preferred direction of orientation, the adult dendritic arbor evolves by continued postnatal lengthening and branching of existing dendrites.

The unmyelinated axons of these interneurons do not begin to develop until the dendrites assume their preferred direction of orientation. The lack of fully developed axons at birth indicates that stalked cells and islet cells and the circuits they mediate are only partially connected to layer I projection neurons.

At birth layers II and III have already reached their adult mediolateral width and the neuropil is already compact. Space for elongating neuronal and astrocytic processes is made available as a result of the disintegration of many dendrites and by an overall fourfold increase in the rostrocaudal length of the dorsal horn of the medulla during postnatal maturation.

At birth lengthening of the plasma membranes of elongating processes occurs as vesicles (addition vesicles) found in aggregates throughout

dendrites, unmyelinated axons and astrocytic processes fuse with and become incorporated into existing plasma membranes. The following sequence is responsible for disintegration of beaded dendrites. Within the beads neurotubules are lost and addition vesicles fuse with each other to form small cavities. These cavities continue to enlarge hollowing out the beads. The cavities ultimately open to the intercellular space as their membranes fuse with the plasma membrane of the beads. Finally, the beads disintegrate as their plasma membranes fragment. The thread-like segments between adjacent disintegrating beads shrivel until they ultimately disappear.

Primary axonal endings synapse on elongating dendrites of stalked cells and islet cells but do not synapse on disintegrating beaded dendrites. Disintegrating beaded dendrites receive synaptic inputs from non-primary sources but these inputs are not sufficient to insure their continued elongation.

Significance to Biomedical Research and Program of the Institute: We have found that although the dorsal horn of the medulla is one of the first regions in the brain to develop, the neural circuitry in local trigeminal pain pathways, which mediate reflex movement of the head and neck in response to noxious stimuli, is only partially formed at birth. This finding coupled with our knowledge of the neural circuitry of local trigeminal pain pathways in the adult provide us the opportunity to make some accurate predictions as to how this neural circuitry develops.

The finding that primary inputs are essential for the development of the dendrites of neurons in trigeminal pain pathways has allowed us to begin a series of studies in which the effects of the loss of primary inputs consequent to peripheral nerve injuries are being assessed. The finding that islet cell and stalked cell dendrites undergo cavitation following the loss of primary input in adult animals illustrates that peripheral nerve injuries result in disruption in the neural circuitry of pain pathways. This finding emphasizes that explanations of chronic pain states undoubtedly involve central nervous system mechanisms as well as peripheral mechanisms.

Proposed Course: With publication of the above studies we will terminate our investigations of the postnatal development of neurons in the substantia gelatinosa of the dorsal horn of the medulla. During the coming year we will evaluate the effects of tooth pulp extirpations in adult cats on the neural circuitry in the substantia gelatinosa. One part of this study will focus on transsynaptic degenerative changes in layer I projection neurons as well as stalked cells and islet cells. We will compare the process of degeneration in this experimental situation with that which occurs in the normal developmental situation. In addition, we also plan to use a combination Golgi-electron microscopic procedure on normal adult neurons in the substantia gelatinosa. The procedure will allow us to examine the morphology of individual Golgi

impregnated neurons at the light microscopic level and then evaluate their cytology and synaptic relations with the electron microscope. This study will show how these neurons are hooked into the neural circuitry of trigeminal pain pathways in the dorsal horn of the medulla.

2. Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE

PROJECT NUMBER (Do NOT use this space)

HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

Z01 DE 00207-02 NA

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Pharmacological Characterization of Axonal Endings in Trigeminal Nucleus Caudalis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Ruda, Maryann T.	Staff Fellow	NIDR NA
OTHER:	Gobel, Stephen	Chief, NEA Section	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH

Neurobiology and Anesthesiology Branch

SECTION

Neurocytology and Experimental Anatomy Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.35	0.95	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This study attempts to pharmacologically characterize axonal endings in the dorsal horn of the medulla (DHM). The methods employed include light and electron microscopic autoradiographic localization of labeled axonal endings in the DHM following application of tritiated [^{3}H] putative neurotransmitters onto the DHM. The transmitters to be investigated include serotonin, norepinephrine and GABA. Our approach relies on the ability of neurons which normally utilize a specific neurotransmitter to take up this [^{3}H] transmitter at their axonal endings. The purpose of these studies is to locate and morphologically characterize pharmacologically distinct axonal endings in the DHM which might be involved in trigeminal pain pathways.

1. Project Description:

Objectives: The purpose of these experiments is twofold: 1) to characterize the ultrastructural morphology of pharmacologically identified axonal endings in the substantia gelatinosa of the dorsal horn of the medulla (DHM); 2) to develop a pharmacologically characterized circuit diagram of potential synaptic interactions in the DHM. The neurotransmitters being investigated include serotonin (5HT), norepinephrine (NE), and gamma-aminobutyric acid (GABA). Data collection on the serotonergic endings in the DHM is completed and is now being prepared for publication. Data on NE and GABA terminals is presently being collected.

Methods Employed: Pharmacological characterization of axonal endings in our study relies on the ability of axonal endings which normally utilize a specific neurotransmitter, to take up this same neurotransmitter when it is exogenously applied in a radioactive form. This tritiated ([^{3}H]) transmitter can then be localized to specific axonal endings using the techniques of light and electron microscopic autoradiography. In our experiments, either [^{3}H] 5HT, NE or GABA was topically applied onto the DHM. Following aldehyde fixation of the tissue, the DHM was processed for light and EM autoradiography. Slides carrying 1μ and EM sections of the experimental tissue were coated with liquid nuclear emulsion, exposed in the dark for several weeks to months and subsequently treated with a photographic developer. This process results in the deposition of silver grains, which can be seen at both the light and EM level, over areas containing radioactivity.

Major Findings: Light microscopic analysis of the distribution of silver grains in 1μ sections of the DHM demonstrate the presence of [^{3}H] 5HT in layers I, II and III of the DHM. Silver grains are found both as individual grains and as aggregates composed of many individual grains. In comparing the distribution of grains in the three layers, layers I and II have about the same amount of single grains and aggregates while both of these decrease in layer III. No [^{3}H] 5HT labeled neuronal cell bodies nor labeled axon bundles have been found in the DHM.

Electron microscopic analysis of axonal endings labeled with [^{3}H] 5HT demonstrates two categories of morphologically distinguishable endings: dome-shaped endings which form a single synapse on dendrites; and scalloped endings which form multiple synapses on dendritic shafts and spines. Each category of ending can be further divided into several types based on morphological criteria which include size, shape and packing density of synaptic vesicles, axoplasmic density and the morphology and location of their synapses. Three types of labeled dome-shaped endings and four types of labeled scalloped endings have been identified. Two of the dome-shaped type endings are found in all three layers while one type is found only in layers II and III. Each type of scalloped ending is found in only one layer.

Since Golgi and horseradish peroxidase studies have shown that most of the dendrites in layer I are derived from layer 1 projection neurons, these experiments suggest a direct axodendritic serotonergic input onto layer I projection neurons. In addition, serotonergic inputs to layers II and III potentially synapse on the dendrites of interneurons in these layers which modulate the transfer of input from primary afferent terminals in these layers, to the projection neurons in layer I. These serotonergic inputs presumably represent descending pathways for the modulation of nociceptive information in the DHM.

Preliminary data on both the NE and GABA terminals indicate a differential pattern of afferent input to the DHM than that described for serotonin.

Significance to Biomedical Research and Program of the Institute: These experiments provide a model of the pharmacological circuitry of layers I, II and III of the dorsal horn of the medulla. The description of 5HT and NE axonal endings in the DHM is of particular significance since the activation of descending serotonergic and noradrenergic pathways are implicated in the production of analgesia. Our experiments demonstrate that the modulation of layer I nociceptive neurons can occur either through direct axodendritic serotonergic synapses on layer I projection neurons, or by input onto layer II and III interneurons. Our pharmacological circuit diagram is also applicable to the spinal cord dorsal horn since organization of both have been shown to be similar.

