PART IV

NATIONAL INSTITUTE OF DENTAL RESEARCH ANNUAL REPORT

Intramural Research

Fiscal Year
October 1, 1979 to September 30, 1980

Dental Research Data Officer
National Institute of Dental Research
National Institutes of Health

PART IV

NATIONAL INSTITUTE OF DENTAL RESEARCH

ANNUAL REPORT

INTRAMURAL RESEARCH

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Compiled By

Dental Research Data Officer

National Institute of Dental Research

National Institutes of Health

Bethesda, Maryland

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Annual Report
Clinical Investigations and Patient Care Branch
National Institute of Dental Research
1980

The Clinical Investigations and Patient Care Branch conducts research related to the diagnosis, prevention, and treatment of oral and dental diseases. It also provides support and facilities for clinical research programs of other Branches and Laboratories within the Institute. In addition, the Branch offers consultation on diagnosis and treatment to Clinical Center patients. Preventive dental care and restorative treatment are offered to selected medically compromised patients.

The Branch, previously known as the Clinical Dental Services Section, has as its major goal the development of programs in which the dental care for the unique patient population of the Clinical Center is integrated not only with the conduct of clinical research, but also with the training of clinicians and other dental staff in the delivery of care and in the methodology of clinical research.

In order for the Branch to perform efficiently and effectively, the objectives of the different functions must be defined and communicated. Also, the staffing of the Branch, its administrative structure, and clinical activities must be brought in concert with the objectives. It is equally important that the physical plant of the Branch be designed and equipped in such a way that the objectives can be fulfilled.

The objectives are presently being defined in detail and preliminary changes are under way to meet the various requirements listed above. Principal among these is a need for the total renovation of the dental clinic which is almost thirty years old. This need has been recognized by the Clinical Center and according to the current construction schedule, the renovation of the dental clinic and the preparation of new laboratories, offices, and conference facilities should be completed by the spring of 1983.

As of this date, preliminary plans have been developed for the renovation of the clinic and the utilization of the office/laboratory space. In the meantime, steps have been taken to upgrade the present equipment and modernize the operation of the existing clinic. Further, through conferences with the other institutes, major progress has been made toward defining the role and responsibilities of the clinic as far as patient care is concerned. Regrettably, our oral surgeon, Dr. J. Sweet, a long-time staff member of the Institute and its most active clinical dental researcher, left the Branch at the end of the year.

The staff of the Branch has been expanded as a first step toward enabling it to better reach its objectives and meet its obligations. The developments which are currently under way or planned should make it possible for the Branch to make substantial progress toward its objectives in the year to come.

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 00213-04 IR

PERIOD COVERED
October 1, 1979 - September 30, 1980 CT 0060123

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

Sweet, James B.	Dental Director	IR	NIDR
Folio, John	Clinical Dental Ser Sect	IR	NIDR
Gracely, Richard H.	Research Psychologist	NA	NIDR
Greenberg, Harold A.	Consultant		NIH
Madero, James	Clin Care Cons Psychologist		NIMH
Macynski, Alice A.	Clinical Nurse (General)	IR	NIDR
Butler, Donald P.	Consultant		NIH

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 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.35	.30	.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.

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 3 months, 12 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 3, 6, 12 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 22 subjects have been accepted for the study. Seven have completed their final two year evaluations. No results are yet available due to the small number of completed cases.

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:

Discontinue accepting new patients to the study until the new staff oral surgeon reviews the protocol. If the oral surgeon is interested in continuing the study under his guidance, new patients will be accepted. A minimum of 25 cases should be completed for the study.

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 00241-03-IR

PERIOD COVERED
October 1, 1979 - September 30, 1980 CT 0060124

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

Sweet, James B.	Dental Director	IR	NIDR
Macynski, Alice A.	Clinical Nurse	IR	NIDR
Butler, Donald P.	Consultant		NIH

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 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.5	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.

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 othr 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 last patient on the study was completed on July 7, 1980. The data is presently being processed for analysis. The final results should be available shortly.

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:

The project was completed clinically on July 7, 1980. The processed data should be completed by mid-July. Final analysis will then be made and if significant results are obtained a manuscript will be presented for publication.

Report of the Microbial Systematics Section

National Institute of Dental Research

The Microbial Systematics Section 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; the Veteran's Administration and numerous academic microbiologists, strain data are being entered into the data bank which provides such services as: data on specific organisms and/or groups of organisms, location of 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 established. These files provide 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.

Extensive files of descriptions of filamentous and pleomorphic organisms are being assembled. The files cover all the described types of Mycobacteria, blend into the Nocardia, then through the Actinomycetes (especially a unique set on oral isolates), and finally, Bacterionema. An extensive cooperative study has been initiated to study the oral pleomorphic bacteria (many of which are associated with disease). The study will provide a standard set of well characterized bacteria for the Dental Research community. The data from this study will be incorporated into the files on pleomorphic organisms. These files are 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.

Other files on non-filamentous oral organisms (streptococci, lactobacilli, veillionella, etc.) are being constructed to study correlations among caries activity, phenetic span of characters, serology, source of isolation, and host descriptions.

One of the long term goals in establishing all these 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 worders for use.

PUBLICATIONS:

Wayne, L.G., Krichevsky, E.J., Love, L.L., Johnson. R., and Krichevsky, M.I.: Taxonomic probability Matrix for use with slowly growing mycobacteria. Int. J. Syst. Bacteriol. 28: 528-538, 1980.

Krichevsky, M.I., Walczak, C.A., Rogosa, M., and Johnson, R.: Interchange of abbreviations and full generic names in computers. Int. J. Syst. Bacteriol. 28:585-593, 1980.

Daggett, P.M., Krichevsky, M.I., Rogosa, M., Corliss, J.O., and Girolami, J.P.: Method for Coding data on protozoa strains for computers. J. Protozoa., In press.

Walczak, C.A., and Krichevsky, M.I.: Computer methods for describing groups of binary data: Preliminary summary and editing of data. Int. J. Syst. Bacteriol., In press.

Walczak, C.A., and Krichevsky, M.I.: Computer methods for describing groupd from binary data: Modification of numerical taxonomy programs to increase flexibility. Int. J. Syst. Bacteriol., 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 000044-10 ODIR

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Handling of Microbial Strains Information by Computers

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

Krichevsky, Micah I.	Research Chemist	ODIR	NIDR
Love, Leslie L.	Information Specialist	ODIR	NIDR

COOPERATING UNITS (if any)

Please see addendum

LAB/BRANCH

SECTION
Microbial Systematics Section

INSTITUTE AND LOCATION
National Institute of Dental Research

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	1.00	.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)

Microbial strain data are being entered into a data bank which provides 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 and 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.

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 00250-03 ODIR

PERIOD COVERED
October 1, 1979 to September 30, 1980

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

Walczak, Cynthia A.	Computer Programmer	ODIR	NIDR
Krichevsky, Micah I.	Research Chemist	ODIR	NIDR
Mercer, Paula	Computer Programmer	ODIR	NIDR

COOPERATING UNITS (if any)

LAB/BRANCH

SECTION
Microbial Systematics Section

INSTITUTE AND LOCATION
National Institute of Dental Research

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.00	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)

A feature frequency analysis program has been written which tabulates features for groups of organisms and the uniqueness of each feature for each group. Since grouping of organisms can be based on any logic, the uniqueness of a feature for organisms for a specific disease may be determined. Ranking the features by their mean deviations measures their ability to efficiently partition the groups. We have developed an algorithm for displaying the level of redundancy of features. The programs aid the microbiologist in choosing the "best" set of features useful in identification by probabilities.

Algorithms are being developed and tested for aiding in numerical taxonomy of feature by strain matrices too large to be analyzed by existing programs. Both segmentation and heuristic approaches are being investigated.

Computer graphic algorithms are being tested to aid microbiologists in visualizing individual similarities as well as hierarchical groups memberships among strains.

Annual Report of the Laboratory of Developmental Biology & Anomalies
National Institute of Dental Research

The major efforts in the Laboratory of Developmental Biology and Anomalies are on the prevention and treatment of inherited and acquired defects in oral-facial development. These projects include studies on the development of facial tissues, the possible roles of environmental agents and of genetic factors in oral-facial malformations, connective tissue formation and the interaction of normal as well as tumor cells with matrix proteins.

Several changes in personnel have occurred in the Laboratory. Dr. Robert Pratt, Chief, Craniofacial Development Section, LDBA, and a Principal Investigator in this Laboratory (Z01 DE 00149-06 DB) has accepted a position in the National Institute of Environmental Health Sciences in North Carolina. Dr. Pratt will continue to study environmental and genetic factors that effect the developing fetus when at the NIEHS. Dr. George R. Martin has become the Principal Investigator on Contract N01 DE 52452 with the Yerkes Primate Center.

Dr. Larry Paglia (Principal Investigator on Z01 DE 00025-13 DB) has left to assume a position in the Department of Biochemistry, Uniform Services University of the Health Sciences. Dr. Mark Sobel who carried out many of the recombinant DNA experiments on the structure of collagen genes while in the Laboratory of Molecular Biology, NCI, has joined the LDBA and taken over the study of the regulation of gene activity during development.

Dr. Hugh Varner is now the Principal Investigator on Project Z01 DE 00253-03 dealing with cartilage development. Ms. Barbara Corcoran, Chemist, with 17 years service at the NIDR has transferred to the Consumer Products Safety Commission. Mr. Joseph Wilczek, Biologist, with 8 years service at the NIDR has transferred to the Food and Drug Administration. Dr. Pamela Gehron Robey completed her doctorate at Catholic University and has left to assume postdoctoral training at the NIAMDD, NIH, with Dr. E. Neufeld.

Craniofacial Development Section

Neural Crest - Avian neural crest cells are pluripotential and will differentiate into a variety of cell types as a result of "inductive" interactions with other tissue. In culture, we have noticed that neural crest cells differentiate into melanocytes in media with calf serum and into cholinergic nerves in media with horse serum. We have assumed that these sera contain factors that are able to direct the differentiation of neural crest cells. Using a sensitive assay for pigmentation as an index of its activity during its purification from serum, we have isolated a protein in nearly homogeneous form that induces the neural crest cells to form melanocytes. Further purification and characterization are under way as is the preparation of antibody to the protein for investigating the origin and importance of the protein in developing tissues.

Teratogen Assays - Since teratogen testing in animals is complicated, slow and expensive, we have developed *in vitro* assays for detecting teratogens, using differentiating neural crest and limb bud cells. In culture, under defined conditions, these cells differentiate into various cell types including chondrocytes, melanocytes and cholinergic nerves. Most teratogens blocked the differentiation of the cells while nonteratogenic compounds were without effect. However, thalidomide did not react positively in these systems. Now we have found that serum from monkeys administered thalidomide inhibits the ability of limb bud mesenchyme cells to differentiate into chondrocytes. These studies suggest that the teratogenic activity of thalidomide is due to a metabolite which interferes with limb development. Further, it is likely that metabolites of drugs with teratogenic potential can be detected in primate serum with the cell culture assays.

Palatal Development - Two environmental agents causing craniofacial anomalies are under study. Jervine is a plant alkaloid that induces cleft lip and palate in developing sheep and cattle which graze on the plant, Veratrum californicus. Now we have found that certain strains of mice show similar sensitivity and also that cultured epithelial cells are altered by the compound.

TCDD is a notoriously dangerous compound of the dioxin class which among other actions causes cleft palate when administered to sensitive strains of mice. Fetal tissue contains a cytoplasmic macromolecule which binds TCDD. We have found that mouse strains sensitive to the teratogenic action of the drug contain higher levels of fetal cytoplasmic ligands than do resistant strains.

Mesenchymal cells from human palate have been cultured in serum-free media supplemented with hormones. These cells show an absolute dependence on epidermal growth factor for proliferation. These cells are much more sensitive than fibroblasts to the inhibitory action of glucocorticoids indicating that they are one of the prime targets for steroid induced malformations.

Connective Tissue Section

Interaction of Environmental and Genetic Factors in Birth Defects - Mutant mice have been identified with defects in development. The CMD (Cartilage Matrix Deficient) mouse is a dwarfed mouse. Our studies have shown that this mouse has abnormal cartilage which lacks the cartilage specific proteoglycan. The defect appears to be due to a failure to synthesize the protein portion of the proteoglycan, since the synthesis of glycosaminoglycan chains in the presence of xyloside was normal. The DMM (Disproportionate Micro Melia) mouse has a similar appearance. However, our studies show that this mouse mutant fails to synthesize normal amounts of type II collagen. These mutants should prove valuable in studies on the synthesis structure and function of cartilage. New methods involving recombinant DNA will be applied to studying the molecular basis of these disorders.

The ER (Repeated Epilation) mouse was originally reported to be born lacking an oral cavity. Subsequent studies here showed that the mucosal surfaces of the mouth had fused. Similarly, the apposing epithelial surfaces of limbs and trunk were also found to be fused. Now we have found that this mutant lacks a 28,000 dalton protein present in normal mouse epidermis. This protein is rich in histidine and believed to participate in the formation of keratin filaments. Normal keratin filaments are not observed in the ER mouse and the epidermis does not keratinze. The lack of the histidine rich protein probably blocks keratinization and accounts for the fusion of epithelial surfaces.

Previous studies suggested that genetic and environmental factors showed a strong synergism where both altered a similar step in development. For example Brachymorphic mice have a defect in proteoglycan synthesis. They are unusually sensitive to the teratogenic effects of glucocorticoids which are known to have inhibitory action on proteoglycan synthesis but they show normal responses to other teratogens such as vitamin A and hydroxyurea. In contrast, OEL mice are highly sensitive to vitamin A but not to glucocorticoids. By determing the interaction of genes with enviromental agents it should be possible to identify the number of sensitive steps in the development of an organ and their biochemical nature.

Cell Attachment - Previous studies have shown that fibronectin binds fibroblasts and certain other cells to collagen. Recently we have found that the interaction of fibronectin with collagen during fibrillogenesis decreases the rate of formation of collagen fibers while increasing their size. These results suggest that fibronectin may regulate fibril diameter in developing tissues. Interestingly, it has been observed that fibronectin inhibits the degradation of collagen by collagenase. It is possible that the binding of fibronectin to collagen blocks the access of the enzyme. These interactions suggest that the coordinated production of degradative enzymes would be required for the removal of collagen.

Recent studies have shown that chondrocytes require a protein factor for attachment that is different from fibronectin. This factor which we have named chondronectin is present in serum, cartilage and the vitreous humor, but not other tissues. Now we have purified serum chondronectin to homogeneity. The factor is an 180,000 dalton protein with disulfide linked chains of about 80-90,000 daltons. Immunological studies demonstrate that chondronectin and fibronectin show no cross-reaction. Antibody to serum chondronectin blocks the spontaneous attachment of chondrocytes to collagenous substrate indicating that the factor produced by chondrocytes and that present in serum are closely related.

Previously we isolated a large glycoprotein, laminin, from the basement membrane produced by the EHS tumor. Immunohistochemical studies showed that laminin was present in normal basement membranes also. Now we have found that laminin is the specific attachment protein for epithelial cells. Further it binds to type IV but not to other collagens. These studies indicate that epithelial cells use laminin to attach to basement membrane collagen. The epithelial cell-laminin-basement membrane collagen interaction is specific and defines the molecular basis for the histological distribution of these cells.

Interestingly metastatic cells derived from a fibrosarcoma have been found to produce and utilize laminin for attachment. Presumably this allows the metastatic cells to pass from one compartment to another during their spread.

Chemotaxis - Progress has been made in characterizing a small substance, produced by tumor cells that inhibits the chemotactic response of phagocytic cells. The antichemotactic factor is less than 400 daltons, is inactivated by chymotrypsin and contains equal amounts of tyrosine and glycine plus an as yet unidentified substance. This factor has been shown to decrease the number of receptors in the phagocyte cell membrane for chemotactic peptides as well as the methylation reactions involved in chemotaxis. The production of this peptide may account for the decreased resistance to infection caused by tumors. The peptide may be useful in suppressing inflammatory conditions.

The chemotaxis of connective tissue cells is under study. Fibronectin has been found to be chemotactic for fibroblasts. Furthermore, a 160,000 dalton fragment of fibronectin containing the cell attachment domain contains the chemotactic activity. Further cleaveage of the peptide inactivates it. These studies suggest that serum fibronectin and fragments of fibronectin may attract fibroblasts into wounded areas to initiate repair and that the chemotactic signal can be ended by proteolytic action.