Proposed Course: The autoradiographic approach of radioactively tagging neurotransmitters for visualization at active sites is currently being extended to include norepinephrine and GABA. The study of 5HT terminals provided anatomical information on the potential modulatory influence of serotonin on trigeminothalamic projection neurons which respond to noxious stimuli. Information on the potential synaptic connections of NE and GABA in the DHM is important because of the role of NE in analgesia and that of GABA as an inhibitory transmitter. The long range goal of these experiments is to elucidate the pharmacological synaptic circuitry of pain pathways through the DHM.

2. Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER ZO1 DE 00248-01 NA

PERIOD COVERED October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Horseradish peroxidase studies of trigeminal pain pathways

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hockfield, Susan J.	Biologist	NIDR NA
OTHERS:	Gobel, Stephen	Chief, NEA Section	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neurocytology and Experimental Anatomy Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.1	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project examines the neural circuits involved in the appreciation of oral-facial sensations, particularly the circuits mediating pain and temperature sensations. Incoming sensory information may be modified by neuronal activity in many areas of the nervous system before being relayed to higher brain centers. Neuron marking techniques, in which a foreign protein (horseradish peroxidase) is picked up by axonal endings and carried back to their neuronal cell bodies, accurately demonstrate which neurons participate in a given neural circuit. Previous experiments in this study identified the neurons that relay incoming oral-facial sensations to higher brain centers. Current experiments will identify the neurons that can modify incoming sensory information and that may, in part, mediate analgesic and hyperalgesic states.

1. Project Description:

Objectives: The objective of this study is to identify the neurons in the central nervous system that project to the dorsal horn of the caudal medulla and may, in part, modify incoming sensory information from the face. Three general locations of these neurons will be analyzed: (1) within the main sensory and spinal trigeminal nuclei; (2) in areas of the brainstem outside of these trigeminal nuclei; and (3) in the cervical spinal cord.

Methods Employed: Horseradish peroxidase (HRP), a protein of about 40,000 molecular weight was microinjected into nucleus caudalis. This protein is picked up pinocytotically by axonal endings and transported retrogradely through the axon to the cell body of origin where it is sequestered in lysosomes. After the appropriate histochemical reaction, cells of origin which send their axons into nucleus caudalis can be clearly visualized in the brainstem.

Major Findings: The initial experiments of this project identified three locations of trigeminothalamic projection neurons in and adjacent to nucleus caudalis. These are: layer I, layer V, and an area just ventral to nucleus caudalis which represents the trigeminal component of the lateral cervical nucleus. The similarities between the morphology of nucleus caudalis and the spinal cord dorsal horn, and between the locations of trigeminothalamic neurons in nucleus caudalis and of spinothalamic neurons in the spinal cord dorsal horn, has led us to regard nucleus caudalis as the medullary continuation of the head of the dorsal horn of the spinal cord. It receives trigeminal sensory input in the same way that the dorsal horn of the spinal cord receives sensory input for the rest of the body.

In current experiments, by injecting horseradish peroxidase into the dorsal horn of the caudal medulla we can identify the sources of input to the medullary dorsal horn which are involved in modifying the output from this nucleus to higher brain centers.

Neurons in many locations in the spinal cord and brainstem send axons to the medullary dorsal horn. In the spinal cord, neurons that project to the caudal medulla are found predominantly in layers I, IV, V and VII. Contrary to classical descriptions, they are not found in layers II and III. In the caudal medulla, neurons in layers I, IV, V, and VII project to the opposite medullary dorsal horn. Neurons in the more rostral trigeminal sensory nuclei and the areas bordering them also send projections into the dorsal horn of the caudal medulla.

Neurons in the raphe nuclei, the locus coeruleus, and the periaqueductal gray matter also contain labeled neurons following injection

of horseradish peroxidase into the dorsal horn of the medulla. These comprise descending pathways and some may provide inhibitory input to nociceptive trigeminothalamic neurons in the caudal medulla.

Significance to Biomedical Research and Program of the Institute: Oral-facial pain is manifested frequently throughout the population in both acute and chronic states. Elucidation of the neural circuits mediating oral-facial pain can demonstrate how acute and chronic pain states occur and may suggest how they can be controlled. Recent evidence indicates that the mechanisms relating to analgesia such as drug therapy, acupuncture, and electrical stimulation of the skin, the dorsal columns, and the periaqueductal gray, may involve some of the circuits demonstrated in these studies. This project will show the neural pathways that are involved in the perception of pain and analgesia, and thereby may suggest new methods for the control of pain.

Proposed Course: During the next year, the analysis of sources of afferents to the dorsal horn of the caudal medulla will be concluded. The precise location and morphology of the neurons giving rise to these afferents will be determined which will allow for more rigorous anatomical delimitation of nuclear areas throughout the brainstem and spinal cord. Injections of horseradish peroxidase into the main sensory nucleus and nucleus oralis will demonstrate the neurons in the caudal medulla that modify the output of neurons in the rostral sensory nuclei, and will further our understanding of the organization of the dorsal horn of the caudal medulla.

2. Publications:

Hockfield, S. and Gobel, S.: Neurons in and near nucleus caudalis with long ascending projection axons demonstrated by retrograde labeling with horseradish peroxidase. Brain Research, 139: 333-339, 1978.

Report of the Clinical Investigations Branch
National Institute of Dental Research
Summary Statement FY 1978

The Clinical Investigations Branch is concerned with the study of factors influencing diagnosis of oral disorders from theoretical to descriptive analyses of known functional relationships.

The Branch is comprised of two sections: the Oral and Pharyngeal Development Section (OPD) under Dr. James Bosma, and the Diagnostic Methodology Section (DMS) headed by Dr. Richard Webber. These sections work largely independently . OPD emphasizes the study of form, development and function of oral and pharyngeal tissues through detailed anatomical description and physiological analyses coupled with psychophysical measurements. DMS approaches the study of oral disorders with more of a systems orientation which is grounded in image processing and information theory. Together the sections complement each other by providing a multidisciplinary approach to diagnostic problems of mutual interest.

A significant effort by OPD is directed toward description of the developmental anatomy of the mouth and pharynx of humans and homologous animals with particular emphasis on the basicranium. This work is usually pursued collaboratively with other institutions and is expressed in the form of atlases and detailed compendia of anatomical information.

A recent undertaking involves the study of mechanisms of taste both from the point of view of clinical deficits and developmental characteristics. This work involves sensory measurements elicited from controlled taste stimuli and is also expressed in terms of salivary biochemistry since salivary constituents play a significant role in the mediation of taste stimuli. Other work involves the study of oral and pharyngeal dysfunction as it relates to speech, respiration, and feeding.

DMS activities are balanced between the investigation of new bases for quantitatively classifying information of diagnostic interest and the demonstration of improved techniques via computer simulation and development of new diagnostic imaging systems. This work includes the development of a new way to describe two-dimensional shapes abstracted from biological material and a variety of image enhancement techniques based on extensions of information theory and three-dimensional representation of two-dimensional displays. This involves the development of new image processing algorithms designed to facilitate visual interpretation of radiographs and the creation of software which provides objective bases for quantifying morphological attributes of growth and development.

Hardware development is done largely in collaboration with other Federal agencies. Recent efforts have been directed toward the assembly

and testing of a prototype self-contained fluoroscopic device which promises to facilitate diagnostic research in a variety of dental applications. The theoretical bases underlying this and related developmental efforts were also explored in an open workshop. Of specific interest is the potential for on-line control of exposure geometry through the use of a feedback system in order to facilitate the detection of lesion changes from one examination to the next.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00065-07 CI

PERIOD COVERED
October July 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Development and Evaluation of Improved Dental Radiographic Systems with Emphasis on Factors Influencing Diagnostic Performance

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Webber, Richard L	Senior Dental Surgeon	CI	NIDR
COPI:	Wiebe, John D.	Sr. Asst. Dental Surgeon	IR	NIDR
OTHER:	Nagel, Roger N.	Senior Staff Fellow	CI	NIDR
COOP:	Rennie, John		NVL	Ft. Belvoir
	Farr, Mack		NVL	Ft. Belvoir
	Yin, Elo I.		NASA	
	Seltzer, Stephen		NBS	
	Wagner, Robert		BRH	FDA
	Gobel, John		RS	NIH
	Talbot, Tom		BEI	NIH

COOPERATING UNITS (if any)
National Bureau of Standards; Goddard Space Flight Center, NASA; Night Vision Laboratory, Ft. Belvoir; Bureau of Radiological Health, FDA.

LAB/BRANCH
Clinical Investigations Branch

SECTION
Diagnostic Methodology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.56	1.10	.46

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A prototype self-contained high-gain dental fluoroscope has been created from a microchannel plate image intensifier proximity coupled to a phosphor screen which is irradiated by a sealed isotopic source. A protocol has been approved to clinically test a suitable prototype to determine its relative utility and comparative dose with conventional radiographic techniques required for screening of surgical patients and endodontic procedures. The theoretical advantages of this approach are being analyzed from model systems simulated by computer. Of particular interest is the effect of scatter on image quality in dose-limited systems and the feasability of on-line feedback control of dental radiographic systems in general.

In vitro studies are also underway to determine the radiographic image quality necessary to perform specific diagnostic tasks. This information provides rational bases for designing improved x-ray systems.

NIDR CLASSIFICATION: 10400, 20400, 33400

Project Description

I. Objectives

The purpose of this project is to integrate theoretical and practical information currently available into prototype dental radiographic systems which can be optimized for specific diagnostic tasks. Emphasis is directed toward improving efficiency by reducing dose and turn-around time for applications requiring less than maximum information capacity.

II. Methods

Promising x-ray sources and image detector configurations are being developed and evaluated in collaboration with other Government agencies and private interests. The idea is to more rationally relate system elements to the diagnostic task to be accomplished.

Previous efforts were directed toward the evaluation of rod-anode sources which could be used either inside the mouth or outside depending on the application. An obvious extension of this work involves the use of sealed sources produced from ^{125}I to provide x-rays from inside the mouth as originally described by Henrikson. Although the relatively short half-life of this isotope precludes practical use of conventional detectors, this problem is minimized when a high-speed, variable-gain image intensifier is employed.

A radiographic system which couples an ^{125}I source to a micro-channel plate image intensifier has been developed in cooperation with scientists at the Laboratory for Solar Physics and Astrophysics, Goddard Space Flight Center, NASA; members of the Systems Development Technical Area, Night Vision Laboratory, U.S. Army; representatives of the Division of Electronic Products Bureau of Radiological Health, FDA; and an investigator representing the Center for Radiation Research, National Bureau of Standards. A special miniaturized shutter release mechanism has been developed by representatives of the Biomedical Engineering and Instrumentation Branch in consultation with the co-principal investigator to permit intraoral placement of the source. Dosimetry studies have been performed in collaboration with the Radiation Safety Branch, NIH, to assure relative safety in radiological applications of diagnostic interest.

A clinical protocol has been developed and approved to compare the prototype system with conventional no-screen film techniques routinely used for screening of dental patients prior to surgery and for implementing endodontic procedures.

Other work done in association with the Bureau of Radiological Health involves the analysis and evaluation of new filters and constant potential x-ray tubes intended for conventional extraoral applications. This effort involves modeling of the systems and determining the relative differences in exposure required to produce signals with comparable detectability. Other in vitro studies have been undertaken to determine the amount of scattered radiation reaching the detector plane for exposure geometries simulating intraoral and extraoral placement of the radiation source. This work involves depth-dose determinations obtained from a water phantom coupled with computer-derived estimates of the back-scatter factor.

The feasibility of using on-line feedback control mechanisms to stabilize exposure geometry is also being explored in association with the American Academy of Dental Radiology, the University of Connecticut School of Dentistry, and the Division of Electronic Products, Bureau of Radiological Health.

III. Major Findings

Theoretical analyses indicate that a fluoroscopic device using an isotopic source can significantly reduce the radiation burden associated with certain routine tasks which currently require the use of x-rays. A prototype system derived largely from "off the shelf" components confirms that these theoretical advantages can be realized in a practical fashion. This developmental effort has already demonstrated some practical limitations which must be taken into account in the design of a clinically useful device. These include the need for an effective source diameter of less than 0.2 mm, a format large enough to image an entire tooth, and characterization of the maximum number of noise equivalent quanta required to permit the dentist to reliably perform endodontic procedures.

Also found to be important is the choice of isotope. Henrikson showed that ^{125}I is ideal for imaging thin structures (teeth, two to three millimeters thick) but that energies approaching 40 keV are required for efficient radiography of thicker specimens. Other radioactive sources which are more penetrating and which have longer half-lives are being explored as a means for extending the performance of such a device to a variety of potential applications.

Other experiments have been performed which substantiate and clarify major findings cited in last year's report. These include a double blind clinical study of the effects of small amounts of photon fogging exposure on panoramic dental radiographs. As theoretically predicted film contrast associated with images of teeth was <u>increased</u> in spite of the associated loss of information produced by the decrease in signal-to-noise ratio. Conversely, the depth-dose studies cited above show

that significant amounts of scatter reach the film plane in dental radiographic procedures in the absence of prefogging exposure. This is true even when an allowance is made for absorption of back scatter. The clinical manifestations of this scatter appear to be minimized by the comparatively low detection efficiency of conventional (no-screen) film so that the signal-to-noise ratio is high in spite of the scatter. It is anticipated that the effects of scatter will become more significant as higher efficiency detectors find their way into dental radiographic systems. Efforts to determine the state of the art in source and detector technology applicable to on-line feedback control systems were manifest in an open workshop on this subject held on May 16 at the University of Connecticut. This meeting was jointly sponsored by the Academy of Dental Radiology, the National Institute of Dental Research, the Bureau of Radiological Health and the University of Connecticut. Information obtained at this meeting provides a broad basis for assessing and extending research in this area.

IV. Significance

These findings demonstrate the potential for improved performance made possible by the elimination of scatter, use of appropriate energies, manipulation of system nonlinearities, and the use of favorable exposure geometries.

V. Proposed Course

Future efforts will continue to emphasize the multidisciplinary approach described above. Collaborative efforts will be coordinated in ways which optimize the resources conveniently available from the many sources and agencies having interests in this area.

Publications:

Lieberman, J. E. and Webber, R. L.: Clinical evaluation of prototype intraoral source x-ray system. Oral Surg., Oral Med., Oral Path. (In Press)

Yin, L. I., Trombka, J. I., Seltzer, S. M., Webber, R. L., Rennie, J., and Farr, M. R.: The lixiscope. SPIE Conf. Proc. (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00158-04 CI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)
Cephalometric Description of Growth Processes Through the Use of Symmetric Axis Coding

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Webber, Richard L.	Senior Dental Surgeon	CI	NIDR
COPI:	Blum, Harry	Physical Scientist	LSM	DCRT
COPI:	Nagel, Roger N.	Senior Staff Fellow	CI	NIDR
OTHER:	None			
COOP:	None			

COOPERATING UNITS (if any)
DCRT, NIH

LAB/BRANCH
Clinical Investigations Branch

SECTION
Diagnostic Methodology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.07	1.05	1.02

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Any closed continuous plane curve can be expressed as an isomorphic transform which relates all points to a unique central description called the symmetric axis. Algorithms have been developed and implemented which permit a digital computer to reversibly transform data derived from cephalometric radiographs into such a description. When applied to the average shape of mandibles extracted from the Broadbent-Bolton series, axis points produce a characteristic shape which appears to be independent of age. Analysis of higher order relationships exhibited by the symmetric axis functions provides quantitative bases for characterizing the curvature of boundaries and segmentation of shape into mathematically tractable elements.