Recent studies indicate that smooth muscle cells as well as fibroblasts are strongly attracted to the platelet derived growth factor (PDGF). PDGF is active in chemotaxis at very low levels while other growth factors show little or no chemotactic activity. PDGF may play a dual role in fibrotic reactions by attracting cells and then stimulating their proliferation.

Ahumada, G.G., Rennard, S.I., Figueroa, A.A., and Silver, M.H.: Fibronectin in rat heart: distribution during development and quantitative comparison of possible sites of synthesis.
J. Cell Biol.

Berman, M.D., Waggoner, J.G., Foidart, J.M., and Kleinman, H.K.: Attachment to collagen by isolated hepatocytes from rats with induced hepatic fibrosis.
J. Lab. Clin. Med., 95: 660-671, 1980

Binderman, I. and Reddi, A.H.: Phosphate-dependent mineralization of cartilage and bone.
Calcified Tissue Research. In press, 1980

Brown, K.S.: Vestibular anomalies as a marker in clefting: Discussion. In Melnick, M. and Bixler, D. (Eds.): Etiology of Facial Clefting. New York, Alan R. Liss. In press, 1980

Brown, K.S.: Genetics of clefting in the mouse: Discussion.
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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 00149-06 DB

PERIOD COVERED
October 1, 1979 - September 30, 1980

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

Pratt, Robert M. Jr.	Research Chemist	DB NIDR
Cannon, Frances B.	Bio Lab Tech (Micro)	DB NIDR
Dencker, Lennart	Guest Worker	DB NIDR
Horigan, Elizabeth A.	Research Biologist	DB NIDR
Leyshon, Webster C.	Biologist	DB NIDR
Mosley, General L. Jr.	Bio Lab Tech (Animal)	DB NIDR
Silver, Michael	Postdoctoral Fellow	DB NIDR
Sim, Russell	Visiting Associate	DB NIDR
Yoneda, Toshiyuki	Visiting Associate	DB NIDR

COOPERATING UNITS (if any)

Emory University

LAB/BRANCH
Laboratory of Developmental Biology & Anomalies

SECTION
Craniofacial Development Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
9.30	5.00	4.30

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 understand the regulation of cellular proliferation and differentiation in craniofacial tissues. At present we are (1) refining an in vitro method for screening teratogens, (2) developing methods to culture craniofacial tissues and cells and (3) determining the biochemical and hormonal factors that regulate palatal development.

I. Teratogen Screening

Animal tests used for determining possible teratogenic activity of a wide variety of chemicals are time consuming and expensive. We have previously developed a rapid in vitro screening assay for teratogens using differentiating neural crest and limb mesenchyme cells. The only teratogen tested that did not react positively in this system was thalidomide, presumably due to its insolubility and spontaneous decomposition in solution. We have administered thalidomide to pregnant Rhesus monkeys during its teratogenically sensitive period and taken serum at various intervals thereafter. In preliminary studies, we have found that serum from treated monkeys demonstrates inhibitory effects on limb mesenchyme cell chondrogenesis in culture. These results suggest that a metabolite of thalidomide, only formed in sensitive species, is the active teratogen which appears to directly affect limb chondrogenesis.

Horigan, Dencker, Mosley

II. Palatal Development

Clefts of the primary and secondary palates are common and serious birth defects of interest to the NIDR. Normal palatal development involves growth, elevation and fusion of the palatal processes. An alteration in any one of these processes could lead to cleft lip and palate.

The steroidal plant alkaloid, Jervine, has been found to be teratogenic in a number of animals including the A/J and C57BL/6J mouse (also see Brown Z01 DE 00024-14 DB). A high percentage of cleft lip fetuses are produced when Jervine is administered on day 8 of gestation. These results suggest that an early phase of development is affected, most likely migration of the primary mesenchyme or neural crest cells. Serum from Jervine-treated pregnant C57 mice interfers with the adhesive capacity of various cell types in culture and may be related to the etiology of Jervine-induced congenital anomalies.

Sim, Leyshon, Cannon

The distribution of fibronectin has previously been reported for the developing primary and secondary palatal shelves and now extended to the developing rodent limb. Recently, we have found that the distribution and presence of fibronectin in the palate in vitro is strongly influenced by epidermal growth factor. Fibronectin is distributed rather uniformily in the mesenchyme of the early limb bud, but appears in higher amounts in the aggregating mesenchyme cells destined to become chondrocytes. Once the developmental comittment to chondrogenesis has been made (i.e., synthesis of Type II collagen), the cells no longer appear to synthesize or accumulate fibronectin. These results suggest that fibronectin may play a key role in the early stages of conversion of mesenchyme cell to chondrocyte.

Silver, Cannon

Previous studies have demonstrated a correlation between the sensitivity to glucocorticoid-induced cleft palate and the levels of glucocorticoid receptors present in the developing facial region. We have examined dermal fibroblasts from a number of adult individuals with cleft palate and also cleft lip and palate tissue taken from the neonate at the time of surgical repair. We find a small, but significant, decrease in the level of glucocorticoid receptors in these affected cells. This suggests that the hormonal regulation of development may be altered in these individuals. To define the hormonal involvement in human palatal development, we have established human secondary palatal mesenchyme cells in culture. These cells are highly sensitive to the growth inhibitory effects of glucocorticoids, in contrast to adult human fibroblasts. These cells can be cultured in a serum-free hormone-supplemented medium and studies have shown that epidermal growth factor (EGF) is essential for their growth in culture. This supports our previous studies suggesting a role for EGF in development of the palatal epithelial as well as mesenchyme cells. The environmental pollutant TCDD is a potent dioxin and cleft palate producing teratogen in various sensitive strains of mice. We have found a correlation between the level of fetal cytoplasmic receptors for TCDD and sensitivity to TCDD induced cleft palate in the mouse. These results suggest that, like glucocorticoids, TCDD interfers with normal palatal development by a receptor dependent mechanism.

Yoneda, Dencker

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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 00257-02 DB

PERIOD COVERED

October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Differentiation of cranial neural crest cells

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

Greenberg, Judith H.	Sr. Staff Fellow	DB NIDR
Jerdan, Janice	Postdoctoral Fellow	DB NIDR
Seppa, Silja	Visiting Fellow	DB NIDR
Varner, Hugh H.	Postdoctoral Fellow	DB NIDR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Developmental Biology and Anomalies

SECTION

Craniofacial Development Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.20	2.20	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)

Cranial neural crest cells migrate in a highly specific manner and differentiate into many craniofacial structures. Neural crest cells in culture differentiate into two of their normal derivatives, neurons and melanocytes. As an approach toward understanding normal and abnormal neural crest development, we are (a) characterizing the factors which induce their differentiation, (b) examining chemotactic responses which may direct their migration, and (c) studying their interactions with extracellular matrix molecules.

I. Characterization of Factors which Affect Differentiation of Neural Crest Cells in Culture

Chick cranial neural crest cells cultured in medium containing horse serum differentiate into neurons containing choline acetyltransferase activity, whereas in fetal calf serum or calf serum (CS) they differentiate into melanocytes. Up until the third day of culture, the pathway of differentiation of neural crest cells can be changed by exchanging the type of serum in the medium.

We have observed that the extent of pigmentation of neural crest cells is less with 2% CS than with 5% CS, suggesting that a specific factor(s) in CS promotes pigmentation. We have developed a sensitive assay for pigmentation which measures melanin synthesis on day 5 in cultures containing as few as 10^4 neural crest cells. The pigment promoting factor has been partially purified from CS by precipitation with ammonium sulfate and chromatography on dye-Sephadex complexes and lectin affinity columns. Activity has been purified over one hundred-fold by this procedure, and SDS-PAGE of the enriched fraction contains approximately four protein bands. Additional chromatographic procedures are being used to purify the pigmentation-promoting activity to homogeneity. Antibodies will then be prepared against this material and should be useful in vivo for immunofluorescence localization of factors which promote melanogenesis of neural crest cells as they migrate in the early embryo.

Greenberg, Jerdan, Varner

II. Neural Crest Cultures as a Screening System for Teratogens

We previously showed that differentiating neural crest cells in culture could detect teratogens based on the ability of teratogens, but not of non-teratogens, to alter the growth, morphology or differentiation of these cells. We have extended these studies to show by biochemical assays that the teratogens 5-bromo-2-deoxyuridine and retinoic acid reduce the accumulation of melanin and the development of CAT activity. These results demonstrate that the effects of teratogenic compounds can be quantitated and provide additional criteria for assessing the effects of potential teratogens in this test system.

Greenberg, Horigan

III. Chemotaxis of Neural Crest Cells

We are investigating the possibility that chemotaxis plays a role in the highly directed migration of neural crest cells in the embryo. The migration of neural crest cells grown in culture has been studied in Boyden chamber assays using a variety of substances as possible attractants. These include the extracellular matrix molecules, proteoglycans, collagen, fibronectin, laminin, and chondronectin, and extracts of embryonic tissues. Fibronectin which is present at the time of neural crest migration enhances cell movement and may be a chemoattractant for these cells.

Greenberg, S. Seppa

IV. Attachment of Neural Crest Cells to Collagen

Other studies have shown that fibronectin and other attachment proteins can alter the phenotype of cells. For this reason we are examining the specificity of attachment of neural crest cells to collagen and the effect of various attachment factors. Neural crest cells attach equally well to substrates prepared from types I-V collagen but less effectively to laminin. The cells require fibronectin for attachment, and optimal attachment is prevented by blocking protein synthesis. This suggests that the cells synthesize an attachment protein or receptor which promotes attachment.

Greenberg, Varner, Hewitt

Future Plans

Future studies will characterize factors that are involved in the differentiation of neural crest cells and relate them to possible inductive or permissive factors in vivo. After purifying the pigmentation-promoting factor and preparing antibodies against it, immunofluorescence studies will be performed to localize possible inductive interactions in vivo. A similar approach will be used to characterize and localize factors which promote neurogenesis of neural crest cells in vitro.

Experiments will also continue to define the attachment requirements of neural crest cells to matrix components and to determine if these requirements change as the cells differentiate. The possible chemotactic role of matrix components and of surrounding embryonic tissues will be studied.

PUBLICATIONS:

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Pratt, R.M., Wilk, A.L., Horigan, E.A., Greenberg, J.H., and Martin, G.R.: Screening for teratogens in vitro. In Melnick, M. and Bixler, D. (Eds.): Etiology of Cleft Lip and Palate. New York, Alan R. Liss. In press, 1980.

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 00006-20 DB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on chemotaxis in phagocytic and non-phagocytic cells

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

Schiffmann, Elliott	Research Chemist	DB LDBA
Corcoran, Barbara	Chemist	DB LDBA
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Seppa, Silja	Visiting Fellow	DB LDBA
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COOPERATING UNITS (if any)

NIMH, ADMHA; NCI, NIH; NCI, NIH; NHLBI, NIH; Medical College of Virginia; University of Connecticut; and University of Washington Medical School

LAB/BRANCH
Laboratory of Developmental Biology & Anomalies

SECTION
Connective Tissue Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.40	3.15	1.25

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)

Our aim is to understand chemotactic migration in cells from a biochemical standpoint. To this end we have investigated this process in both phagocytic cells and connective tissue-type cells. Studies on the regulation of phagocyte chemotaxis, using formylated peptide attractants, have indicated that a tumor-derived peptide inhibits migration by reducing receptor availability, inhibiting hydrolysis of the attractant, and decreasing membrane lipid turnover. Receptor mediated internalization of attractant may be required for chemotaxis since inhibitors of transglutaminase affect both processes in a well-correlated manner. Glucocorticoids, anti-inflammatory agents, appear to exert their effect by inducing the synthesis of a phospholipase A_2 inhibitor. Actual locomotion involving microtubules may depend upon attractant-stimulated addition of C-terminal tyrosine to tubulin. Studies on chemotaxis in connective tissue-type cells have shown that fibroblasts respond to the cell binding domain of intact fibronectin and to platelet-derived growth factor; that angiogenesis factors induce endothelial cell migration, and that transformed endothelial cells respond to angiogenesis factors, platelet-derived growth factor, and fibronectin.

Introduction - At sites of tissue injury resulting from infection or trauma there occurs a well described course of cell accumulations. Initially phagocytes appear to destroy bacteria and debride the wound. Later, connective tissue-type cells such as fibroblasts, arrive at the site to effect repair. It is likely that blood phagocytes arrive at the site of inflammation mainly in response to attractants generated at the affected area. We have been studying the biochemistry of the leukotactic response in vitro using potent synthetic formylated peptides that may be related to naturally occurring bacterially-produced attractants. The synthetic compounds, which interact with a high affinity receptor on the leukocyte, are being used to probe receptor-mediated events in leukotaxis, such as regulation of receptor availability, transduction of the signal from the activated receptor to motile elements of the cell, molecular alterations in the cytoskeletal components, and the manner in which the chemotactic response is modulated. In the latter regard we are characterizing a material from tumors which inhibits chemotaxis.

Our studies now include in vitro chemotaxis of non-phagocytic cells. These cells probably accumulate at wound sites by chemotaxis since it has been shown in vitro that fibroblasts respond to lymphokines and to the cell attachment factor, fibronectin (our findings).

Methods - Neutrophils as typical phagocytic cells were obtained from rabbit peritoneal exudates and from peripheral blood of human volunteers. Assays for in vitro chemotaxis in Boyden chambers and for peptide binding to receptors were carried out using standard procedures. Anti-chemotactic material derived from tumors was obtained by extraction of tumor cells, followed by gel filtration and subsequent liquid chromatography of the extract to achieve purified fractions. With non-phagocytic cells, standard tissue culture procedures were employed to maintain stocks. Fibroblast chemotaxis was carried out essentially according to Postlethwaite et al., (J. Exp. Med., 144, 1188, 1976) with incubation for 4 hr at 37^{o} in blind well Boyden chambers using 8 μ polycarbonate filters precoated with collagenous substrates. Measurement of migration by the other cells involved similar procedures.

Results - Phagocyte Chemotaxis - We have studied molecular events in phagocyte chemotaxis at a number of levels. Recent findings on structure-activity relationships using the model peptide FMet-Leu-Phe have shown that substitution of norvaline, valine, isoleucine, or norleucine for leucine in position #2 resulted in little loss of chemotactic potency. This contrasts with replacements of Met and Phe in positions #1 and #3 respectively, where more stringent requirements for activity exist. Also esterification of Phe with benzyl alcohol yields a more potent agonist. The significance of these results may be in their application to the synthesis of potent chemotactic antagonists, since replacing formyl with t-Boc in the model peptide has resulted in an antagonist.

Receptor-mediated internalization of chemotactic peptides has been investigated. Substrates for transglutaminase (Dansylcadaverine), have, as in other cells, inhibited internalization of ligand. In addition, chemotaxis

was reduced, but the latter occurred at much lower levels of dansylcadaverine than those which were required to inhibit internalization. However, the use of certain covalently binding reagents which inhibit transglutaminase did produce a similar inhibition of both ligand-internalization and chemotaxis. The significance of these results may be that internalization is required for efficient chemotaxis and that transglutaminase may have a role in this process.

The regulation of chemotaxis has been under study. A material derived from a murine fibrosarcoma, or tumor factor, was found to inhibit phagocyte chemotaxis. This material is small ($\sim$ 400 daltons), is anionic at neutral pH and is destroyed by chymotrypsin. Amino acid analysis of a purified fraction of the tumor factor revealed the presence of equimolar amounts of N-terminal tyrosine and glycine. Estimates of the potency of the factor indicate that it is active at 0.05 μM. The partially purified material inhibits the methylation of both protein carboxyl groups and membrane phospholipids of the leukocyte, components which are believed to have roles in transmitting the chemical signal from receptor to motility elements of the migrating cell. In addition, hydrolysis of attractant is inhibited and receptor availability decreased by this factor. The tumor material stimulates angiogenesis. The significance of these findings may be in providing insight on the tumor cells' ability to subvert the host's immune defense and to ensure their own blood supply.

In other studies on the regulation of chemotaxis it has been found that glucocorticoids induced in leukocytes the synthesis of a phospholipase A_2 inhibitory protein and depressed chemotaxis in these cells. Chemoattractants have been shown to stimulate phospholipase A_2 in leukocytes, resulting in the production of prostaglandins and other inflammatory mediators from the arachidonate released. Since inhibition of the stimulated phospholipase A_2 reaction by reagents such as quinacrine also results in inhibition of chemotaxis, these findings may provide insight into the nature of the anti-inflammatory effects of glucocorticoids. In this respect, samples of sera from rheumatoid arthritic patients have been shown to contain antibodies to the phospholipase A_2 inhibitor.