NIDR CLASSIFICATION: 41410, 41420, 43400

Project Description

I. Objectives

Biological forms characterized by growth develop in predictable ways which cannot be efficiently described using conventional geometric primatives, i.e., points and lines. An alternative geometry of form developed by Blum characterizes both two-dimensional and three-dimensional objects by means of a central, as opposed to peripheral, description. This geometry capitalizes on the redundancy of linear growth processes and therefore provides a meaningful way to describe biological shapes which are of interest in studies of growth and development of oral structures. The purpose of this investigation is to explore the utility of this new geometry in the description of biological shape, and to use it to study growth and other functionally constrained changes in biological shape.

II. Methods

Any closed continuous plane curve can be expressed as an isomorphic transform which relates all points to a unique central description called the symmetric axis. This axis has two components;

> The locus of center points of maximally inscribed discs, called the axis points, and the ordered collection of disc radii, called the radius function.

Algorithms have been implemented which permit a digital computer to transform a given two-dimensional figure into its unique symmetric axis representation and conversely reconstruct it from its symmetric axis description in a reversible fashion. Additional algorithms have been constructed to analyze the symmetric axis components and produce a set of shape descriptors. These descriptors provide a rich environment based on the symmetric axis which permits quantitative analysis of the biological shape under study. By comparing these shape descriptors obtained from biological structures at various stages of development, it is possible to identify and analyze developmental changes.

III. Major Findings

A preliminary investigation of several mandibles from the Broadbent Bolton series of sex-averaged mandibles has produced interesting results. Using nine mandibles which represent children at ages 1,3,...,17 it was found that the symmetric axis points had a characteristic shape which was preserved across the development from the one-year-old to the seventeen-year-old child. The axis points and the radius function were analyzed by computer and it was found that the shape of the mandible

could be subdivided into parts corresponding to the superior ramus, inferior ramus, and body. It was further possible to formulate a growth rate for each of these parts as well as the mandible considered as a whole. While these results are still at the preliminary stages, the early experiences with symmetric-axis-based analysis is considered very encouraging. Accordingly, work is in progress on new analysis algorithms to compute a variety of shape measures based on the properties of the symmetric-axis points and the radius function. These new properties are based on the analysis of the first, second, and third derivatives of the two symmetric-axis functions, and their joint occurrences. Theoretical analysis of these factors shows that it is possible to characterize the curvature of the boundary, segment the original object into parts, and produce a rich variety of shape descriptors which permit a quantitative object description.

IV. Significance

This project has produced interesting preliminary results, however it is still too early to determine the ultimate significance. This year the transformation algorithm has become available, and several test cases have been studied in our laboratory and by others on the N.I.H. campus. Investigations at the National Bureau of Standards have also used the transformation to describe microscopic fibers. Results of these early trials and theoretical analyses have led to the formulation of additional algorithms to be implemented on the computer. These new algorithms compute the feature measurements on biological objects as represented in the symmetric axis transform.

A reverse symmetric axis transform has also become available this year, and experiments are under way to determine the error, if any, between the original object and the reconstructed symmetric axis object. An estimate of this quantity is considered to be an important factor in evaluating the significance of the symmetric-axis approach to shape description and the analysis of growth and development.

V. Proposed Course

The development of new shape and feature analysis algorithms will continue, and a series of experiments on cephalometric data will be conducted. The currently available algorithms will be documented and released to the N.I.H. community in order to obtain a wider basis for evaluating the utility and significance of the symmetric axis approach to shape analysis.

Publications:

Blum, H. and Nagel, R. N.: A Symmetric Axis Basis for Object Recognition and Description. Pattern Recognition (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00211-02 CI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Enhancement of Diagnostic Images

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Webber, Richard L.	Senior Dental Surgeon	CI	NIDR
COPI:	Nagel, Roger N.	Senior Staff Fellow	CI	NIDR
OTHER:	None			

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Investigations Branch

SECTION
Diagnostic Methodology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.41	.60	.81

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project involves the creation, development, and testing of image processing techniques designed to improve diagnostic performance. Of continued interest is a computer implemented transformation which generates an isometric three-dimensional "pseudo-solid" from any two-dimensional array of grey levels. This redundant coding scheme permits an image to be viewed as a projection of an opaque solid whose shape is determined by the original distribution of grey levels. When applied to dental radiographs induced interproximal lesions were enhanced in a way which is relatively independent of the information capacity of the image. Projection angle of the simulated source of illumination and viewing geometry were found to be task-dependent and sufficiently independent to permit rational bases for optimization.

A new enhancement scheme derived from second and third order estimates of image redundancy is being explored as a possible basis for extending the principle of histogram equalization. This scheme is a logical extension of information theory which may aid in image abstraction as well as analysis.

NIDR CLASSIFICATION: 10400, 20400, 33400

Project Description

I. Objectives

This project is concerned with transformations which lead to improved diagnostic performance obtainable from images. Previous work has shown that image processing can improve diagnostic performance obtainable from dental radiographs when the task is not limited by the information capacity of the system. This work also demonstrated that environmentally familiar modes of display provide promising bases for extending the perceptual capabilities of the interpreter.

Current efforts involve further evaluation of the utility of this approach by determining the effects of reduced information capacity on the new class of image-enhancing transformations reported on last year, and the introduction of a totally new enhancement scheme based on second order extension of histogram equalization techniques.

II. Methods

A co-occurrence matrix of image grey-levels is used to determine the relative frequency of grey-level pairs in a digitized image containing information of diagnostic interest. Grey-level configurations are determined from adjacent picture elements expressed digitally. The relative rarity of grey-level configurations manifest between nearest elements expressed as a sum provides a basis for ranking paired grey-levels in terms of manifest redundancy. The result is an irreversible transformation having grey-levels determined by the second order spatial statistics of the original image. This technique can be used independently or as a pre-processing scheme for other enhancement methods.

Algorithms have been created to test this technique and others reported last year by introducing controlled amounts of image degradation (blurring, data sampling, etc.) in order to determine the extent to which enhancement is independent of information capacity. These algorithms are being developed to permit efficient display and compilation of psychophysical data for more rigorous analysis of diagnostic performance. The "pseudo-solid" algorithm described last year has been improved and rewritten to more efficiently use the capabilities of a PDP-1140 computer.

III. Major Findings

The large number of projection geometries and illumination angles made possible by the "pseudo-solid" transformation preclude comprehensive bases for rigorously testing of diagnostic performance obtainable from processed images. However, a limited number of pilot studies using performance measures derived from previous work suggest that this mode of display can enhance the detectability of induced interproximal lesions

from bitewing radiographs. Performance of this task appears to be influenced by both the apparent viewing angle and the direction of the simulated light source relative to the "pseudo-solid". These effects also behave as relatively independent factors in terms of their perceptual impact. As a result a desired perceptual effect may be obtained by manipulating either light angle or view angle independently.

When this transform is applied to comparable images subjected to image degradation (reduced information capacity), preliminary observations confirm that the enhancing effect is preserved in the presence of a four-fold reduction in information capacity. This assures that performance of this task is not limited by the information capacity of this system, and conversely, a reasonable potential exists for preserving diagnostic performance while reducing the total amount of information recorded.

The "pseudo-solid" and the new co-occurrence transformations applied to a variety of other images of diagnostic interest including nuclear medicine scans and ultrasonic displays, yield outputs which appear rich with easily perceived contours of potential interest. These transformations thus provide comprehensive bases for future research in this area.

IV. Significance

Preliminary findings based on image degradation studies confirm that image processing can be used to reduce the radiographic exposure required to perform diagnostic tasks of dental interest. The demonstrated independence of factors influencing the display of images enhanced via the "pseudo-solid" transform provides a rational basis for systematically optimizing performance.

V. Proposed Course

Further efforts to characterize diagnostic performance obtainable from these image enhancement techniques will involve evaluation by unbiased human interpreters of processed images associated with a variety of diagnostic tasks. The results will be objectively compared with findings obtained from unprocessed controls. A specific effort will be made to compare results obtained from simple isometric display with those produced by the more complex "pseudo-solid" transform in order to assess the perceptual importance of nonlinearities associated with geometric projection of opaque solids.

Publications:

Webber, R. L. and Nagel, R. N.: Image preprocessing as an aid to visual interpretation. IADMFR Proc. (1978) (In Press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00048-07 CI

PERIOD COVERED

October 1, 1977 to September 30, 1978

N01 DE 22401

TITLE OF PROJECT (80 characters or less)

Anatomical Studies of the Head

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bosma, James F.	Chief, Oral Pharyn.	OPD CI NIDR
COPI:	None		
OTHER:	Murayama, Diane	Illustrator	OPD CI NIDR
COOP:	Pierce, Robert	Acting Chaman of Department of Radiology, University of Illinois, Rockford, Illinois	
	Mainen, Michael	Private Practice, Minnesota	
	Bartner, Howard	Division of Research Services, MAPB	
	Munger, Bryce	Department of Anatomy, Pennsylvania State University School of Medicine at Hershey	
	Schön, Miguel	Department of Anatomy, the Johns Hopkins Medical Center	

COOPERATING UNITS (if any)

LAB/BRANCH

Clinical Investigations Branch

SECTION

Oral and Pharyngeal Development

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.50	.30	.20

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This is the ninth year of anatomical studies of the face, pharynx and cranium of the human fetus at term. The Cranium of the Newborn Human: An Atlas of Tomographic and Anatomic Sections under the authorship of Robert Pierce, Michael Mainen and James Bosma was published by the U.S. Goverment Printing Office in September, 1977. This printing was technically unsatisfactory. The volume is being printed again, by Government Printing Office arrangement. A general anatomy book, The Head of the Human Infant, is in preparation of text matching the completed illustrations. Portions of the book are now in critical review. An international Symposium on Development of the Basicranium, held June 23-25, 1975, was edited by J. Bosma and published by the U.S. Government Printing Office; publication date: December, 1977. In a collaborative contract project, Dr. Bryce Munger is in the fifth year of a study of development of oral mucosal sensory receptors in the rhesus. Standard site samples from caesarean fetuses, neonates and postnates are examined histologically by light and electron microscopy. Journal publications of this project continue to appear.

NIDR CLASSIFICATION: 41240, 41250, 41450, 42210, 44210

PHS-6040
(Rev. 10-76)

Project Description

I. Objectives

To provide a base of descriptive anatomy of the human infant and child for studies of normal development and for definition of malformations.

II. Methods

Routines of dissection and illustration which are standard in the field of anatomy, supplemented by specialized radiography, particularly tomography and cineradiography.

Arrangement, conduct and publication editing of symposia on strategic items of anatomical development.

III. Major Findings

The Cranium of the Newborn Human: An Atlas of Tomographic and Anatomic Sections. This includes matching tomograms and anatomical sections at 27 levels in the coronal plane, 17 levels in the transverse plane and 25 levels in the sagittal plane. For printing technical reasons, this volume is being reprinted, by arrangement of the Government Printing Office.

The Head of the Newborn Infant, a general descriptive anatomy, is in the process of expansion of its original manuscript, with 30 additional illustrations, to total of approximately 220, and corresponding enlargement of text. Its critique is simultaneously in progress; 214 of its approximately 220 illustrations are completed.

A Symposium on Development of the Basicranium edited by J. Bosma, was published by the U.S. Government Printing Office in December, 1977.

As a separate collaboration under contract sponsorship, with Bryce Munger of the Department of Anatomy, University of Pennsylvania Medical Center at Hershey, has continued histological studies of oral sensory receptors in developmental perspectives. Mucosa from standard sites on lip, tongue and palate is sampled in caesarean fetal, infantile and later immature and mature monkeys. The development of specific receptors is compared per age and per site, to afford reference description data of this maturation in normals. This work has been reported in part (see bibliography); full report will be in a monograph which has been invited to publication in Advances in Anatomy, Histology and Embryology.

IV. Significance

These studies provide information for description of normal development of anatomical form and structure and of the surface sensory

resource of the mouth. Such information is requisite for description of abnormalities of development, including those originating in the peripheral structures and those which evolve secondarily in these structures as effects of central neurological disease.

The Symposium on Development of the Basicranium is strategic in relating recent advances in information and understanding in the fields of developmental anatomy and developmental biochemistry of connective tissue.

V. Proposed Course

Publication of the anatomical description of the infant head and of the oral mucosal sensory development. Publication of monograph by M. Munger, Patterns of Maturation of Primate Oral Sensory Receptors.

Publications:

Bosma, J. F. (Ed.): Symposium on Development of the Basicranium. U.S Dept. of Health, Education, and Welfare, Publ. No. (NIH) 76-989, Wash., D.C., Govt. Print. Off., 1977, 700 pp.

Publications from Contract Project at University of Pennsylvania, in Hershey:

Biemesderfer, D., Munger, B. L., Binck, J., and Dubner, R.: The Pilo-Ruffini complex: a non-sinus hair and associated slowly-adapting mechanoreceptor in primate facial skin. Brain Res., 142: 197-222, 1978

Munger, B. L.: Neural epithelial interactions in sensory receptors. J. Invest. Dermatol. 69: 27-40, 1977

Munger, B. L. and Ide, C.: The ultrastructural basis for the identification of primate golgi-mazzoni corpuscles (abstract). Anat. Rec. 190: 487, 1978

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00079-05 CI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Conferences and Publication of Symposia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bosma, James F.	Chief, Oral Pharyn.	OPD	CI	NIDR
COPI:	None				
OTHER:	Sapperstein, Selma	Secretary	OPD	CI	NIDR
COOP:	Fogarty International Center				
	National Institute of Child Health and Human Development				

COOPERATING UNITS (if any)

Fogarty International Center
National Institute of Child Health and Human Development

LAB/BRANCH
Clinical Investigations Branch

SECTION
Oral and Pharyngeal Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.15	.10	.05

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Symposium on Development of Upper Respiratory Anatomy and Function, in Relation to Sudden Infant Death, James F. Bosma and Jane Showacre Eds.

NIDR CLASSIFICATION: 41240, 41323, 41440

Project Description

I. Objectives

To design, arrange and sponsor interdisciplinary exchanges on topics relevant to the description of oral and pharyngeal performance areas in man and other mammals.

II. Methods

These conferences and publications have been carried out in standard routine, with early invitations. The complete conference recording and typescription, the presentations and each item of discussion are edited by participants. All publication manuscripts are prepared in retrospection of the conference. Publication of the 1973 and 1976 Symposia have been by the U.S. Government Printing Office.

III. Major Findings

The Symposium on Development of Upper Respiratory Anatomy and Function, in Relation to Sudden Infant Death is in print, the Government Printing Office, since July, 1976.

IV. Significance

Information and understanding of human and general mammalian development is being dispersed among the specialized disciplines of developmental anatomy, physiology and psychology. This dispersion is the basic rationale of our Symposia. Persons from these disciplines are selected for their expertise, their perspicacity and their capacity to communicate within the broad milieu of biology and clinical work.

V. Proposed Course

This series of symposia on development of oral and pharyngeal performances is concluded. A prospective Fifth Symposium on Oral Sensation and Perception: Development of Feeding has been cancelled.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00124-05 CI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Oral Reflexes of the Infant

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Weiffenbach, James M.	Research Psychologist	OPD	CI	NIDR
COPI:	None				
OTHER:	Cowart, Beverly	Psychologist	OPD	CI	NIDR
	McCreary, Dawn	Technician	OPD	CI	NIDR

COOPERATING UNITS (if any)

Department of Pediatrics, National Naval Medical Center, Bethesda, Maryland

LAB/BRANCH
Clinical Investigations Branch

SECTION
Oral and Pharyngeal Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.95	.50	.35

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The discrete elicited lateral tongue movement of the human newborn first described under this project continues to be used in psychophysical studies of oral sensation. The transverse tongue reflex described previously can be elicited by separate 5 microliter drops of fluid. The reflex adapts to repeated fluid elicitations. Responding returns when suprathreshold glucose or sodium chloride is added to the stimulus fluid after adaptation to water. This experimental sequence thus indicates taste discrimination. Current studies address the question of the relation of responsivity to sweet and salty tasting stimuli.