Studies on cytoskeletal elements in chemotaxis have provided the novel finding that peptide chemoattractants in a receptor-mediated process stimulated a post-translational addition of tyrosine to the C-terminus of the α-chain of tubulin. Agents that inhibit agonist-receptor binding, attractant-stimulated phospholipid methylation, and calcium influx all inhibited the tyrosylation reaction. Since it is conceivable that this modification prepares tubulin for assembly into tubules, these results may lead to elucidation of the sequence of events in the organization of microtubules in the migrating cell.

Chemotaxis in connective tissue-type cells - Fibroblasts - We have previously shown that fibroblasts require both a collagen substratum and fibronectin for adherence prior to migration in the Boyden chamber. Fibronectin was also found to be a potent attractant, and the involvement of methylation reactions and cytoskeletal elements in migration was suggested. We have extended these observations. Fibronectin, a multifunctional protein, contains

specific domains which can be separated by limited proteolysis. We have now found that an 160,000 dalton fragment from fibronectin containing the cell-binding site is chemotactic, while a 40,000 dalton fragment containing the collagen-binding site is not. Further degradation of this fragment causes loss of activity. Since the 160K fragment does not promote attachment to collagen in contrast to the intact protein, it is evident that the cell can respond to a gradient of soluble fibronectin acting as a true attractant. Similarly chemotactic fragments might be produced at sites of injury, enhancing fibroblast migration. In addition, with G. Grotendorst, it has been shown that a growth factor derived from platelets (PDGF) is an exceedingly potent attractant for fibroblasts. The PDGF released by platelets as they aggregate may induce fibroblasts into the wound to begin tissue repair.

Endothelial cells - These cells are involved in angiogenic responses. Cell motility, or chemotaxis, may be a crucial element in such behavior. In collaboration with Dr. B. Glaser, Dept. of Ophthalmology, Johns Hopkins School of Medicine, it has now been shown that bovine aortic endothelial cells are attracted to an angiogenic material derived from bovine retina. The activity is in a fraction whose molecular weight range is between 10K and 100K daltons and is inactivated by boiling, suggesting its protein nature. These cells also respond to fibronectin and conditioned medium from sarcoma 180 cells. A tumor cell line, HB_4, presumably derived from endothelial cells, responded chemotactically to the retinal angiogenesis factor, PDGF, conditioned medium from sarcoma 180 cells and fibronectin. Studies of the requirements for endothelial cell migration could shed light upon neovascularization processes in wound healing, various retinopathes, and cancer.

Proposed course of project - We shall concentrate upon the characterization of the tumor derived inhibitor of chemotaxis. Material purified by liquid chromatography will be analyzed by high resolution mass spectrometry and for amino acid content. Additionally, we will label tumor cells with tyrosine and glycine, the two amino acids detected in the tumor preparation, to see if the active material contains these residues.

Chemotaxis in connective tissue-type cells - It will be determined in fibroblasts whether collagen synthesis or secretion is required for migration. Also cells from patients with diseases such as diabetes will be tested for chemotactic defects. We will determine whether PDGF promotes adherence since, like fibronectin, it is a potent attractant.

In pursuit of studies on endothelial cell chemotaxis, we shall determine whether protein synthesis (as in fibroblasts) and methylation reactions are involved. Also, a variety of angiogenic materials will be tested for chemotactic activity to determine whether there is a correlation between these activities for a given material.

We shall initiate studies on epidermal cell migration. We shall test the chemotactic activity of the protein laminin which is an attachment factor for these cells and, like fibronectin, may be multifunctional.

PUBLICATIONS:

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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 00009-19 DB

PERIOD COVERED

October 1, 1979 - September 30, 1980

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

Martin, George R.	Ch, Lab. Dev. Bio. & Anomalies	DB NIDR
Rohrbach, David	Postdoctoral Fellow	DB NIDR
Somerman, Martha J.	Staff Fellow	DB NIDR
Terranova, Victor P.	Sr. Asst. Dental Surgeon	DB NIDR
Yaar, Mina H.	Visiting Fellow	DB NIDR

COOPERATING UNITS (if any)

Johns Hopkins University; NEI, NIH; NCI, NIH; University of Minnesota and Max-Planck-Institute for Biochemie

LAB/BRANCH

Laboratory of Developmental Biology & Anomalies

SECTION

Connective Tissue Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.35	4.50	1.85

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)

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) characterization of the matrix components in a tumor which produces basement membrane, (2) the role of collagen in diseases and (3) interaction of tumor cells with collagens.

Recent studies from this and other Laboratories have shown that extracellular matrices have specific interactions with cells and in part regulate tissue form and function. In the best understood systems, fibrous connective tissue and cartilage, it has been shown that specific glycoproteins bind the cells to the extracellular matrix. Not only are the collagens and proteoglycans in these tissues unique but also the glycoproteins. Fibroblasts utilize fibronectin to bind to collagen while chondrocytes use chondronectin. These cells require each component for normal function and significant abnormalities are observed in tissues where the components are altered.

Considerable effort is now being directed toward characterizing basement membranes. Basement membranes arise early in embryonic development and appear to have an important morphogenic function during development and in wound healing. The basement membranes underly epithelial cells and seperate these cells from stroma. Further, basement membranes have been shown to restrict the passage of macromolecules through capillary walls and glomeruli. Basement membranes are altered in a variety of diseases including blistering diseases of the skin and mucosa, in diabetes and in cancer. Epithelial cells have been difficult to culture, presumably because they lack interaction with the basement membranes.

In normal tissues, basement membranes are minute structures composed of largely insoluble components. To circumvent the problem, we have used a tumor that produces an abundant basement membrane matrix as a model for normal basement membranes. So far we have characterized the collagenous proteins (type IV) in this tumor matrix; laminin; a large glycoprotein and BMI, a large heparan sulfate proteoglycan. Using specific antibodies to these proteins, we have shown that they occur in the tumor matrix as well as in the basement membranes of normal tissues. Ultrastructural studies with purified antibodies indicate that the type IV collagen is present in the lamina densa zone, laminin in the lamina rara zone next to the epithelial cells and BMI proteoglycan is imbedded in the edges of the lamina densa.

Laminin Is the Epithelial Attachment Protein - We have found that epithelial cells attach preferentially to type IV collagen substrates over types I-III collagen. In contrast, fibroblastic cells bind to all collagen types. While serum and fibronectin enhance the attachment of fibroblasts, they have no effect on epithelial cell attachment. Cyclohexamide inhibits epithelial cell attachment indicating that the cells synthesize a protein necessary for attachment. Purified laminin was found to increase the attachment of epithelial cells even in the presence of cyclohexamide. Laminin was found to bind to type IV collagen and then to the cells. Further, antibodies to laminin were found to prevent the spontaneous attachment of epithelial cells, indicating that they produce this protein and require it for attachment. Some ten different epithelial cells have been shown to require laminin for attachment while fibroblasts and chondrocytes do not. These studies indicate that laminin is the cell attachment protein for epithelial cells.

Metastatic cells from fibrosarcomas have been found to produce and utilize laminin for attachment. These cells must bind to basement membranes as they cross from one tissue compartment to another. These studies suggest that the ability of a tumor cell to produce laminin may help to predict its metastatic potential.

Terranova, Rohrbach, Martin

Epidermal Cells in Normal and Disease States - Factors effecting the differentiation of epidermal cells are under study. Enzyme liberated epidermal cells keratinize, cornify and die when cultured in suspension culture. It is likely that the detachment of the cells from the basement membrane starts these steps in terminal differentiation. Initially there is an increase in the permeability of the epidermal cell membrane. Vitamin A delays these events apparently by decreasing the permeability of the membrane. Calcium ionophores reverse the vitamin A effect indicating that the penetration of calcium into the cell is sufficient to induce differentiation.

Circulating antibody exists in several blistering diseases that reacts with material in the basement membrane of the epidermis. Using antibody from patients with bullous pemphigoid, we have found that the antibody precipitates a 225,000 dalton protein produced by the epidermal cells. While this protein does not correspond to any known protein, it elicits an antibody response in these patients which induces an autoimmune disease.

Yaar, Stanley

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. Various acquired disorders such as diabetes, certain dermatological conditions and cancer may induce and involve alterations in connective tissue components. Our approach should develop new methods for the diagnosis and detection of diseases and reveal the etiology of such disorders.

PUBLICATIONS:

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Wilk, A.L., Greenberg, J.H., Horigan, E.A., Pratt, R.M., and Martin, G.R.: Detection of teratogenic compounds using differentiating embryonic cells in culture. In Vitro, 16: 269-276, 1980.

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 00024-14 DB

PERIOD COVERED

October 1, 1979 - September 30, 1980

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

Brown, Kenneth S.	Medical Director	DB NIDR
Harne, Leslie C.	Bio Lab Technician (Animal)	DB NIDR
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COOPERATING UNITS (if any)

Howard University; St. Agnes Hospital, Baltimore; NCI, NIH; NEI, NIH and NIAMDD, NIH

LAB/BRANCH

Laboratory of Developmental Biology and Anomalies

SECTION

Connective Tissue Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.45	1.95	3.50

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)

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 of 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 objective is to determine the biochemical bases of genetic differences in malformations. Inherited differences in mice of different genotypes are being used to detect the biochemical mechanisms of birth defects. Mutants with generalized defects of growth resulting in facial clefts and dwarfism are being explored in detail at the biochemical level. Mutants with differing sensitivity to teratogenic agents are being used as probes for the cellular and biochemical basis of abnormal development.

Methods - Selected stocks of mice carrying mutations producing malformations and highly uniform inbred laboratory mice differing in metabolic responses are our test systems. Timed pregnancy and genetic uniformity permit specific studies of critical periods of development and the specific effect of gene action on morphogenesis. Timed treatments with appropriate drugs, hormones or growth factors act as detailed probes of the biochemistry of the developmental process as assayed by rates of defect and biochemical changes in critical tissues.

Findings - Different skeletal dysplasies, disproportionate micromelia (Dmm), cartilage matrix defeciency (cmd), brachymorphic (bm), foreleg defect (fld), and hypochondroplasia (hcp) are under investigation. The Dmm/Dmm and cmd/cmd are severe dysplasias which are lethal at birth and have associated cleft palate. Brachymorphic (bm/bm) and hypochondroplastic mice (hcp/hcp) are viable. Our studies show that the Dmm animals have a defect in the synthesis of type II collagen, cmd animals have a defect in the synthesis of the core protein of the cartilage specific proteoglycan, and bm animals have a defect in the synthesis PAPS (active sulfate) used to sulfate the proteoglycan. These three dysplasias involve the synthesis of cartilage matrix in the growth plate with reduced bone growth. Defect in type II collagen appears to greatly reduce the structural strength of the cartilage with resulting deformity whereas the deficiencies of proteoglycan synthesis in cmd and bm reduced the volume without greatly changing the strength of the matrix. Based on characteristic properties of the cartilage it appears that another skeletal dysplasia, chondrodysplasia (cho) is also a defect of type II collagen. The fld/fld mouse has a defect of bone in which the amount of mineralized matrix is reduced. The bone is structually weak and is deformed and fractured although the cartilage growth plate is apparently normal. The collagen of the cartilage and bone is normal. This mutant is a possible model for certain forms of osteogenesis imperfecta or brittle bone disease. It also represents a possible test system for assaying drugs to stimulate bone formation.

The Er gene results in changes in the development of the cornified layer of skin. Opposing surfaces of skin in the Er/Er embryo adhere causing among other phenomena a closure of the oral cavity and fusion of limbs and tail to trunk. The development of the digits is also arrested. The Er/+ embryo has no defects but the skin in late gestation is thinner than normal. We have found in collaboration with Beverly Dale, (Univ. of Washington, Seattle) that Er/Er animals lack the histidine rich protein which normally occurs in association with the appearance of mature keratohyalin granules and the

proximal stratum corneum layer of the fetal mouse epidermis. This 27,000 MW protein was first described by Balmain et al., 1979, in mouse, and has been found by Steinert et al. to be involved in self assembly of ordered keratin macrofibrils in the stratum corneum. Presumeably the lack of this protein prevents the formation of the cornified layer of the epidermis and opposing epithelial tissues are able to attach to one another.

The oel gene also changes epithelium. The oel gene has been found on chromosome 4 by linkage analysis. The oel/oel mouse has cleft palate and abnormally keratinized cornea and eyelids. The oel/+ mouse develops abnormal corneal keratinization. These defects are associated with abnormal response to retinol. Retinol is a much more effective teratogen in oel/+ mice than in +/+. Both oel/+ and +/+ are sensitive to the teratogenic effects of retinoic acid, a normal metabolite of retinol. The cytosol receptor for retinoic acid is detectable at the critically sensitive period for retinol although the retinol receptor is not. We conclude that retinol acts as a teratogen by metabolism to retinoic acid and that oel/+ is sensitive because it favors that pathway. Conversely the epithelial defects of the eyes of oel/+ mice seem to be due to a lack of retinol which is the only retinoid to which the eye is sensitive.

The plant alkaloid Jervine causes cyclopia in developing lambs and in cattle, rabbits, and hamsters. Rats and mice were thought to be resistant. We have found that some mouse strains are sensitive. The effect appears to be on the proliferation or differentiation of neural crest cell derivatives and can produce micrognathia cleft lip and cleft palate in mice as well as otocephaly and cyclopia. The cranioschesis strain (crn/+) which carries a gene that results in cranioschesis when homozygous appears uniquely sensitive to this drug and the crn/+ fetus develops cranioschesis when treated rather than the typical responses of other strains. The (crn/+) strain is also sensitive to cytochalasins.

Future Plans - Future studies of the skeletal dysplasies will extend to the analysis of the mRNA and DNA with Dr. Mark Sobel, who has recently joined this laboratory, of the defective homozygous animals in the cases where specific structural defects occur such as Dmm/Dmm and cmd. The studies of other dysplasias such as hcp/hcp will be carried out to expand our understanding of the role of collagen and proteoglycan in cartilage matrix. The bone of fld/fld will be examined biochemically for changes in extracellular matrix. Studies of phosphatase isozymes and vitamin D metabolites will be carried out. Effects of changes in dietary calcium and possible reduction in GLA protein will be studied. The specific defects of the epithelium of Er/Er and the role of epithelial adhesion in malformation will be followed in collaboration with Drs. Dale and Stienert. Specific antibodies to the histidine rich protein of the epidermis are being prepared to assess whether the protein is synthesized but not processed. The differences in retinoid effects in OEL/+ and +/+ will be further studied in collaboration with Drs. Hassell, Newsome, and DeLuca. Possible specific effects of retinoid response differences measured by ornithine decarboxylase assay will be examined. The action of Jervine will be followed using isotopically labeled Jervine and studies of extracellular matrix in connective tissue of treated

and control mice. Jervine effects on developing chondrosarcoma will also be examined.

PUBLICATIONS:

Hubert, H.B., Fabsitz, R.R., Feinleib, M., and Brown, K.S.: Olfactory sensitivity in humans: Genetic versus environmental control. Science, 208: 607-609, 1980.

Pratt, R.M., Salomon, D.S., Diewert, V.M., Erickson, R.P., Burns, R., and Brown, K.S.: Cortisone-induced cleft palate in the brachymorphic mouse. Teratogenesis, Carcinogenesis, Mutagenesis. In press, 1980.

Brown, K.S.: Genetics of clefting in the mouse: Discussion. In Melnick, M., Bixler, D. (Eds.): Etiology of Facial Clefting. New York, Alan R. Liss. In press, 1980.

Brown, K.S.: Vestibular anomalies as a marker in clefting: Discussion. In Melnick, M., Bixler, D. (Eds.): Etiology of Facial Clefting. New York, Alan R. Liss. In press, 1980.

Brown, K.S.: Implications of the general physiology of receptors for teratology. In Melnick, M., Bixler, D. (Eds.): Etiology of Facial Clefting. New York, Alan R. Liss. In press, 1980.

Pennypacker, J.P., Kimata, K., and Brown, K.S.: Brachymorphic mice (bm/bm): A generalized biochemical defect expressed primarily in cartilage. Dev. Biol. In press, 1980.

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 00025-14 DB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Regulation of collagen gene expression in chondrocytes and fibroblasts

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

Sobel, Mark	Research Associate	DB NIDR
Kaul, Ravi	Visiting Fellow	DB NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Developmental Biology & Anomalies

SECTION
Connective Tissue Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.83	.58	.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Chondrocytes derived from chick embryo sternae are differentiated cells typical of cartilage which exhibit several unique biochemical characteristics when grown in vitro. They synthesize a cartilage-specific proteoglycan and a unique collagen (type II) which is readily distinguished from collagens synthesized by other cell types. Previous studies have shown that such specific chondrogenic expression is inhibited when chondrocytes are exposed to 5-bromodeoxyuridine. In particular, such dedifferentiated chondrocytes no longer synthesize type II collagen; type I collagen, typical of chick embryo fibroblasts, is produced instead. As an approach to understanding normal and abnormal cartilage development, we are examining the molecular basis for the loss of phenotypic traits of chondrocytes after exposure to 5-bromodeoxyuridine. Using recombinant DNA technology, we are (a) measuring the levels of type I vs. type II collagen RNA in different cell populations, (b) examining the genomic structure of such cells, and (c) determining if 5-bromodeoxyuridine alters the transcriptional state of the different collagen genes.

Characterization of Type II Collagen mRNA - Previous studies have shown that specific chondrogenic expression is inhibited when chondrocytes are grown for a prolonged time in vitro or when chondrocytes are exposed to a variety of agents, e.g., 5-bromodeoxyuridine, embryo extract, calcium, Rous sarcoma virus, the tumor promoter phorbol myristate acetate, and preparations of bovine testicular hyaluronidase. Specifically, there is a switch in synthesis from type II to type I collagen. To study the molecular basis for the loss of phenotypic traits of chondrocytes, it is first necessary to establish an accurate method of distinguishing the type II collagen mRNA typical of chondrocytes from type I collagen mRNA of fibroblasts. Specific type I collagen DNA probes have previously been constructed and characterized in the Laboratory of Molecular Biology, DCBD, NCI. A cDNA clone derived from chick embryo sternae was recently constructed in that laboratory. Initial studies suggest that this cDNA clone contains sequences of the type II collagen gene. DNA sequence analysis performed by P. Fietzek and B. Olson (Dept. of Biochemistry, Rutgers Medical School) demonstrated nucleotide sequences similar but not identical to the DNA of the $\alpha 1$(I) collagen gene. Our initial studies are aimed toward definitively demonstrating that the sternal cDNA clone is specific for type II collagen RNA. When total cellular RNA from chick embryo chondrocytes is analyzed by the Northern hybridization technique, two major RNA species are detected by the sternal cDNA probe. The sizes of these molecules are similar to those observed with pro $\alpha 1$(I) probes RNAs. This cDNA clone also detects two RNA species in chick embryo fibroblasts. However, the relative proportion of the RNA molecules differs in the two different cell types. Current experiments involve the purification of type II collagen mRNA by hybridization to the sternal cDNA clone, its translation in an in vitro reticulocyte system, and identification of the translation product.

Future Plans - The effect of 5-bromodeoxyuridine and other agents that cause dedifferentiation of chondrocytes will first be studied by assessing the relative quantities of type I vs. type II collagen RNAs in Northern hybridization experiments. The DNA of cell populations at different stages of dedifferentiation will be examined by restriction endonuclease analysis and Southern blotting. The nucleosome structure of the collagen gene loci will also be examined in nucleolytic cleavage experiments and by in vitro transcription studies. These experiments will lead to a better understanding of the mechanism by which normal connective tissue develops and by which the abnormal expression of connective tissue genes causes various disease states.

PUBLICATIONS:

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Salomon, D., Paglia, L., and Verbruggen, L.: Hormone dependent growth of a rat chondrosarcoma in vivo. Cancer Res., 39: 4387-4395, 1979.

Breitkreutz, D., Diaz de Leon, L., Paglia, L., Gay, S., Swarm, R., and Stern, R.: Histological and biochemical studies of a transplantable rat chondrosarcoma. Cancer Res., 39: 5093-5100, 1979.

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Gottesman, M.M. and Sobel, M.E.: Tumor promoters and Kirsten sarcoma virus increase synthesis of a secreted glycoprotein by regulating levels of translatable mRNA. Cell 19: 449-455, 1980.

Yamamoto, T., Sobel, M.E., Adams, S.L., Avvedimento, V.E., DiLauro, R., Pastan, I., de Crombrugghe, B., Showalter, A., Pesciotta, D., Fietzek, P., and Olsen, B.: Construction of a recombinant bacterial plasmid containing pro-α1(I) collagen DNA sequences. J. Biol. Chem., 255: 2612-2615, 1980.

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 00230-04 DB

PERIOD COVERED
October 1, 1979 - September 30, 1980

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

Kleinman, Hynda K.	Research Chemist	DB NIDR
Grotendorst, Gary R.	Postdoctoral Fellow	DB NIDR
Hewitt, Arthur T.	Expert	DB NIDR
Martin, George R.	Ch, Lab. Dev. Bio. & Anomalies	DB NIDR
Murray, J. Clifford	Visiting Fellow	DB NIDR
Wilkes, Charlotte M.	Biologist	DB NIDR

COOPERATING UNITS (if any)

NEI, NIH; NCI, NIH; NIAMDD, NIH; NHLBI, NIH, University of Minnesota, Washington University School of Medecine; and Veterans Administration Hospital, San Francisco

LAB/BRANCH
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SECTION
Connective Tissue Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.05	3.50	1.55

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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 attachment properties of various cultured and non cultured cells and whether cells synthesize or utilize attachment proteins different than fibronectin, (2) the nature of the attachment protein(s) specificity for different collagens, reconstituted collagen, and the cell surface, (3) the nature of the interaction of fibronectin with native collagen and its role in collagen fibrillogenesis and (4) the migration of cells on collagen substrates.

Introduction 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. Fibroblastic cells do not bind directly to collagen but are linked to the collagen by a large glycoprotein, fibronectin. On the other hand, cartilage lacks fibronectin and chondrocytes utilize another factor for adhesion. We are currently investigating the interaction of fibronectin with native collagen and the role of the fibronectin-collagen complex in mediating the attachment and migration of various cell types. Our studies indicate that many cells attach to collagen via fibronectin but different modes of cell adhesion exist for other cell types.

Methods - The interaction of fibronectin with collagen and the adhesion of cells to collagen are studied in vitro. Collagens and fibronectin are prepared by standard methods while the cells are obtained either as cell lines or from tissues after proteolytic dissociation. Human skin fibroblasts and CHO cells were obtained from American Type Culture Collection, 3T3 and NRK cells from S. Aaronson (NCI), hepatocytes from adult liver, smooth muscle cells from sheep embryonic aorta, chondrocytes from chick embryonic sternum, ACT cells from mouse skin, TACT cells arose from ACT after 15 passages in culture and PMT cells from a highly metastatic tumor.

Attachment Properties of 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 fibroblastic cells such as human skin fibroblasts, CHO, 3T3, show enhanced attachment to collagen over plastic and require serum fibronectin for attachment. Cells such as hepatocytes derived from normal liver also show serum dependence while hepatocytes derived from fibrotic liver are poorly adherent even with serum or fibronectin. Smooth muscle cells require fibronectin to adhere to collagen types I, II, and III but can maximally adhere to type V collagen in the absence of fibronectin or any added factors. These cells apparently are not synthesizing an attachment factor since cyclohexamide does not inhibit their attachment. Soluble type V collagen blocks smooth muscle cell adhesion while soluble type I collagen has no effect. Others have reported that type V is made in relatively large quantity by smooth muscle cells and is localized by immunofluorescence at the cell surface. These data suggest that smooth muscle cells have a cell surface receptor for type V collagen.

Preliminary studies suggest NRK (normal rat kidney) cells also adhere preferentially to type V collagen suggesting they may be of mesangial origin.

Grotendorst, Wilkes, and Kleinman

Attachment Properties of Tumorigenic and Metastatic Cells - Metastatic cells must break their attachments with the extracellular matrix of the primary tumor in order to enter the circulation and invade other tissues. We have therefore, compared the attachment properties of metastatic murine cells to tumorigenic (but not metastatic) cells and to normal cells. The metastatic

cells (PMT) attached preferentially to type IV (basement membrane) collagen in the absence of serum or fibronectin. In the presence of serum, these cells adhered well to both type I and type IV collagens. The normal (ACT) and spontaneously transformed (TACT) fibroblasts adhered well to both these substrates in the presence of serum but the TACT adhered more slowly. The tumorigenic cells (TACT and PMT) synthesize little or no fibronectin or collagen while the normal cells (ACT made measurable amounts of both proteins. These data demonstrate that the metastatic cells a possess unique mechanism for binding to type IV collagen and that this preferential adhesion could be important in the metastatic process where cells must bind and penetrate basement membranes.

Murray, Liotta

Binding of Fibronectin to the Cell Surface - Fibronectin is present on the surface of cells and in the serum used to culture the cells. Using a quantitative immunologic assay (ELISA) which can distinguish between murine and human fibronectin, we have been able to quantitate and investigate the origin of the fibronectin present on the cell surface of human fibroblasts cultured in murine serum. Although the cells are cultured in a 10-fold excess of fibronectin (in serum), greater than 80% of the fibronectin on the cell surface originates from the cells. Soluble collagen stimulates the binding of fibronectin to the cell surface 2- to 6-fold in a concentration- and time-dependent manner. Although cells maintained in suspension bind much less fibronectin than cells in monolayer, cells in suspension can also 2- to 6-fold more fibronectin in the presence of soluble collagen. Gangliosides which have previously been shown to inhibit fibronectin-mediated cell adhesion (Kleinman et al., 1979. Proc. Natl. Acad. Sci. USA 76: 3367-3371) also block the binding of fibronectin to the cell surface even in the presence of soluble collagen. These data demonstrate that the fibronectin present in the cell layer originates from the cells and that collagen facilitates the incorporation of fibronectin into the cell layer. Such data may explain why transformed cells which synthesize reduced amounts of collagen also have reduced amounts of fibronectin in the cell layer.

Rennard, Hewitt, Wilkes, and Kleinman

Interaction of Fibronectin with Collagen - The nature of the fibronectin-collagen interaction is being explored. Studies on the interaction of fibronectin with denatured collagen demonstrated that a specific sequence of approximately 8 amino acids including the collagenase sensitive site on the collagen α1(I) chain recognizes a specific 40,000 dalton domain of the fibronectin molecule located near the amino terminus. Few studies have characterized the interaction of fibronectin with native collagen. Furcht has found that the collagen fibrils (as seen by immunoelectronmicroscopy) have fibronectin on their outer surface in a 70 nm periodic arrangement. When fibronectin is present during collagen fibrillogenesis fibronectin binds to the forming fibers. Binding is blocked by denatured collagen and collagen fragments which contain the specific fibronectin binding site, but not by Ascaris collagen which lacks this site. Fibronectin also inhibits fiber formation. The collagen binding fragment of fibronectin (40,000 dalton) was

as active on a molar basis as the intact molecule in inhibiting fibrillogenesis, while the cell binding fragment (160,000 daltons) and various other proteins (albumin, chondronectin, and laminin) had no effect. Heating the fibronectin to 80° for 30 minutes abolished its ability to mediate cell adhesion but did not affect its ability to bind to collagen or delay fibrillogenesis, suggesting an unusual stability to the collagen binding region in the fibronectin molecule. These data demonstrate that fibronectin binds to native collagen probably through the same site as already determined for denatured collagen. In addition, since fibronectin delays collagen fibrillogenesis, it may have a role *in vivo* in determining fibril size.

Since fibronectin binds to denatured collagen at the site cleaved by mammalian collagenase, it may block the enzyme from cleaving collagen. We are studying the influences of fibronectin on the cleaveage of collagen by this collagenase.

Wilkes and Kleinman

Migration of Smooth Muscle Cells on Collagen Substrates - Fibroblasts have been shown to migrate in the Boyden chamber on filters coated with collagen. When tested in this system, fibronectin is a chemoattractant for fibroblasts (Gauss-Muller *et al.*, 1980. J. Lab. and Clin. Med., in press). Smooth muscle cells migrate to fibronectin on type I collagen but not on a type V collagen substrate. The growth factor released from platelets (PDGF) has been demonstrated to be an attractant for smooth muscle cells. The continued proliferation of these cells produces the atherosclerotic plaque. Smooth muscle cells migrate to PGDF at concentrations (0.1 unit/ml) below that required for proliferation. Other growth factors (FGF, NGF, or EGF) do not promote cell migration. These data suggest that smooth muscle cells could migrate to the site of endothelial injury in response to platelet derived growth factor.

Grotendorst, Seppa, Kleinman, and Schiffmann

Significance - Various genetically distinct collagens exist in association with a specific cell type such as fibroblasts with type I collagen and smooth muscle cells with type V collagen. Such cells in vitro interact with their tissue specific macromolecules. Our studies explain the molecular basis of this sorting out and explore the manner in which the extracellular matrix proteins interact and regulate the function and activity of the cells.

PUBLICATIONS:

Kleinman, H.K., Hewitt, A.T., Murray, J.C., Liotta, L.A., Rennard, S.I., Pennypacker, J.P., McGoodwin, E.B., Martin, G.R., and Fishman, P.H.: Cellular and metabolic specificity in the interaction of adhesion proteins with collagen and with cells. J. Supramol. Struct., 11: 69-78, 1979.

Mosher, D.F., Schad, P.E., and Kleinman, H.K.: Inhibition of blood congulation factor XIII^{a}-mediated cross-linking between fibronectin and collagen by polyamines. J. Supramol. Struct., 11: 227-235, 1979.

Berman, M.D., Waggoner, J.G., Foidart, J.M., and Kleinman, H.K.: Attachment to collagen by isolated hepatocytes from rats with induced hepatic fibrosis. J. Lab. Clin. Med., 95: 660-671, 1980.

Hewitt, A.T., Kleinman, H.K., Pennypacker, J.P., and Martin, G.R.: Identification of an adhesion factor for chondrocytes. Proc. Natl. Acad. Sci. USA, 77: 385-388, 1980.

Kleinman, H.K., Pennypacker, J.P., Foidart, J.M., Hewitt, A.T., and Martin, G.R.: Biology of matrix proteins-the interaction of cells with collagen and fibronectin. Ital. J. Gastroenterol., 12: 27-30, 1980.

Murray, J.C., Liotta, L.A., Rennard, S.I., and Martin. G.R.: Adhesion characteristics of murine metastatic and nonmetastatic tumor cells in vitro. Cancer Res., 40: 347-351, 1980.

Gauss-Muller, V., Kleinman, H.K., Martin, G.R., and Schiffmann, E.: Role of attachment and attractants in fibroblast chemotaxis. J. Lab. Clin. Med. In press, 1980.

Hewitt, A.T., Kimata, K., Pennypacker, J.P., Wilkes, C.M., Martin, G.R., and Kleinman, H.K.: The attachment of chondrocytes and fibroblasts to collagen. In Gaspan, H. (Ed.): Biology of the Articular Cartilage. Munich, Germany. In press, 1980.

Kleinman, H.K., Hewitt, A.T., Grotendorst, G.R., Martin, G.R., Murray, J.C., Rohrbach, D.H., Terranova, V.P., Rennard, S.I., Varner, H.H., and Wilkes, C.M.: Role of matrix components in adhesion and growth of cells. In Pratt, R.M. and Christiansen, R. (Eds.): Current Research Trends in Prenatal Craniofacial Development. New York, Elsevier North-Holland. In press, 1980.

Kleinman, H.K. and Wilkes, C.M.: Interaction of fibronectin with collagen. In Weiss, J.B. and Jaysen, M.I.V. (Eds.): Collagen in Health and Disease. Churchill Livingstone. In press, 1980.

Rennard, S.I., Wind, M.L., Hewitt, A.T., and Kleinman, H.K.: Effect of collagen and cell shape on binding of fibronectin to cells. Arch. Biochem. Biophys. In Press, 1980.

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 00253-03 DB

PERIOD COVERED
October 1, 1979 - September 30, 1980

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

Varner, Hugh H.	Postdoctoral Fellow	DB NIDR
Hewitt, Arthur T.	Expert	DB NIDR
Kimata, Koji	Visiting Associate	DB NIDR
Lee, Willard A.	Chemist	DB NIDR
Somerman, Martha J.	Staff Fellow	DB NIDR

COOPERATING UNITS (if any)
NCI, NIH

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SECTION
Connective Tissue Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

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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 toward the extracellular matrix of developing cartilage and its relationship to normal cellular function. Factors are being characterized which perturb the phenotype of chondrocytes.