NIDR CLASSIFICATION: 105BO, 41323

Project Description

I. Objectives

This project explores the oral sensory competence of the newborn human infant. A novel reflex technique, developed as part of this project, is equally well suited to measuring the local sensitivity of the tongue to touch and to taste stimuli. Early project investigations were primarily concerned with developmental changes in touch sensitivity. Major emphasis is now on studies of the sensory mechanisms underlying neonatal taste experience. Since infants are sensitive to taste at birth it is possible, as it has been contended on the basis of animal studies, that early taste experience shapes later food preferences and dietary habits.

II. Methods

An easily observed reflex displacement of the tip of the newborn's tongue toward the side of stimulation serves as the basis for assessing the sensitivity of the tongue to both touch and taste. For touch stimulation, the frequence of response occurrence is a function of stimulus intensity. An analogous relation of strength of taste stimulation (concentration of tastant in solution) to frequency of response is obtained providing that responding to the solvent alone has been first reduced by repeated stimulation. Post-adaptation increments in responding above that found with continued water stimulation have been demonstrated for both glucose and sodium chloride test solutions.

III. Major Findings

Infants adapted to glucose and subsequently stimulated with saline respond significantly more frequently during the post-adaptation test phase than infants similarly adapted to glucose and tested with glucose. This separation of sensitivity of the neonate's tongue to sweet and salty tastes is analogous to findings arising from studies of cross quality adaptation of adult humans. It is in marked contrast to the ambiguous findings of previous studies of neonates that have attempted to measure salt sensitivity by preference rather than reflex responding.

IV. Significance

Studies of neonatal taste in the human, and of the normal development of taste in infancy and childhood will make an important contribution to understanding the development of those destructive food intake patterns that have been implicated in the etiology of caries. Investigations of the impact of "normal" variation in the pattern of early taste experience on the development of taste sensitivity and food preferences will permit the design of effective strategies for modifying eating habits.

V. Proposed Course

Studies of the sensory mechanisms of taste reception in the human neonate will continue. The lateral tongue reflex technique will be used to further characterize the mechanisms underlying differential responding to sweet and salty solutions and to explore the nature of taste adaptation in the neonate.

Publications:

Weiffenbach, J. M.: The Development of Sweet Preference: Proceeding "Sweeteners and Dental Caries", Eds. Shaw, J. H. and Roussos, G. G., Sp. Supp. Feeding, Weight and Obesity Abstracts, 1978, pp. 75-91.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00181-03 CI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Postnatal Development of the Rat Skull

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bosma, James F.	Chief, Oral Pharyn.	OPD	CI	NIDR
COPI:	None				
OTHER:	Murayama, Diane	Illustrator	OPD	CI	NIDR
	Sapperstein, Selma	Secretary	OPD	CI	NIDR
COOP:	Baer, Melvyn J.	Department of Orthodontics, School of Dentistry University of Michigan			
	Ackerman, James L.	Department of Orthodontics, Philadelphia Children's Hospital, School of Dental Medicine, University of Pennsylvania			

COOPERATING UNITS (if any)

Extramural Programs, National Library of Medicine

LAB/BRANCH
Clinical Investigations Branch

SECTION
Oral and Pharyngeal Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.70	.30	.40

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The cranium and mandible of the rat are observed by drawings and photographs at successive postnatal ages and, in a separate series, by alizarian vital staining and photographs of sections. This combination of methods affords comprehensive demonstration of the development of individual bones, of skeletal composites and of the skull as a whole.

This process of review of this volume by comparative developmental anatomists continues. We hope to receive their criticisms and, correspondly, adapt the publication prior to October 1, 1978.

NIDR CLASSIFICATION: 41240

Project Description

I. Objectives

Normative demonstration of postnatal development of the individual bones and teeth and the cranial composite and mandible in the rat.

II. Methods

Rat skulls of days 1, 15 and 60 were dissected; after papainization the individual bones were photographed and drawn.

Rats at 8 postnatal ages were injected alternately with Alizarin Red S or Alizarin Blue BB and sacrificed at intervals after last injection. Defleshed crania and mandibles were mounted in Bioplast and sawn in sections. Patterns of development in separate areas of each bone and tooth and of development of composites of related bones and teeth were worked out by comparison study of specimens stained on various calenders.

III. Major Findings

Normative illustrations are prepared of the cranium and its bones and of the mandible. These constitute the basis of a textual description of the postnatal development of the rat skull on an individual bone and a regional basis.

The patterns of apposition and resorption at the margin and in the interior of the various portions of individual bones and teeth have been defined and further illustrated by schematics. The study materiel, as prepared for publication, combines color photographs (in 120 projection slides) with tracings of the corresponding anatomical transsections. Each atlas unit includes a projection slide, derived illustrations and textual commentary.

This volume also includes a review of vital marking of skeleton by alizarin and other stains which are chemically incorporated into ossifying tissues.

IV. Significance

This is the first comprehensive description of skull development of a laboratory mammal in this extent of age range, in this inclusion of entire skull and all teeth, and in this extent of detail. The descriptions and associated interpretations are significant contributions to information and understanding of normal mammalian development. And they are of normative value in definition of variations of rat skull and tooth development.

V. Proposed Course

Currently, this volume is under review by three qualified developmental anatomists. We anticipate completion of reviews, and related adaptations of the volume, by October 1, 1978. Prospective publication of book: Postnatal Development of the Rat Skull in 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00182-03 CI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Studies of Sensorimotor Impairments of the Mouth and Pharynx

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bosma, James F.	Chief, Oral Pharyn.	OPD	CI	NIDR
COPI:	Weiffenbach, James M.	Research Psychologist	OPD	CI	NIDR
	Wolf, Robert O.	Dental Investigator	OPD	CI	NIDR
	Geoffrey, Virginia	Speech Specialist	OPD	CI	NIDR
OTHER:	Murayama, Diane	Illustrator	OPD	CI	NIDR
	Sapperstein, Selma	Secretary	OPD	CI	NIDR
	Koback, Ann	Secretary	OPD	CI	NIDR

COOP: National Institute of Child Health and Human Development, Intramural Program
National Institute of Neurological and Communicative Disorders and Stroke, Intramural Program
Diagnostic Radiology Branch, Clinical Center
Department of Radiology, The Johns Hopkins Medical Center

COOPERATING UNITS (if any)

LAB/BRANCH
Clinical Investigations Branch

SECTION
Oral and Pharyngeal Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.65	.90	.75

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with oral and/or pharyngeal impairments of feeding, either of neurological disease or following cancer surgery, are studied, particularly by cineradiography. Patients with sensory impairments of mouth and/or pharynx are studied by sensory testing, cinephotography, cineradiography and speech analysis.

NIDR CLASSIFICATION: 41440, 42310, 42400

-402

Project Description

I. Objectives

The study of clinical variations which are associated with primary abnormalities or surgical or inflammatory modifications of oral and pharyngeal structure and form, or with peripheral sensory or motor impairments, or with abnormalities of the representations of the oral and pharyngeal performances in the brain.

These studies are for the purposes of definition of the anatomical abnormalities and their physiological correlates. And, for the purpose of devising and applying therapies for disabilities of feeding, of speech, and of subjective sensibilities.

II. Methods

Standard procedures of cinephotography, and cineradiography, as well as acoustical and other physiological recordings are used in the study of primary motor or coordinative disorders of the mouth and pharynx. The regional performances of airway maintenance and posture, of speech and other respiratory actions, and of feeding are described in infants, children and adults. Selected patients having sensory disorders of the oral and pharyngeal regions are also evaluated by sensory testing. Currently, we are concerned with the relation of primary peripheral sensory abnormalities to disabilities of feeding and/or speech. Patients who have surgical excision for cancer are studied as examples of the modification of sensory input by ablation.

III. Major Findings

The disorders of position, of speech and of feeding which result from impairment of peripheral motor performance can be meaningfully described by currently available clinical methods. It is possible to predict certain disabilities of progressive motor disorders. In some circumstances, the impairments are accessible to prevention or to specific therapy.