In these investigations, use is being made of limb and mesenchyme cell cultures, mature chondrocytes in culture and a cartilage producing tumor.

Chondronectin as an Adhesion Factor for Chondrocytes - We have previously reported that chondrocyte attachment to collagen is mediated by a serum factor other than fibronectin, which we have name chondronectin (Hewitt et al., Proc. Natl. Acad. Sci. USA, 77: 385-388, 1980). The existence of a separate adhesion molecule is based on several findings: (1) after fibronectin is removed from serum by passage over a collagen-Sepharose column, chondrocyte attachment activity is retained, while fibroblast attachment is lost, (2) purified fibronectin does not promote chondrocyte attachment, and (3) chondronectin can be separated from fibronectin by ion exchange chromatography. Chondronectin has now been purified by various ion exchange and affinity chromatographic procedures. It is present in serum, in cartilage, in a chondrosarcoma and in vitreous fluid. It has a molecular weight of 180,000 daltons and yields chains of about 70,000 daltons on reduction. Chondronectin is more heat labile than fibronectin ($t_{1/2}$ for 30 minutes: 51^{o} vs 57^{o}), and is active in cell adhesion at 50 ng/ml (while fibronectin is active at 1-5 µg/ml). Antibodies against chondronectin do not cross-react with fibronectin but inhibit the attachment of chondrocytes to cartilage. A quantitative immunoassay is also being developed. These studies demonstrate that a specific attachment factor distinct from fibronectin exists for chondrocytes. The effect of the molecule on cartilage differentiation and its function in various pathological conditions including the chondrodystrophies will be investigated.

Hewitt, Varner, Wilkes, and Silver

Studies on Chondrogenic Expression In Vitro - Previous studies have shown that a contaminant in certain preparations of bovine testicular hyaluronidase causes a reversible inhibition of chondrogenic expression in vitro. The component responsible for this effect has been purified to near homogeneity and appears to be a 160,000 MW protein with an isoelectric point of 6.6. A similar inhibition of chondrogenesis has been reported upon addition of the tumor promotor phorbol myristate acetate (PMA) to cultured chondrocytes. For this reason, the protein was tested in other systems in which PMA is known to have an effect. No inhibition of PMA binding was observed. However, the protein blocked the binding of epidermal growth factor (EGF) to its receptors, an effect also obtained with PMA. Additional studies are in progress to further characterize the protein and its effects on chondrocytes in culture.

Varner and Hewitt

Development of a Chondrocytic Cell Line

I. The development of a stable cell line with the biochemical characteristics of chondrocytes would facilitate studies aimed at understanding the regulation and development of normal chondrocytes. Previous studies have shown that cells isolated from a transplantable rat chondrosarcoma and selected by their preferential growth on a type II collagen substrate will exhibit the biochemical and morphological characteristics of chondrocytes in culture. However, these cells grow poorly and fail to maintain a chondrocytic phenotype in long term cultures. However, it was found that these cells would maintain

their phenotype in culture when cis-hydroxyproline was included in the culture medium for at least four days after passage. This effect was assessed by morphology and the continued predominance of cartilage-specific proteoglycans and collagen. Studies are in progress to further define in vitro conditions compartable with growth and maintenance of phenotype of these cells.

Kimata and Somerman

II. Chondrocytes from normal rat articular cartilage and the rat chondrosarcoma were compared on the basis of collagen type synthesized, ability to produce cartilage-specific proteoglycan and the presence or absence of fibronectin in situ and in vitro. One striking difference was the continued presence of fibronectin in tumor but not normal tissue observed by immunofluorescence after treatment of sections with hyaluronidase. Chondrosarcoma chondrocytes in culture continued to stain for fibronectin even after such staining had disappeared in normal cultures which had begun to produce cartilage matrix. The fibronectin-positive staining in cultures of tumor cells was localized in areas which had obvious chondrocyte morphology as well as in more fibroblastic areas.

Attachment properties of the two types of chondrocytes were also different. Both types showed a preference for type II collagen although chondrosarcoma cells also exhibited some attachment to other collagens. Attachment of normal chondrocytes was stimulated by chondronectin but not fibronectin. Tumor chondrocytes showed greater attachment with fibronectin but there was still some activity with fibronectin-free serum. Chick limb mesenchymal cells do not show a collagen preference and require fibronectin for attachment (Pennypacker and Hewitt, unpublished observations). This suggests that tumor cells may resemble more immature chondrocytes or be in a transitional state between a mesenchymal and a differentiated state.

Kimata and Hewitt

PUBLICATIONS:

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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 00275-02 DB

PERIOD COVERED

October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biological testing of fluoride

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

Martin, George R.	Ch, Lab. Dev. Bio. & Anomalies	DB NIDR
Brown, Kenneth S.	Medical Director	DB NIDR

COOPERATING UNITS (if any)

University of Minnesota; Litton Bionetics; and NCI, NIH

LAB/BRANCH

Laboratory of Developmental Biology & Anomalies

SECTION

Connective Tissue Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.00	.50	.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)

The purpose of this project is to study the action of fluoride on various systems used to detect clastogenic or mutagenic substances. To date fluoride has been examined in several systems used to detect mutagens and found to be non mutagenic. No effects on chromosome structure were noted in animals given widely different levels of fluoride. DNA repair after X-ray was unchanged by fluoride. No genetic effects of fluoride were noted in a recessive lethal test of fluoride on drosophila. The data indicate that fluoride has no mutagenic activity.

Fluoride has been widely used to suppress caries. At the levels recommended (0.5-1 ppm), no detrimental effects of fluoride on the health of individuals have been noted. At very high fluoride intakes, alterations in bone metabolism can occur and are associated with degenerative changes in bone.

It is the purpose of this project to examine fluoride in some of the more recently developed tests which detect mutagenic or clastogenic activity. Considerable epidemiological data indicate that fluoride consumption does not increase the incidence of cancer or birth defects. The tests described below provide corraborative experimental information.

In our initial studies, we measured the numbers of abnormal chromosomes in mice from colonies raised on very different levels of fluoride. Bone levels of fluoride differed some 450 fold. The rate of sister chromatid exchange was similar in animals from the two groups. The rate of sister chromatid exchange was similar in animals from the two groups. Mice given low (1 ppm) to high levels (100 ppm) of fluoride had similar levels of chromosomal abnormalities which did not differ from the level of abnormalities present in animals receiving no fluoride supplement.

A repeat of the study of mouse bone marrow and testing chromosomes after six weeks of treatment with a range of 0-100 ppm fluoride in water using sample numbers sufficiently large to detect a doubling of the rate of chromosome malformations confirmed the earlier study that no change in chromosome abnormality rate was produced by these levels of fluoride.

Slight and not statistically significant increases in XY chromosomal dissociation were noted in the range 2-4% in animals supplemented with fluoride. Great variations in this parameter (up to 40%) have been noted in previously published studies and the XY chromosome dissociation is believed to arise during preparation of the chromosomes for examination.

No effect of fluoride levels was found in a sex-linked recessive lethal test in Drosophila melanogaster. This is an *in vivo* test in an eukaryotic organism which measures the frequency of mutation of approximately 1000 loci on the X-chromosome. It has been used as an effective assay for many mutagens.

Fluoride was found to be not mutagenic in the Ames test using microbial cells *in vitro*. It was found to have no effect on the ability of mouse leukemia cells to repair x-ray produced damage.

Taken together the data show no genetic or mutagenic actions of fluoride.

PUBLICATIONS:

None

ANNUAL REPORT OF THE LABORATORY OF BIOCHEMISTRY
NATIONAL INSTITUTE OF DENTAL RESEARCH

The Laboratory of Biochemistry contains three Sections, the Proteoglycan Chemistry Section, the Enzyme Chemistry Section and the Protein Chemistry Section. All are of similar size and composition. Laboratory personnel total about 25 with a ratio of research to support staff of about 2:1. Within the research staff the ratio of temporary (postdoctoral fellows and visitors) to tenure staff is also about 2:1. Of the temporary scientists about one-half are supported by mechanisms (mainly postdoctoral fellowships) that do not count against our position ceiling.

The size of the Laboratory programs is limited largely by our position ceiling and our ability to recruit through other mechanisms. However, space becomes increasingly more limiting each year without much hope for any major relief. Measurements of program size in practice should also include collaborative arrangements. All of the scientific staff collaborate closely with other scientists within and outside NIDR and NIH, sometimes with several different groups. In this manner productivity is mutually increased. In addition, we utilize some space in building 2 (NIAMDD) where the nuclear magnetic resonance instrumentation is located. We also have access to electron microscope facilities in the Laboratory of Biological Structure.

The product produced by the Laboratory is, of course, original research in several areas of biochemistry related to normal biological function and to disease. The unifying theme is molecular structure and function. The conventional measure of productivity is the number and quality of publications. Without trying to assemble numbers or judge individual papers, the Laboratory undoubtedly meets or exceeds whatever standards would apply. In addition to laboratory research, senior personnel participate in a variety of other professional activities of broad importance to science including participating in national and international meetings, reviewing of manuscripts for various scientific journals, evaluating applications for granting agencies and foundations, teaching in the Graduate School at NIH, lecturing to groups within and outside NIH, serving on commitees, and organizing meetings. Increasing emphasis in recent years on management responsibilities including performance appraisals and EEO also takes time and effort of Laboratory personnel.

To produce original research is, however, the primary effort of the Laboratory. Progress during the past year is summarized below according to Section.

Proteoglycan Chemistry Section

Proteoglycans are complex macromolecules which contain glycosaminoglycans and often other oligosaccharides covalently attached to distinct core proteins. They are critical structural elements of connective tissue. For example: (a) cartilage proteoglycans directly influence shape and form of the developing skeleton and provide the resiliency and resistence to compressive load required for proper physical function of adult cartilages; (b) corneal proteoglycans are essential components for maintaining the normal, highly organized matrix of the stroma and for transparency of the tissue; (c) proteoglycans are important constituents of basement membranes which serve as filtration barriers in kidney and are essential for morphogenesis in branching epithelial systems. The Proteoglycan Chemistry Section continues to focus on structure and

biosynthesis of cartilage proteoglycans, and to collaborate with other programs primarily interested in proteoglycans in other tissues including cornea, kidney glomeruli and aortic smooth muscle. This past year, utilizing chondrocyte cultures, immunological methods have been developed for identifying and isolating the newly synthesized core protein prior to biosynthetic addition of glycosaminoglycans. The structures and general locations of other complex carbohydrates on the completed proteoglycan, namely the O-linked oligosaccharides related to keratan sulfate and the N-linked oligosaccharides, have also been determined. Preliminary data indicate that the core protein already contains these oligosaccharides in various stages of processing before glycosaminoglycans are added. The rates of synthesis and turnover of proteoglycans and the final structures of the molecules are the result of many complex processes regulated by the chondrocyte and its interaction with its immediate chemical and mechanical environment. Information about how these processes are regulated is necessary for understanding both normal cartilage function and debilitating cartilage diseases such as osteoarthrosis and rheumatoid arthritis.

Less is understood about proteoglycans of other tissues, but it is becoming increasingly more apparent that they vary widely in kind and relative amounts of polysaccharide and protein, and in size and form. A major challenge is to understand the structure-function relationships of this important group of molecules.

Enzyme Chemistry Section

The study of the transglutaminases was taken up by the group a number of years ago primarily as an interesting subject to study enzyme mechanisms. The only biological function of transglutaminase known at that time was the stabilization of the fibrin clot through ε-(γ-glutamyl)-lysine crosslinks. It is now evident that these enzymes play many roles in structural and cellular processes including crosslinking of hair proteins, clotting of seminal fluid, possibly internalization of extracellular proteins, and several intracellular processes. Crosslinking occurs not only directly between lysine and glutamine but may be mediated by polyfunctional amines bridging glutamine residues. The modification of proteins by the introduction of amines (without crosslinking) may also be a biological mechanism regulating function. There is evidence that transglutaminases are involved in interactions between collagen, fibronectin, fibrin and possibly other extracellular proteins. Thus, a bridge has been provided to other connective tissue studies in the Laboratory of Biochemistry and in other laboratories at NIH.

This past year emphasis has been on several aspects of the broad problem. Synthetic peptide substrates have been prepared to study the mechanism of action. It has been demonstrated that the enzyme is a useful tool in protein chemistry studies by using it to introduce artificial crosslinks. A new cell membrane form of transglutaminase was isolated and shown to have several cellular protein substrates (of as yet unknown function). An uterine form of the enzyme which may function during fetal development was shown to arise from blood platelets. Finally, turnover of proteins crosslinked by transglutaminase was studied and a new enzyme, γ-glutamylamine cyclotransferase, was found which can carry out the last step, cleavage of the crosslink.

These studies of the transglutaminases provide an excellent example of how basic biochemical studies, originally undertaken for their own sake, have led to the development of new biological concepts and stimulated new areas of biological research.

Protein Chemistry Section

The protein chemist works with the collagen molecule which contains three polypeptide chains in a rod-like triple helix. In vivo, however, collagen is present as an aggregate of molecules arranged in an orderly array, the native fibril (types I, II and III collagen). The major interest in structure is currently at the fibril level; how are molecules arranged and how are fibrils formed? A modified model proposed this past year contains molecules arranged helically in a microfibril 4 nm in diameter; microfibrils are packed laterally to form fibrils. Within large fibrils, the microfibrils are compressed so that molecular cross sections lie on a disordered hexagonal lattice while maintaining a covalently defined filamentous substructure. This model explains available data from several sources but is not proven. Present efforts are directed to preparing specimens suitable for high resolution electron microscopy in an attempt to obtain definitive data. Both conventional and scanning transmission electron microscopy are being used. In vitro assembly studies also support this type of model. Studies this year have shown that molecules assemble very rapidly into an intermediate aggregate containing up to about 100 molecules. The intermediate first grows linearly and the resulting filaments then assemble laterally to form the native fibril. In vivo, the steps must be somewhat different although the product is the same.

A technique that has revolutionized the study of molecular interactions in solid state material is nmr. Studies in the Section in recent years using ^{13}C nmr have shown that collagen molecules in the native fibril are in rapid anisotropic motion about their axes. This motion has now been further defined by ^{2}H nmr to show that oscillation occurs through an angle of at least 30° about a preferred orientation or among a set of preferred orientations. In addition, amino acid side chains are in motion. Thus, interactions between molecules must be fluid while at the same time must be sufficiently restricting to define some degree of three-dimensional order.

The same nmr techniques applied to proteoglycans show similar motion in both the polysaccharide and the protein chains that is, however, essentially isotropic. This motion allows rapid changes in shape that must be important to function.

Hemoglobin S, somewhat like collagen, forms a fibrous aggregate in the deoxygenated state that is responsible for the distortion of red blood cells in sickle cell anemia. The interactions that occur between hemoglobin S molecules in solution and aggregated states can be distingusished by nmr, which thus provides a nondestructive technique to follow the process and to test inhibitors of the transition in red blood cells. This is an example of how techniques and concepts developed for one project find applicability in other projects and advance science in unpredictable ways.

Thus, the Laboratory has had a stable year with no major changes in program direction. It has, however, been a productive year with new results leading to new projects.

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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-00134-06 LB

PERIOD COVERED
October 1, 1979, to September 30, 1980

N01 DE 72403

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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De Luca, Silvana M.	Senior Staff Fellow	LB	NIDR
Kimura, James H.	Staff Fellow	LB	NIDR
Caputo, Claudia B.	Arthritis Foundation Fellow		
Thonar, Eugene J-M.A.	Visiting Associate	LB	NIDR
Fellini, Steven	NIH Postdoctoral Fellow		

COOPERATING UNITS (if any) Arnold Caplan, Case Western Reserve University; John Hassell, NEI; Bo Nilsson, NCI; Robin Poole, Shriners Childrens Hospital, Montreal, Canada; Peter Nissley, NCI; Yoshpal Kanwar and Marilyn Farquhar, Yale University; Thomas Wight and Russell Ross, University of Washington.

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Proteoglycan Chemistry Section

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NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
8.00	5.50	2.50

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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 purpose of the project is to study the chemical and physical properties of proteoglycans and their biosynthesis. Topics of present interest include: 1) chemistry of proteoglycans isolated from the Swarm rat chondrosarcoma, 2) biosynthesis of the protein core of proteoglycans and the link proteins by chondrocyte cultures, 3) characteristics and biosynthesis of proteoglycans in corneal stroma, 4) characteristics and biosynthesis of proteoglycans in aortic smooth muscle cells in culture, 5) characteristics and biosynthesis of proteoglycans in kidney glomeruli. The proteoglycan of cartilage is best understood, but increasing knowledge of other proteoglycans shows a wide diversity in size, form and function.

1. Project Description

Background

Cartilage proteoglycans are large macromolecules (MW 1-5 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. N- and O-linked oligosaccharides are also present. Such a molecular architecture yields macromolecules which occupy large hydrodynamic volumes in solution 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 glycosaminoglycans and which interacts in a highly specific way with both hyaluronic acid (HA) and a protein (MW of 45,000) 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. This latter region has a variable molecular weight (from a few thousand to 200,000) and contains more than 90% of the CS chains but less than 35% of the KS chains present in the intact molecules. The variable length of this region appears to be proportional to the number of CS chains present on any individual proteoglycan molecule. The average cartilage proteoglycan molecule contains about 80 CS chains (average MW about 20,000 per chain) and 100 KS chains.

Proteoglycans are found in all connective tissues. As our understanding of cartilage proteoglycans increases, the knowledge and experience gained are being applied to proteoglycans of other tissues. They all have the same basic chemistry, but differ widely in size, relative proportions of protein, glycosaminoglycan and oligosaccharide and, of course, in biological function.

Within NIDR, this program collaborates with: 1) 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 00157-05), 2) Dr. Gretchen Hascall, Laboratory of Biological Structure, in studies on the morphology of the Swarm rat chondrosarcoma (Project # Z01 DE 00163-03).

The following sections describe our ongoing projects.

1. Characteristics of proteoglycans isolated from the Swarm rat chondrosarcoma

(a) Immunology

Large amounts of proteoglycans can be purified from the transplantable Swarm rat chondrosarcoma without prior dissociation of aggregates. The protease, clostripain, was used to remove chondroitin sulfate-peptides from the aggregate, thereby allowing the isolation of a complex containing hyaluronic acid, the hyaluronic acid-binding region (65,000 MW) of the proteoglycan core protein, and the link protein (reduced from 45,000 to 40,000 MW by the digestion). Procedures were used to separate and purify the HA-binding region and the link protein. Antisera were raised in rabbits against both of these purified components as well as against the intact complex (in collaboration with Dr. Robin Poole). Highly specific and sensitive ELISA procedures were developed capable of detecting 10-100 ng/ml concentrations of HA-binding region and link protein. Immobilized HA-binding region and link protein affinity columns were developed to isolate monospecific antisera. These procedures were used in structural and biosynthetic studies described below.

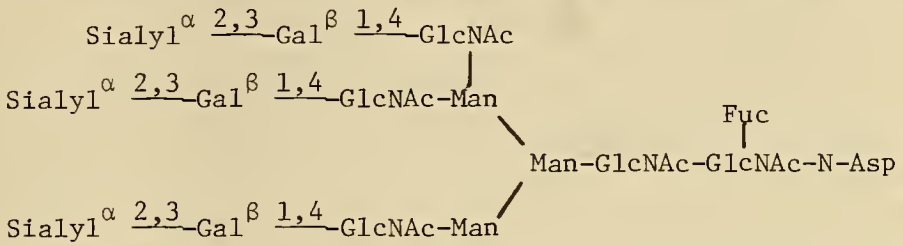
(b) Isolation of aggregate

A band velocity sedimentation procedure using preformed, shallow cesium sulfate gradients was developed in order to separate aggregate from the non-aggregated proteoglycans normally present in purified proteoglycan samples (in collaboration with Dr. Koji Kimata, LDBA). Monomers were then isolated from the purified aggregates and partitioned into several size classes. Chemical analyses suggested that the average size of a proteoglycan molecule correlated primarily with the average size of its chondroitin sulfate chains rather than the size of the protein core. The velocity gradients were formed in a variety of solvent conditions to study the effects of solvent parameters on stability of the aggregate. As the concentration of guanidine-HCl increases beyond 2 M, the three components of the aggregate--monomer, link protein and hyaluronic acid--separate from each other. On the other hand, as the pH decreases below 3.5 the monomer and link stay associated with each other but dissociate from hyaluronic acid. This provides a method for isolating and studying a purified monomer-link complex which appears to be a probable intermediate in aggregate formation.

(c) Characteristics of N-linked and O-linked oligosaccharides

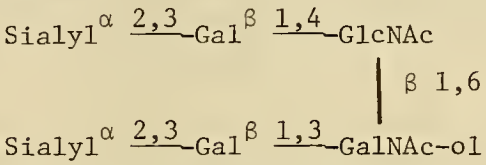
The mannose-containing oligosaccharide in the proteoglycan was isolated after alkaline-borohydride digestion and molecular sieve chromatography. Sialic acid residues were removed with neuraminidase and oligosaccharide was cleaved from the N-asparagine

residue by trifluoroacetolysis. In collaboration with Dr. Bo Nilsson, methylation analyses and gas liquid chromatography-mass fragmentation were used to determine the following structure which constitutes at least 75% of the mannose oligosaccharides present in the proteoglycan:



Approximately 10-15 of these triantennary oligosaccharides are present on the core protein and predominantly located in the 65,000 MW hyaluronic acid-binding region.

The largest of the O-linked oligosaccharides was purified and its structure determined:



This structure is closely related to the attachment region of keratan sulfate. The keratan sulfate chain would be elongated on the circled galactose unit prior to addition of the non-reducing terminal sialic acid residue.

These studies are being extended to proteoglycans isolated from bovine fetal cartilage which contain keratan sulfate as well as both O-linked and N-linked oligosaccharides. The linkage region of the keratan sulfate chains to the core protein has been prepared for chemical studies following removal of the keratan sulfate chain by treatment with an endogalactosidase specific for keratan sulfate.

2. Characteristics of proteoglycans synthesized in culture by chondrocytes from the Swarm rat chondrosarcoma

(a) Isolation of the core protein of proteoglycans

When protein synthesis in the chondrocytes is blocked with either cycloheximide, puromycin or pactamycin, ^{35}S-sulfate incorporation into proteoglycans continues with a decreasing, nearly first order exponential rate having a $t_{1/2}$ of about 60 minutes. This suggests that the cells contain a large pool of core protein which do

not contain chondroitin sulfate chains and that the rate limiting step in processing to the final proteoglycan structure is the assembly of the chains on the core in the Golgi apparatus. Amino acid precursors, ^{3}H-serine and ^{35}S-methionine, were used to label the protein. The intracellular macromolecules were solubilized by a zwitterionic detergent-guanidine HCl procedure. Immunoprecipitation using rabbit anti-HA-binding region and a second anti-rabbit antibody was used to isolate the putative core which has an apparent molecular weight on SDS-PAGE of about 350,000. The identity of the core was verified by (1) pulse-chase experiments in which the kinetics of disappearance of core and appearance of completed proteoglycan were monitored and (2) demonstration of the ability of the core protein to reaggregate with carrier proteoglycan aggregates. Experiments are underway to isolate and characterize the core protein further.

(b) Biosynthesis of oligosaccharides

^{3}H-Glucosamine and ^{3}H-acetate were used as precursors for synthesis of complex carbohydrates. Completed monomer proteoglycans were isolated and the glycosaminoglycan chains and the oligosaccharides were released by treatment with alkaline-borohydride. Molecular sieve chromatography and chondroitinase digestion procedures were used to demonstrate that about 6% of the radioactivity in each case was present in the N-linked and O-linked oligosaccharides. The proportions of the different O-linked oligosaccharides were nearly the same as that found chemically for monomers isolated directly from the tumor. Immunoprecipitation techniques were used to isolate the core protein prior to the addition of the chondroitin sulfate chains. Preliminary data show that the core contains both N-linked and O-linked oligosaccharides indicating that these oligosaccharides are at least partially assembled on the core protein prior to adding the glycosaminoglycan chains. Experiments are underway to determine the kinetics of synthesis of these oligosaccharides and to compare their structures before and after the glycosaminoglycan chains are added.

(c) Polydispersity of monomers

In cartilages, the proteoglycans which have accumulated in the matrix are polydisperse and, in many cases, this polydispersity is reflected in part by a variability in the length of the core protein in the chondroitin sulfate-attachment region. Thus, larger proteoglycans appear to have longer core protein in this region and hence more chondroitin sulfate chains than smaller proteoglycans. It is unknown whether this polydispersity results during synthesis of the proteoglycans or from long-term catabolic processes occuring in the extracellular matrix. A series of experiments were done to determine the extent and causes of polydispersity for newly synthesized proteoglycans. Double-label protocols using either ^{3}H-serine and ^{35}S-methionine or ^{35}H-serine and ^{35}S-sulfate were used to label

proteoglycans. The two amino acids were shown to be differentially located in the core protein: the ^{3}H-serine is primarily in the chondroitin sulfate-rich region (about 80%) whereas the ^{35}S-methionine is primarily in the HA-binding region (about 70%). Labeled aggregate was purified by the cesium sulfate velocity band sedimentation technique and monomer then purified from aggregate with a dissociative density gradient to isolate only that population of proteoglycans which contain a functional HA-binding region. These monomers were then partitioned into different class sizes by molecular sieve and velocity gradient procedures. The ratio of ^{35}S to ^{3}H for the ^{35}S-methionine plus ^{3}H-serine monomer was constant for all size classes whereas the ^{35}S to ^{3}H ratio for the ^{35}S-sulfate plus ^{3}H-serine monomer decreased with decreasing monomer size. It was then shown that the chondroitin sulfate chains on the larger proteoglycans had larger average molecular weights. The data suggest that the length of the core protein in the newly completed monomers is constant whereas the average chondroitin sulfate chain size varies from monomer to monomer. Polydispersity of newly synthesized monomers in these cultures, then, is primarily related to average chondroitin sulfate chain lengths rather than to variability in the length of the core protein.

(d) Effect of insulin, multiplication stimulating activity (MSA) and insulin-like growth factors (IGF-I and IGF-II) on proteoglycan synthesis

Insulin at 1 ng/ml and MSA at 100 ng/ml stimulate proteoglycan synthesis to nearly half maximal values obtained when chondrocytes are maintained in 15% fetal calf serum; 10 ng/ml insulin stimulates synthesis to the same extent or even higher than the 15% serum. The stimulation appears to be a general increase in anabolic pathways since total protein synthesis is stimulated to nearly the same extent as proteoglycan synthesis. Levels of insulin and MSA sufficient for nearly maximum stimulation are not sufficient to stimulate cell division. A series of binding experiments (in collaboration with Dr. Peter Nissley) were done using ^{125}I-labeled insulin, MSA, IGF-I and IGF-II. The results demonstrated that the chondrocytes contain an insulin receptor and at least one, and possibly two, additional receptors with affinity for IGF-I, IGF-II and MSA. Proteoglycan synthesis is stimulated by insulin at the same concentrations as those which displace ^{125}I-insulin from the cells. Stimulation of proteoglycan synthesis by IGF-I, however, occurs at much lower concentrations than required to displace ^{125}I-insulin

from the cells indicating that the IGF-I is most likely acting through a different receptor. Experiments are underway to study further the relationship between hormone binding and proteoglycan synthesis by chondrocytes.

3. Characterization of proteoglycans of cornea (in collaboration with Dr. John Hassell)

The characteristics of the keratan sulfate proteoglycan present in cornea and synthesized by corneal stromacytes in explant cultures is being studied further. Chondroitinase digestion, followed by density gradient, ion exchange and molecular sieve chromatography are being used to purify the keratan sulfate proteoglycans from the dermatan sulfate proteoglycans also present in cornea. The experiments suggest that there may be two closely related keratan sulfate proteoglycans. ^{35}S-Sulfate and ^{3}H-mannose labeled proteoglycan were combined with unlabeled keratan sulfate proteoglycan and the keratan sulfate chains isolated by molecular sieve chromatography after papain digestion. The keratan sulfate specific endogalactosidase is being used to remove the chains from the mannose-rich linkage oligosaccharide-peptide region and trifluoroacetolysis to remove the peptide for subsequent determination of the structure of the linkage region.

4. Characterization of proteoglycans of glomerular basement membrane (in collaboration with Drs. Marilyn Farquhar and Yoshpal Kanwar)

The lamina rarae of the basement membrane of kidney glomeruli contain heparan sulfate. Rat kidneys were maintained in vitro by perfusion in a medium which contained ^{35}S-sulfate. The glomeruli and then the purified basement membrane were isolated by a combination of techniques involving homogenization, band velocity centrifugation and deoxycholate extraction. A large proportion of the ^{35}S-labeled macromolecules in the purified glomeruli were subsequently shown to be present in the basement membrane by autoradiographic and chemical techniques. Extraction procedures were developed using detergents and guanidine HCl to solubilize about 70% of the labeled proteoglycans in the basement membrane. About 80% of the solubilized proteoglycans contained heparan sulfate with the remainder containing chondroitin sulfate. Both classes had average molecular weights of about 120,000. The heparan sulfate proteoglycan molecule was shown to contain about 4 chains of 20,000 molecular weight attached to the protein core. Experiments are underway to isolate and purify sufficient quantities of the heparan sulfate proteoglycan for further chemical and immunological characterization.

5. Synthesis of proteoglycans by aortic smooth muscle cells. (In collaboration with Drs. Thomas Wight and Russell Ross)

Smooth muscle cells have been isolated from monkey aorta and grown in culture. The proteoglycans synthesized and secreted into

the medium were studied using ^{35}S-sulfate as a precursor. Kinetics of synthesis and accumulation in the medium were nearly linear over 20 hours after refeeding. Two size classes of labeled proteoglycans were identified by molecular sieve chromatography in dissociating solvents. The larger (50-60% of the total) has a similar size distribution to cartilage proteoglycans and was shown to be able to aggregate with hyaluronic acid. This class, then, appears to be functionally similar to the major proteoglycan of cartilage. The second class of labeled proteoglycan was smaller and did not aggregate. Experiments are underway to determine the size and types of glycosaminoglycans in each class of proteoglycan, to study the details of the aggregation process for the larger proteoglycans and to study kinetics and structures of proteoglycans synthesized by these cells following stimulation with a platelet derived growth factor known to stimulate smooth muscle cells to divide.

Significance

Proteoglycans are major structural components of connective tissue. This is most obvious in cartilage where they in part determine the physical properties and form of the tissue 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. We can already gain a general understanding from the diversity of form of proteoglycans attained by varying the amount and kind of protein and polysaccharide.

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 aging. A broad approach will be continued emphasizing not only basic physical and chemical studies but also the role of proteoglycans in biological systems.

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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-00001-28-LB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Transglutaminases: Specificities, Physiological Functions and Catabolism of Products

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

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Gorman, Jeffrey J.	Visiting Fellow	LB	NIDR
Seelig, Gail F.	NIH Postdoctoral Fellow	LB	NIDR
Park, Myung H.	Visiting Fellow	LB	NIDR

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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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)

Specificity and catalytic mechanism studies on transglutaminases are underway. The relationships to cellular control processes and other physiological processes of polyamine-protein conjugates and biogenic amine-protein conjugates produced by these enzymes in both cells and body fluids is under investigation. Knowledge has been obtained concerning the catabolism of these amine-protein conjugates.

1. Project Description

Objectives

Studies carried out over the past several years have been directed toward characterization of the transglutaminases and elucidation of their physiological functions. These enzymes catalyze post-translational modifications of proteins through the formation of γ-glutamyl amide bonds. The sites of enzymic attack are the carboxamide groups of glutamine residues. In the presence of acceptor amines, substituted amides are formed ($-CONH_2 + RNH_2 \longrightarrow -COHNR + NH_3$).

Among the products of transglutaminase action found in proteins of cell and body fluids are ε-(γ-glutamyl)lysine bonds, γ-glutamyl-putrescine, γ-glutamylpolyamines, γ-glutamylhistamine and bis(γ-glutamyl)polyamine bonds. There has been increasing evidence for the formation and importance of these transglutaminase products in fibrin clots, cell membrane, myofibrils of muscle, proteins of seminal plasma, wool keratin, citrulline-containing proteins of hair and numerous unidentified cellular proteins. The wide occurence of transglutaminase products in biological systems and the recent suggestions for the involvement of these enzymes in such diversified events as receptor-mediated endocytosis and cell blastogenesis has led us to focus attention not only on the enzymes themselves, but on their biological roles and the mechanisms by which they express their cellular and extracellular functions.