Certain cleft lip and palate infants have asymmetrical deficiencies and distortions of sensory elicitations of the lips and tongue.

When portions of the pharynx and mouth sensory resource are surgically removed, the functions of this region which depend for elicitation and guidance upon the sensory resource are impaired, as may be expected. But we have recently demonstrated that some of the residual sensory resources may be exaggerated in effect in the subject's experience and also in the elicitation of particular responses such as cough.

IV. Significance

Adaptations of certain diagnostic methods, including sensory evaluation, have enhanced the definition of sensorimotor disorders. This has contributed to the recognition of related elements of separate disorders, to anticipation of some impairments in progressive diseases, and to therapy of disorders which are shown to have common pathogenic mechanisms. Examples:

1. The further identification of motor coordinative disorder has lead to recognition of a common medication in different patients.

2. The identification of mechanism of feeding disabilities in familial dysautonomia subjects has lead to routine control of content and circumstance of oral feeding, or to utilization of nasoesophageal or gastrostomy tube feeding which may prevent aspiration and/or malnutrition.

3. The identification of mild or moderate degree of pharyngeal dysphagia in certain forms of progressive motor impairment, such as muscular dystrophy or myotonia, can occasion patient instruction, by which a severe, life-threatening episode of choking may be avoided.

4. Recognition of particular sensory deficiencies and distortions in post-surgical subjects facilitates their rehabilitation therapy. Potentially, surgery of the pharyngeal and oral area may be specifically designed to diminish impairments of local sensory inputs.

V. Proposed Course

Clinical studies of subjects having the above categories of disorders will continue with current methods.

Our studies of sensorimotor pharyngeal impairments are currently under accumulation into a general volume, under authorship of James F. Bosma and Martin Donner, of the Department of Radiology, the Johns Hopkins Medical Center.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 DE 00212-02 CI

PERIOD COVERED
October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Taste and Its Disorders

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Wolf, Robert O.	Dentist	OPD CI NIDR
PI:	Weiffenbach, James M.	Research Psychol.	OPD CI NIDR
OTHER:	Cowart, Beverly	Psychologist	OPD CI NIDR
	Bennett, Kathleen	Technician	OPD CI NIDR
	Arensen, Stacy	NSF/American University Program	

COOPERATING UNITS (if any)

Hypertension - Endocrine Branch, NHLBI
John B. Pierce Foundation Laboratory, New Haven, CT

LAB/BRANCH
Clinical Investigations Branch

SECTION
Oral and Pharyngcal Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.20	.90	.30

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The selection or development of appropriate psychophysical methods for the separate measurement of the various aspects of taste perception is a primary and continuing concern of this project. Laboratory studies of taste are directed both toward defining the normal developmental course of taste perception and understanding the mechanisms by which specified circumstances or disease entities have an impact upon it. Clinical studies examine the taste perception of individuals reporting abnormalities of taste experience such as deficiency or absence of taste, persistent taste, or exaggeration of perception of certain taste qualities.

NIDR CLASSIFICATION: 105B0, 41323

Project Description

I. Objectives

This project employs carefully selected modern psychophysical methods to define the relation of taste perception to oral and to systemic disease. Cases in which disorders of taste perception are the presenting symptom are also studied.

II. Methods

Currently, a "sip and spit" procedure is employed to provide whole mouth stimulation with room temperature fluids. All tasting is done with an immediately prior rinse of double-distilled water. Numerous variant methods for determining the order of stimulus presentation and calculating measures of perception have been rejected in favor of a combination of the "up-down" procedure for determining stimulus presentation order with the forced-choice procedure for measuring response.

III. Major Finding

Two major findings requiring further investigation have emerged. First, measurement of both detection and recognition thresholds in our subjects yields much greater overlap than would be expected from comparisons of thresholds measured on independent groups. Second, direct scaling of taste pleasantness of sucrose yields a much higher than expected frequency of "sweet aversion" in normal young adults.

IV. Significance

The human taste system that is the focus of this program is a major oral sensory system. Through its role in the control of ingestion, taste affects both systemic nutrition and oral exposure to cariogenic substances. The taste system is liable to dysfunctions which are both debilitating and poorly understood. Variation in taste as a correlate of systemic disease suggests that it may serve as a diagnostic indicator.

V. Proposed Course

Current laboratory studies of individuals with oral disease and their matched controls will be expanded to include measurement of suprathreshold aspects of oral experience. Studies comparing detection and identification of taste stimuli using completely analogous techniques will explore the relation of these two functions, in the absence of method differences. Studies of modified taste perception in the circumstance of head and neck X-radiation and of endocrine disorder exemplify our pattern of cross-institute collaborations.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 DE 00217-02 CI

PERIOD COVERED

October 1, 1977 to September 30, 1978

TITLE OF PROJECT (80 characters or less)

Salivary Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Wolf, Robert O.	Dentist	OPD	CI	NIDR
OTHER:	Wright, William E.	Dentist	IR		NIDR
	Hubbard, Van Saxton	Physician			NLAMD
	Graykowski, Edward	Physician and Dentist		LOM	NIDR
	Hamosh, Margit	Biochemist	Dept. Anatomy, Georgetown Univ.		
	Bennett, Kathleen	Technician	OPD	CI	NIDR

COOPERATING UNITS (if any)

S. Mishkin, Physician, Division of Gastroenterology, Royal Victoria Hospital, Montreal, Canada; T. C. Pomeroy, Physician, ROB, NCI; J.E. Galleli, Pharmacist, PHARM., CC

LAB/BRANCH

Clinical Investigations Branch

SECTION

Oral and Pharyngeal Development

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.90	.70	.20

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☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Extrinsic (i.e. saliva) and intrinsic (e.g. serum salivary isoamylase) salivary gland products, mechanisms of production and control constitute the subject matter of this study. Part A. Human (primarily parotid) saliva chemical constituents and mechanisms are studied as related to health and disease. An emphasis has been placed on extrinsic secretion study of lysozyme. amylase, lipase, electrolyte content and flow rate in both normal and disease states such as Sjögren's syndrome and apthous stomatitis. Part B. The intrinsic secretion of salivary isoamylase in serum of cystic fibrosis of the pancreas and Sjögren's syndrome as well as normal monkey serum and monkey gland homogenates are being studied. Diagnostic application and an understanding of the mechanisms of hyperamylasemia are being sought. Part C. Fluoride is being studied as a sialogogue for alleviating and preventing xerostomic symptoms.

NIDR CLASSIFICATION: 34200, 34400

Project Description

I. Objectives

Part A This part of the project involves the study of saliva and associated extrinsic glandular function in normal and diseased states. It includes analysis of normal and abnormal salivary constituents and their effect on oral mucosa. Emphasis has been placed on enzymes and proteins. Parotid salivary lysozyme is currently being studied to find out if the parotid saliva lysozyme level changes during the ulcerative and non-ulcerative stages of apthous stomatitis (AS). An AS population is currently being maintained by the Institute. This study parallels and was suggested by a report that in herpes labialis a decrease in salivary lysozyme occurred during the ulcerative stage.

Part B Salivary glands also function intrinsically by secreting products internally. This process is analogous to and sometimes referred to as an endocrine function. This portion of the project involves the study of this function and associated products with emphasis on the effects of salivary gland trauma (e.g., infection, hypoxemia, manipulation, duct ligation or obstruction), in order to identify the mechanisms involved.

Part C The mechanisms of stimulation and control of both intrinsic and extrinsic salivary gland secretions are the concern of this part of the project. Subjective clinical evidence indicates that a fluoride (NaF) mouth rinse can alleviate xerostomic symptoms accompanying head and neck radiation by acting as a unique salivation stimulus. The current study attempts to demonstrate, by the use of a standard fluoride mouth rinse, decreased xerostomic symptoms in patients (head and neck radiation, Sjögren's syndrome, drug therapy) expected to have "dry mouth".

II. Methods

Part A Five consecutive two minute parotid saliva samples are collected from (AS) patient during and after ulceration. Lysozyme activity is assessed by radial diffusion in agar gel containing substrate.