This project is in part collaborative with S.I. Chung, Project #: Z01 DE-00049-09 LB

Major Findings

The specificity of activated human blood transglutaminase (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 the use of factor XIIIa and the labeled casein was subjected to chemical and enzymic degradation. Glutamine 167 was identified as the site of enzymic modification. Solid phase synthesis of a 15-member peptide containing the sequence of β-casein surrounding this glutamine residue was carried out. This peptide was found to be an excellent substrate for factor XIIIa. Approximately 20 peptides containing variation in this sequence were prepared and tested as substrates. The findings supply evidence that several aliphatic hydrophobic amino acid residues on each side of the substrate

glutamine are essential for optimum substrate properties and that a lysine residue close to the glutamine is also essential. These results form the basis for the design of excellent low molecular weight substrates for factor XIIIa.

A transglutaminase-mediated procedure for photochemical labeling of peptides and for production of cleavable crosslinks between protein molecules has been devised. Bifunctional amine substrates of the general structures:

$$H_2N - (CH_2)_2 - R_1 - (CH_2)_2 - R_2$$

where R_1 = -S-S-, $\overset{O}{\overset{\|}{C}} - \overset{OH}{\overset{|}{C}}H - \overset{OH}{\overset{|}{C}}H - \overset{O}{\overset{\|}{C}}$ - or ---

and R_2 = NH_2 or -NH- NO_2 $-N_3$

have been employed to prepare photolabeled peptide hormones for receptor studies and have been used for reversible crosslinking of model proteins. These reagents should extend the usefulness of transglutaminases as tools in protein and peptide chemistry.

Catalytic mechanism studies conducted on factor XIIIa have revealed that this two catalytic subunit enzyme reacts with chemical active-site directed reagents in either a half-of-the-sites or an all-of-the-sites fashion, depending upon the conditions used. This finding supports an hypothesis of cooperativity, both positive and negative, between subunits and is apparently the first example of an enzyme in which a change from one type cooperativity to another has been demonstrated.

Inactivation studies on the proenzyme forms of factor XIII and on the enzyme factor XIIIa, in the absence of the essential cation Ca^{2+}, have supplied evidence for preformed catalytic sites within these forms of enzyme, as is the case with several pancreatic proteases and with blood coagulation factors VII and X. The findings are consistent with a conclusion that proteolytic activation of zymogen does not itself result in sufficient conformational reorganization in the molecule to generate the substrate binding site, but that only after addition of Ca^{2+} is the enzyme capable of efficient binding of substrates.

A fragment of plasma factor XIII ($\underline{a}_2\ \underline{b}_2$) noncatalytic $\underline{b}$ subunits has been isolated from cyanogen bromide digests of this subunit. This fragment retains the structural features essential for binding to $\underline{a}$ chains, but has a molecular weight only about one-half that of native $\underline{b}$ chain,

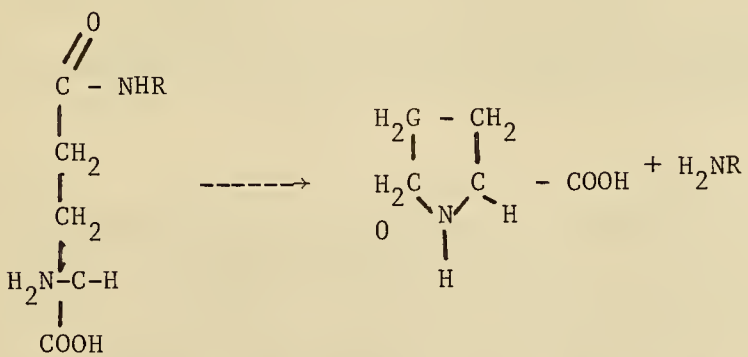
contains only a portion of the original carbohydrate, and is converted to several peptides upon reduction. A recombinant formed with a subunits is more liable to chemical modification than is native factor XIII.

Evidence has accumulated that putrescine and the polyamines, spermidine and spermine, serve as physiological substrates for transglutaminases in both cells and body fluids. Crosslinks between proteins through polyamines occur in one extracellular system tested, clotting of rat seminal fluid. In human peripheral lympocytes, although no evidence of crosslinking was obtained, protein conjugates of both putrescine and spermidine were found after treatment of cells with mitogens. In both systems the polyamines were found to be complexed through covalent γ-glutamyl linkage, strong evidence for their transglutaminase-catalyzed incorporation.

The finding of the covalent attachment of histamine to intracellular proteins in lymphocyte preparations is the first evidence that this important biogenic amine functions as a physiological substrate for transglutaminases. Cell fractionation studies indicate that lymphocytes are the cells involved.

During the course of studies with lymphocytes, a post-translational modification resulting in formation of an unusual amino acid, hypuscine, was recognized. Spermidine was found to be the precursor of this amino acid which occurs in only three small proteins of lymphocytes.

An enzyme that catalyzes the breakdown of ε-(γ-glutamyl)lysine, N-(γ-glutamyl)polyamines, N,N-bis-(γ-glutamyl)polyamines, γ-glutamylhistamine, and a variety of other γ-glutamylamines has been found in numerous mammalian tissues and cell types. This enzyme, called γ-glutamylamine cyclotransferase, was first observed in and partially purified from rabbit kidney. It has been shown to catalyze the following reaction:



Preliminary studies indicate that proteins that have been modified by transglutaminases are rapidly degraded by cell-free ATP-dependent

protein degradation systems to release γ-glutamylamines, the substrates for this enzyme and suggest that this enzyme is essential in the catabolism of products of transglutaminase action.

Significance

Knowledge of the molecular characteristics of the transglutaminases and of their catalytic mechanisms is vital to determination of the function of these enzymes in normal and diseased tissues as well as an understanding of the physiological and pharmacological control and regulation of their activity.

The minimal substrate structural requirements for transglutaminases have been defined over the past several years through studies such as those described. We now have a better understanding of the mechanisms 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.

Amines, both polyamines and biogenic amines, and the transglutaminases are widely distributed. Both the amines and the enzymes may play important roles in control of growth and other biological processes. The finding that these amines are natural substrates for transglutaminases is an important first step in determining the role each plays in these vital processes. That there may be differences in the manner in which the amines serve as substrates in cells and in body fluids may contribute to our understanding of their possible function in control of cellular and extracellular processes.

Until now little has been known about the catabolism of products of transglutaminase action. The characterization and determination of the specificity of an enzyme, γ-glutamylamine cyclotransferase, that catalyzes breakdown of ε-(γ-glutamyl)lysine and other γ-glutamylamines is certainly vital in understanding the turnover of transglutaminase-modified proteins.

Proposed Course

Specificity and catalytic mechanisms studies will be continued with special emphasis placed on the transglutaminases involved in hemostasis and wound healing. Principal objectives will be to determine the importance of amino acid side chains in close vicinity to substrate glutamines, and to continue investigation of the importance of multiple catalytic subunits, the role of proteolytic modification and the mechanism of cation-induced conformational alterations in enzyme activation.

Now that strong evidence has been obtained that the polyamines and histamine function as transglutaminase substrates, attempts will be made to determine how the products of these reactions participate in cellular and extracellular processes. First steps will involve characterization of the protein conjugates, identification of their cellular or extracellular location, and estimation of their metabolic rates and characteristics.

Knowledge of the specificity and distribution of the enzyme that breaks down γ-glutamyl derivatives should reveal much information about the basic catabolism of the products of transglutaminase action. For example, can crosslinks be hydrolyzed in intact proteins or must the proteins be largely degraded before the crosslinks can be cleaved? The answer to this question is of prime importance to a thorough understanding of the physiological role of these important enzymes.

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PERIOD COVERED

October 1, 1979 to September 30, 1980

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

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The physiological function and the mode of regulation of the transglutaminases are being studied. The roles of individual transglutaminases in cell membranes, in stimulation of specific cellular processes, and in connective tissue matrix stabilization are under investigation. A new transglutaminase apparently present in all cell membranes has been identified. It may function in chemotaxis and other cellular processes. The transglutaminase in uterus is derived from platelets. Together with fibronectin produced by uterine epithelia it may function in tissue stabilization.

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 membranes. A further function of the transglutaminases may be modification of proteins by the introduction of a biogenic amine (without crosslinking).

The present objectives are to characterize several different transglutaminases with special emphasis on their physiological function and regulation. The following summary is divided into three projects: (1) Uterine transglutaminase; (2) Cell membrane transglutaminase; and (3) Plasma and platelet transglutaminase (factor XIII).

Major Findings

1. Uterine transglutaminase

In continuing efforts to understand the biological role of the transglutaminases, utero-placental tissue was chosen as a system for study of the mode of synthesis and for identification of the proteins involved in enzyme-catalyzed cross-linking. A vital function of transglutaminase in this tissue has been suggested from observations of repeated spontaneous abortion and severe decidual bleeding in congenital factor XIII deficiency. Factor XIII, the transglutaminase in platelets, was found by the use of a monospecific antibody to the platelet zymogen to be distributed between the epithelial and stromal cell layers of rabbit uterus endometrium. Isolated epithelial and stromal cells, however, were shown to be essentially devoid of factor XIII providing substantial evidence that this zymogen is not synthesized in the uterus. The immunoidentity between the uterine and platelet zymogens suggests that blood platelets are the source of uterine factor XIII.

Since fibronectin is an important element in cell-matrix interactions and transglutaminase is believed to stabilize these interactions, the uterine system was examined for fibronectin production. From uterine epithelial cells in culture, fibronectin was isolated by immunoprecipitation from both the cell layer and the medium. Whereas the molecular weight of cellular fibronectin was identical to that of plasma fibronectin, the fibronectin released from cells was surprisingly of slightly higher molecular weight. Uterine epithelial cell fibronectin, together with the presence of factor XIII may be an important element in matrix stabilization during fetal development.

2. Cell membrane transglutaminase

A new transglutaminase has been identified and partially characterized from the membrane fraction of many cell types. These cells include various lines of fibroblasts, myoblasts, endothelial cells, embryonic cells, lymphocytes, polymorphonucleocytes, macrophages, CHO cells and chondrosarcoma cells, as well as both epithelial and stromal cell of rabbit uterus. Rat chondrosarcoma was chosen as the source of cells for study of this enzyme. In these cells, as in others, enzyme activity is increased significantly by treatment with mitogens or with trypsin or thrombin. The enzyme has been partially purified and shown by immunochemical and physical methods to be distinct from other known transglutaminases. Treatment of the purified enzyme with thrombin results in concomitant increases in enzymic activity and reduction in molecular weight from ∿94,000 to ∿89,000. One possible role for the membrane transglutaminase is suggested by the observation that several amines, among them the fluorescent amine, dansylcadaverine, inhibit chemotaxis in rabbit polymorphonuclear cells. (These are collaborative studies with Dr. E. Schiffmann, see project # Z01 DE-00006-20 DB.) During incubation of these cells with dansylcadaverine two proteins become labeled with fluorescent amine. Because this amine prevents transglutaminase-catalyzed cross-linking by acting as a substitute acceptor substrate, it is believed that the proteins identified may participate in some manner in chemotaxis. Studies done elsewhere have also implicated the enzyme in endocytosis.

3. Factor XIII

We have shown that animals rendered deficient in plasma factor XIII by the use of multiple injections of purified antibody to the zymogen display retarded wound healing. A single injection of antibody results in almost complete loss in plasma factor XIII for up to 72 hours and wound healing is delayed. The platelet count and fibrinogen levels were unaffected by this treatment. These findings are strong evidence that only plasma factor XIII, and not the platelet zymogen, participates in normal wound healing, that plasma factor XIII is not an accute phase protein, and that the platelet zymogen is not converted to plasma factor XIII within a 72 hour period.

Significance

The transglutaminases are ubiquitous enzymes involved in many biological processes. Except for fibrin crosslinking, very little is known about mechanism or regulation. The findings so far made, however, make it clear that a whole new area of biological investigation has been opened. The results will have important implications in stabilization of biological structure, in cell-matrix interactions, and in perhaps several cellular processes.

Proposed Course

The mode of activation and of involvement of membrane-bound-transglutaminase in such cellular processes as chemotaxis and receptor-

mediated endocytosis will be further investigated using a variety of cells. Attempts will be made to correlate the products of enzyme action with the cellular processes. Studies on the biosynthesis, release, and catabolism of platelet factor XIII will be carried out using guinea pig megakaryocytes. Regulation of factor XIII in rabbit uterus epithelial cells and its role in ovum implantation is under investigation. Attention will be focused on the in vivo covalent interactions between fibrinogen, fibronectin, and collagen as catalyzed by transglutaminases and their possible relationships to cell adhesion and proliferation in wound healing.

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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-00002-30 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structural Studies on Collagen

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

Trus, Benes L.	Senior Staff Fellow	LB NIDR
Piez, Karl A.	Chief	LB NIDR

COOPERATING UNITS (if any)
R. Feldmann, DCRT; A. Steven, M. Navia, V. Nikodem, NIAMD; M. Elzinga, Brookhaven National Laboratory, Upton, New York; Dr. Michael Beer, Johns Hopkins University; Dr. Joseph Wall, Brookhaven National Laboratory.

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.25	1.50	.75

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The primary goal of this project is an understanding of collagen structure from the molecular to the fibril level. Emphasis is presently on two main aspects of the general problem. 1) Conventional and scanning transmission electron microscopy and analysis of micrographs to obtain quantitative data and reveal detail not obvious to the eye. 2) Three-dimensional computer models to study and illustrate structural features of collagen and molecular aggregates. Each of the above two approaches has led to the development of computer software that is being applied to other problems in protein chemistry. These include myosin structure and the computer analysis of two-dimensional gels of protein mixtures. A third aspect of the problem recently initiated is to analyze x-ray diffraction data as a means of selecting from among possible molecular packing models of collagen.

1. Project Description

Objectives:

The primary objective of this project is to determine the structure of the collagen molecule and of aggregates of collagen including the native collagen fibril. Several approaches are being taken. 1) Scanning transmission (STEM) and conventional electron microscopy (CEM) of collagen aggregates to reveal details not obvious to the eye and to provide quantitative data. 2) Computer modeling of collagen molecules and aggregates to illustrate and test proposed structures. 3) Analysis of x-ray diffraction patterns of collagen in relationship to proposed models. Secondary objectives are to extend the computer modeling programs developed for collagen to myosin and to use image processing procedures developed for electron micrographs to other kinds of images.

Methods Employed:

For CEM, negatively stained specimens have been used. Procedures have been devised to obtain micrographs under minimal beam exposure to decrease radiation damage. STEM is being done in collaboration with scientists at Johns Hopkins using single-atom staining procedures and at Brookhaven using direct visualization of unstained specimens and mass measurements. Computer methods have been developed in collaboration with scientists in NIAMDD and DCRT to process electron micrographs (and other images).

Major Findings:

Early aggregates formed during collagen assembly (see Z01 00215-04 LB) have been visualized both by CEM of negatively stained specimens and by STEM of unstained specimens. Under favorable conditions single collagen molecules can be seen. These aggregates are long thin structures containing many collagen molecules in a characteristic overlapping array. The number of molecules and their relationship is being investigated. In these studies type I collagen has been used.

Another approach to determining structure by STEM is to stain specific functional groups with single heavy atoms which can then be seen by STEM. From the known amino acid sequence, the staining pattern can be predicted. Preliminary experiments have been done in collaboration with scientists at Johns Hopkins on SLS aggregates of collagen and on single collagen molecules as control experiments. It has been possible to specifically stain methionine residues with a Pt reagent and carbohydrate residues with an Os reagent.

The image processing system is now being used as more and better electron micrographs of collagen are becoming available. The system is also being used by other investigators at NIH with similar research problems. A new application of the system is to analyze and compare two-dimensional patterns of protein mixtures.

The molecular modeling programs have been used to produce a thirteen minute color movie of collagen structure. The movie starts by illustrating molecular structure, shows the relationship between two molecules, and concludes by depicting the 5-stranded microfibril which is believed to be a substructure of the native fibril. A final version of the movie has been produced and a sound track has been added. It has been shown to various audiences at meetings and seminars around the world.

The myosin computer program has been used to study the pitch of myosin along two segments of available sequence. The pitch can be changed dynamically along the partial sequence to accomodate systematic local variations and thus predict structure and, later, intermolecular relationships.

To resolve a conflict between a recently proposed model of the collagen fibril based on x-ray diffraction evidence and the microfibril model, supported by other data, we have reinterpreted the x-ray diffraction data. We have proposed a revised model which contains "compressed" microfibrils placing molecules on a pseudohexagonal lattice. The unit cell provides an accurate fit of the observed reflections on the x-ray diffraction pattern and is consistent with other data.