Part B Serum and urine pancreatic and salivary amylases are measured by first separating the isoenzymes by polyacrylamide gel electrophoresis (PAGE) and then determining the enzyme activity in serial slices of the gel. Various clinical populations (cystic fibrosis, Sjögren's syndrome, and heart failure) are being assessed.

Part C The fluoride mouth rinse was first shown to be a sialogogue. Administration of a course of fluoride mouth rinse or placebo is being done in a double-blind manner and the efficacy is being determined longitudinally by time-flow analysis of total extrinsic secretions as well as by subjective questionnaire.

III. Major Findings

Part A All five patients completed to date have demonstrated lower lysozyme levels during ulceration by 29%. Comparable time points (e.g. midway during ulcer-free period) during ulceration or during non-ulceration have not been compared.

Part B Our previous studies of diseased patients have shown a mean decrease of serum salivary or pancreatic isoamylases which is dependent on the disease (i.e. Sjögren's syndrome or cystic fibrosis of the pancreas (CFP) respectively). In both diseases there were observed several individuals with exceptionally high isoamylase values. In CFP those exceptions were patients with functioning pancreata. Now, three years later, two of the three of those high serum pancreatic isoamylases CFP patients have diminished serum pancreatic isoamylase values.

Hyperamylasemia has been found coincident with hypoxemia in the absence of pancreatitis or renal failure. The low blood oxygen tension observed in specific pathologies (e.g. myocardial infarction, cervical fracture dislocation. cranial contusion) with evidence of deprivation of oxygen via the blood sypply to major salivary glands or pancreas was reflected in increased serum salivary or pancreatic isoamylases, strongly suggesting that the trauma of hypoxia increases the involved gland amylase intrinsic secretion.

Part C Testing of 20 normal subjects has shown that the fluoride mouth rinse is a sialogogue. Patients with Hodgkins disease, Sjögren's syndrome, and patients therapeutically irradiated in the head and neck region (six subjects in each category) are currently under study.

IV. Significance

Part A The etiology of AS is unknown. The decrease of lysozyme in parotid saliva during ulceration in AS patients is a finding without a clear explanation. The source of the change is somewhat removed from the ulcer site. The finding parallels that of herpes labialis. Histologically the glandular source of lysozyme is thought to be the ductal cells, however macrophages and monocytes are known to constantly produce lysozyme. The cytoplasm of granulocytes also contain lysozyme and the explanation might well be in the dynamics of these cell populations and salivary gland membrane permeabilities.

Part B The transition of high serum pancreatic amylase in CFP to lower levels of enzyme substantiates our previous hypothesis that the high serum pancreatic amylase patients are undergoing pancreatic trauma parallel to the much more rapid increase/decrease observed in radiation of the parotid glands.

Hypoxemia is a heretofore unknown cause of increased intrinsic secretion of salivary and pancreatic amylase and may play a role in hyperamylasemia where the normal serum pancreatic-to-salivary amylase ratio is not changed. The serum salivary amylase decrease in Sjogren's syndrome is in keeping with salivary gland involvement in this disease.

Part C If administered fluoride is found to be an effective sialogogue in the initial six radiated patients, and if the finding is verified in our expanded series we will have identified a unique sialogogue of considerable clinical application which will alleviate the dry mouth symptom most universally complained about among these patients. This in turn should diminish the caries experience associated with radiation therapy involving salivary glands by direct action of the fluoride on the teeth as well as by stimulating saliva.

V. Proposed Course

Part A Complete a series of 12 AS patients. If a statistically significant difference is found it will be necessary to assess the temporal dynamics of lysozyme with blood parameters and with all pertinent clinical observations. We have previously reported lysozyme isoenzymes in parotid saliva and will study the isolysozymes of the AS population with and without ulcerations and as compared to a normal population.

Part B Evaluation of the oxygen tension and isoamylases in anterior cervical veins during systemic hypoxemia and following oral surgical manipulations may conclusively show that hypoxemia increases serum amylase selectively with respect to source and that the increase in serum amylase reported with oral manipulations is due to vasoconstriction in major salivary gland areas.

We plan to continue the study of populations commonly experiencing hypo- or hyperamylasemia and to follow up those few cases having abnormally high serum pancreatic and serum salivary values in comparison with the cystic fibrosis and Sjogren's syndrome populations already studied. More efficient methods of serum isoamylase quantitation assessment are being developed.

(1) Electrophoretic separation on cellulose acetate, though not possessing the resolving power of PAGE, can potentially give percentage result by simple densitometric analysis.

(2) Purification and utilization of a specific salivary amylase inhibitor from wheat as a non-electrophoretic assay system.

Part C If orally administered fluoride is found to be an effective sialogogue in the initial small series, an expanded series of experiments will be undertaken to discover the action of fluoride supplementation by seeking similarities with related ions and by seeking evidence of ion specific receptors. Exploration of total salivary flow and other possible factors in the maintenance of gland function will continue.

Publications:

Jam, I., Shoham, M., Wolf, R. O. and Mishkin, S.: Elevated serum amylase activity in the absence of clinical pancreatic or salivary gland disease. Amer. J. of Gastroenterol. (In Press)

Gratwhol, A. A., Moutsopoulos, H. M., Chused, T. M., Akizuki, A., Wolf, R. O., Sweet, J. B. and Deisseroth, A.: Sjögren's-type syndrome after allogenic bone-marrow transplant. Ann. of Intern. Med. 87(6): 703-706, Dec. 1977

Hubbard, V. S., de Saint Agnese, P. A. and Wolf, R. O.: Letter to the Editor: Acute pancreatitis in children. J. Pediat. 92(4): 685-686, 1978

Henkin, R. I., Lippoldt, R. E., Bilstad, J., Lum, C. K. L., Wolf, R. O. and Edelhock, H.: Fractionation of human parotid saliva. J. Biol. Chem. (In Press)

Boehm-Truitt, M., Harrison, E., Wolf, R. O. and Notkins, A. L. Radioimmunoassay for human salivary amylase. Analytical Biochem. 85: 476-487, 1978

GERL and the Golgi apparatus, and the neurosecretory granules form directly from the Golgi saccules. Comparable studies of other secretory cells will be carried out to determine if similar structural and functional alterations in these organelles can be induced by physiological or pharmacological stimulation.

In addition to the progress made on the individual research projects, as reviewed above, the past year has also witnessed a number of significant events for the Laboratory as a whole. In March of this year, the Third International Symposium on Tooth Enamel was held in Washington, D.C. Drs. John D. Termine and Marie Nylen served as organizers of the Symposium, and their efforts contributed greatly to the success of the meeting. Additionally, the efforts of the secretarial staff of the Laboratory, particularly Mrs. Patricia Youmans, played an essential role in the organization and smooth running of the conference. Several members of the Laboratory were honored this past year for their contributions to the NIH research effort and for significant advances in their own research field. Dr. E.D. Eanes was elected as a Fellow of the American Association for the Advancement of Science, and also received the NIH Director's Award for his studies of calcium phosphate chemistry and mineralization processes. Mrs. Betty Ho was awarded the NIH Merit Award for her contribution to the Laboratory's research program. Dr. Arthur R. Hand received the Basic Research in Oral Science Award of the International Association for Dental Research for studies of salivary gland structure and function.

Two significant personnel changes also occurred during this last fiscal year. Mrs. Elaine Lenk joined the Laboratory as a NIH Visiting Associate; she will collaborate actively with the rest of the Laboratory members, as well as with investigators from other NIDR laboratories, providing expertise in all aspects of electron microscopy. It is anticipated that this arrangement will significantly benefit the Laboratory's research efforts, and will foster closer cooperation and increased collaboration with the other NIDR laboratories. Secondly, a premanent successor to the position of Laboratory Chief was appointed in June of this year. Dr. Arthur R. Hand, who succeeded Drs. E.D. Eanes and J.D. Termine as Acting Chief, and served in that capacity for 11 months, was selected as the new Chief.