Significance:

Collagen is the major protein of connective tissue and is found in various forms throughout the body. Through interactions with other macromolecules such as proteoglycans, mineral, and cells, it plays an important role in many biological processes during development and in pathological states. These studies on collagen structure are important to understanding these interactions.

Proposed Course:

The principal investigator on this project will transfer to DCRT in the coming fiscal year. He will continue to develop and apply computer procedures for image processing and molecular modeling in collaboration with us and with other investigators.

Emphasis will continue to be placed on the development of procedures for specimen preparation of collagen aggregates, CEM and STEM of these aggregates and analysis of electron micrographs. As this project progresses, collagen types other than type I and interactions of collagen with other components such as fibronectin and proteoglycan will be studied by the same techniques.

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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-00157-05 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

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

Torchia, Dennis A.	Biophysicist	LB	NIDR
Jelinski, Lynn W.	Staff Fellow	LB	NIDR
Batchelder, Lynne S.	NIH Postdoctoral Fellow	LB	NIDR

COOPERATING UNITS (if any)
Dr. A. N. Schechter, NIAMDD

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Laboratory of Biochemistry

SECTION
Protein Chemistry Section

INSTITUTE AND LOCATION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.40	3.05	1.35

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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 and proteoglycans, and to study intracellular gelation of Hemoglobin S. The structural information obtained will be correlated with function. Areas of present interest are 1) Molecular structure and dynamics of collagen. ^{13}C and ^{2}H magnetic resonance techniques are being used to study the structure and interactions in collagen 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) ^{13}C magnetic resonance is being used to study the extent and mechanism of hemoglobin S gelation in erythrocytes. For these studies, magnetic resonance spectrometers have been assembled which give ^{2}H, ^{13}C and ^{31}P spectra of solids. High power decoupling, cross-polarization, magic angle spinning, and solid echo experiments are all performed.

Introduction

The goal of this work is to determine aspects of the molecular structure and interactions of macromolecules in connective tissue and to elucidate structure-function relationships.

Methods

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 rigid structures having high molecular weights 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 is 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. In addition, cross-polarization can be combined with rapid spinning of the sample about the magic axis (an axis making an angle of 54.7° with the external magnetic field) to produce spectra having small linewidths, comparable to those obtained for samples in solution. In the case of ^{2}H nuclei, decoupling is not required and spectra can be measured directly using a solid echo technique.

We have built two pulsed nmr spectrometers which provide solid state spectra of ^{2}H, ^{13}C and ^{31}P. 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, and magic angle spinning spectra can be obtained for ^{31}P. 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. Deuterium quadrupole coupling constants and lineshapes are obtained from ^{2}H spectra and are sensitive to molecular motions that occur on the 10^{4} - 10^{6} timescale.

Unlike ^{31}P, the natural abundances of ^{2}H and ^{13}C are low (0.016 and 1.1%, respectively). Hence, it is advantageous to incorporate labeled amino acids into proteins under study. The presence of the label greatly simplifies interpretation of the spectra since the ^{2}H and ^{13}C resonances can readily be assigned to the labeled sites. We have used biosynthetic techniques to incorporate ^{2}H and ^{13}C labeled amino acids into collagen and proteoglycans.

Progress

1. Molecular Structure and Dynamics of Collagen. Spectra of chick calvaria collagen fibrils containing a ^{13}C-labeled amino acid (Gly, Ala, Lys, Glu, or Met) have provided strong evidence that rapid, anisotropic molecular motion occurs in the helix backbone and in the labeled sidechains. The emphasis has now shifted to ^{2}H nmr studies of collagen fibrils. The calvaria culture system has been used to incorporate ^{2}H labeled Ala, Pro and Leu into collagen. Analysis of lineshapes has confirmed the presence of backbone motion in the fibril, through an azimuthal angle of at least 30°. The Pro and Leu sidechains exhibit local motions in addition to the backbone reorientation. We are currently elucidating these motions via analysis of the deuterium spectra.

2. Proteoglycan Dynamics and Structure. We have prepared samples of the chick limb bud proteoglycan monomer containing serine ^{13}C-labeled at C^{β} and glycine labeled at C^{α}. Linewidths, signal intensities and relaxation times measured for the labeled carbons indicate that the protein backbone and the point of attachment of the polysaccharide to the core protein undergo isotropic reorientation with a rate that is 3-4 orders of magnitude faster than the motion calculated for the proteoglycan monomer as a whole. This result implies that the core protein is segmentally flexible. Chain flexibility is non-uniform in the intact monomer since computer analysis shows the observed signal to be composed of broad and narrow components. Heterogeneity in flexibility is consistent with the complex chemical structure of the proteoglycan, and we have assigned the broad and narrow signal components to the various regions of the molecule on the basis of the chemical structure. (N. B. This is a collaboration with Dr. V. C. Hascall, see project #Z01 DE-00134-05 LB.)

3. ^{13}C Nmr of Hemoglobin S Gelation. We showed earlier that ^{13}C nmr provides a reliable means to quantitate the amount of polymer in a cell-free deoxygenated hemoglobin S gel. Using the nmr technique we have now shown that the amount of deoxyhemoglobin S polymer within red cells increases almost linearly with decreasing oxygen saturation. Due to the excluded volume effect of oxyhemoglobin in the cell, polymer is detected at oxygen saturation values above 95%. Theoretical curves for the relationship of polymer fraction to oxygen saturation fit the nmr data well. (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 ^{2}H and ^{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 the nature of the molecular interactions that determine the structure and function of the macromolecules investigated. In addition, ^{13}C nmr is a promising method for investigating the mechanism of gelation within intact red cells and the activity of potential inhibitors.

Future Plans

The general strategy of using solid state nmr to study labeled macromolecules will be followed. Our goal is to elucidate in detail the nature of sidechain motions within fibrils. Emphasis will also be placed upon solution studies of collagen using the high field proton nmr spectrometers that have recently become available at the NIH. In particular we will investigate the structure of the non-helical regions of the molecule at various stages during fibril formation. Hemoglobin S studies will continue to concentrate on investigating hemoglobin gelation within the red cell.

Publications

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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 ZO1 DE-00215-04 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

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

Lee, Sandra L.	Staff Fellow	LB NIDR
Piez, Karl A.	Chief	LB NIDR

COOPERATING UNITS (if any)

None

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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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) The mechanism of collagen fibril formation in vitro. 2) The size and structure of early intermediates in assembly. 3) The effect of noncollagenous molecules on collagen assembly in vitro. We have developed a reproducible in vitro assembly system and shown that collagen fibril formation is a multi-step process. The first step leads to an aggregate of 5-100 molecules <8 nm wide and >1500 nm long. This aggregates grows in length and may reorganize to form a D-periodic thin filament. The filaments then assemble laterally to form native collagen fibrils.

Project Description

Objectives

1. The mechanism of collagen fibril formation *in vitro*.

The development of a well-characterized, reproducible system for the study of collagen fibril formation *in vitro* and of models of fibril structure has made it possible to investigate the mechanism of collagen assembly in greater detail than previously possible.

2. The size and structure of early intermediates in assembly.

Quasielastic light scattering affords a technique for the study of the size and shape of macromolecules in solution and is well suited for studies of the early stages of collagen assembly where small intermediates form. Another approach recently initiated is to examine the location of covalent crosslinks that form spontaneously. Identification of the positions of the crosslinks places constraints on possible models.

3. The role of noncollagenous molecules in collagen assembly and fibril structure.

In vivo, collagen assembles in the presence of a variety of macromolecules and low molecular weight substances. Although none of these (except perhaps phosphate) appears to be necessary for *in vitro* assembly, it is likely that they may be involved *in vivo*, perhaps to regulate the process. Of particular interest are proteoglycan, lysyl oxidase and fibronectin. Preliminary experiments are being done to study the effect of proteoglycan on *in vitro* assembly.

Methods Employed

Type I collagen is prepared from 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, turbidity, quasielastic light scattering and chemical determination of crosslink location.

Progress

1. 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. We have found that collagen assembly occurs *via* a mechanism of at least three steps. Step 1, initiation, involves a temperature-dependent change which leads to an intermediate aggregate. Step 2 is linear growth of thin filaments by a temperature-independent process. The mechanism of growth is not know but may require a reorganization of molecules. Step 3 is lateral association

of filaments by a temperature-dependent process. The concentration dependence of the rates of steps 2 and 3, and the lack of a measurable critical concentration suggest assembly by accretion. Collagen treated with pepsin, which removes the nonhelical ends, makes distorted fibrils by a similar mechanism. However, assembly is very much slower and step 1 is markedly altered. The nonhelical ends therefore are critically involved in assembly.

2. Size and structure of early intermediates in assembly.

Quasielastic light scattering has shown that an aggregate <8 nm is diameter and >1500 nm long is an early intermediate and a product of Step 1. It has been seen by both conventional electron microscopy and by scanning transmission electron microscopy (see project Z01 DE-00002-30 LB) but its structure has not yet been determined. Chemical methods are currently being devised to determine structure.

3. The effect of proteoglycan on collagen assembly in vitro.

We confirmed last year that the rate of type I collagen assembly is retarded in the presence of a cartilage-type proteoglycan from a rat chondrosarcoma. Proteoglycan also binds tightly to the collagen but does not appear to alter the structure of the fibril. On the other hand, proteoglycan slightly accelerates the assembly of type II collagen and does not bind. Since type I and II collagens are basically similar molecules and form similar fibrils, this result argues for a highly specific interaction between collagen and proteoglycan. It seems unlikely that the effect of proteoglycan depends on some nonspecific property such as its polyanionic character. Additional preliminary studies were undertaken in the current year, but this aspect of the project has had a low priority. A related study on the effect of phosphoprotein on the kinetics of fibril formation was done. An interaction between phosphoprotein and collagen was suggested by a change in the kinetics.

Significance

The basic properties of connective tissues can be described in terms of the macromolecules of which they are composed. Of these, collagen is the major structural protein. Collagen is present as native fibrils which vary in diameter and in their higher level organization as a function of species, tissue and developmental stage. Information regarding the mechanism of assembly and interactions with other macromolecules provides a basis for continued research on development in tissues and on the relationship of structure to disease.

Proposed Course

1. Emphasis will be placed on determining the structure of intermediates in assembly as a means of understanding mechanism. Chemical and electron microscopic methods will be developed further and applied to our fibril forming system.

2. Our studies of the mechanism of in vitro assembly of collagen will be extended to collagen types other than type I. We will also investigate the role of other macromolecules in the process.

3. Because of personnel and space constraints, the quasielastic light scattering instrument has been transferred to another Institute at NIH where it will be used on other projects.

Publications

Gelman, R. A., Poppke, D. C. and Piez, K. A.: Collagen fibril formation In Vitro. The role of the nonhelical terminal regions. J. Biol. Chem. 254: 11741-11745, 1979.

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Piez, K. A., Gelman, R. A., Williams, B. R. and Poppke, D. C.: Assembly of collagen In Vitro. Proceedings of the Seventh Aharon Katzir-Katchalsky Conference. in press.

Gelman, R. A., Conn, K. M. and Termine, J. D.: The effects of phosphoproteins on collagen self-assembly in tail tendon and incision dentin from rats. Biochim. Biophys. Acta. 630: 220-224, 1980.

ANNUAL REPORT
Laboratory of Biological Structure
National Institute of Dental Research

The research efforts of the Laboratory of Biological Structure are focused on the elucidation of the structural, chemical and functional characteristics of the hard and soft tissues of the oral cavity. Of the four research groups comprising the laboratory, the activities of three are directed toward skeletal and dental tissues, with the objectives of understanding their normal growth and development, characterizing their synthetic products, and determining the physicochemical and biological mechanisms of mineralization. The pursuit of these common goals through widely divergent approaches forms the basis for fruitful collaborative efforts between these groups. While the program of the fourth group deals primarily with secretory tissues, its morphological orientation also lends itself to collaborative studies with the other research groups. As is evident from the individual project reports, LBS thus has the capacity to deal with significant biological problems employing an integrated investigative approach.

The programs of the laboratory were reviewed by the Board of Scientific Counselors in April 1980. Apart from their assessment of the scientific merit of our activities, they were unanimous in their recognition of our need for additional space and personnel. Through the cooperation of the Director of Intramural Research, we are attempting to achieve some relief from our overcrowded conditions. It is hoped that the recent trend towards reduction in scientific staff will soon be halted or reversed, so that the continuity, quality and productivity of our research programs can be maintained.

Bone Cell Biology

The Bone Cell Biology program of LBS has continued to make important contributions to our understanding of the cellular and molecular events involved in cartilage and bone differentiation. Fibronectin, a cell surface glycoprotein known to mediate cell-substratum interactions *in vitro*, has been shown for the first time to play a similar role in the *in vivo* development of cartilage and bone. Circulating fibronectin binds to the implanted particles of demineralized matrix, and appears to initiate the attachment and subsequent proliferation of mesenchymal cells. Antibodies to fibronectin inhibit the matrix-mesenchymal cell interaction, reducing cell proliferation and chondroprogenitor cell differentiation. Biosynthetic studies revealed that fibronectin is synthesized and present throughout the development of the bone plaques. Localization of fibronectin by immunofluorescent techniques revealed that, in addition to fibroblasts and mesenchymal cells, it is associated with chondrocytes and cartilage matrix, osteoblasts, and developing hematopoietic colonies.

The synthesis and accumulation of other specific proteins during matrix-induced bone differentiation has also been followed. The synthesis of type III collagen is maximal during mesenchymal cell proliferation, while type I collagen is synthesized during bone formation and remodeling. The accumulation of osteocalcin, a γ-carboxy glutamic acid-containing protein, begins during cartilage mineralization and continues throughout bone formation.

In a continuation of studies initiated last year on the effects of diabetes and insulin on cartilage and bone differentiation, the local effects of insulin were studied. Insulin has a direct effect on skeletal tissues, since in diabetic animals, local insulin corrects the impaired mesenchymal cell proliferation, and in non-diabetic animals, local injections of insulin antibodies inhibit cell proliferation. These clear-cut results further emphasize the utility of the matrix-induced system for studies of the normal mechanisms of cartilage and bone development, as well as its potential for increasing our understanding of a number of skeletal diseases.

Skeletal Matrix Biochemistry

Continued progress has been made in studies on the matrix proteins of teeth and bones. Comparative studies of enamel and dentin matrix proteins have revealed many similarities between several species, as well as some striking differences. For example, the composition of the guanidine-EDTA soluble glycoproteins of adult human enamel is similar to that of the fetal bovine enamelins. In contrast, a portion of the enamelin-like human protein forms a composite with enamel apatite which is insoluble in guanidine-EDTA. The neonatal hamster molar amelogenins have been found to parallel their fetal bovine counterparts in regard to the specific classes present, their electrophoretic and chromatographic profiles, and their shift in molecular size as tooth maturation progresses. The enamelin fraction, however, contains some much larger components, while the dentin phosphoprotein appears to be somewhat smaller, than their respective fetal bovine counterparts. Several amelogenin components, ranging in size from 32,000 - 6,000 daltons have now been isolated from different species, and their properties and interrelationships are being sorted out. It has been found that three of these components have identical amino terminal residues, and the largest and smallest share common cyanogen bromide fragments.

In a related morphological study, the organic coats on enamel crystals have been examined by electron microscopy in thin sections of rat and hamster teeth. The organic envelopes are closely apposed to the crystals, and appear to be 20-70 Å in thickness. The envelopes appear similar in chemically-fixed as well as freeze-dried material, suggesting that they are not an artefact of preparation. Presumably, this material represents the crystal-bound enamelin proteins. The location of the amelogenins has not been clearly identified, but examination of teeth extracted with 4M guanidine, which removes amelogenins, suggests a possible loss of material from the area between the organic envelopes in regions of newly secreted matrix.

A major new long-term effort has been undertaken to isolate and characterize the noncollagenous proteins and proteoglycans of fetal bovine subperiosteal bone. At present, 4-5 apatite crystal-specific proteins have been identified, based on their solubility in guanidine-EDTA. It is anticipated that these studies will provide significant advances in our understanding of the structural and functional relationships between the various constituents of bone matrix, and their respective roles in mineralization.

Mineral Chemistry and Structure

Work continued during the past year on the characterization of the effects of various organic and inorganic ions on apatite formation in synthetic calcium phosphate solutions. Citrate ion, a constituent normally present in mineralizing tissue, decreases the rate of conversion of the octacalcium phosphate intermediate to the more apatite-like crystalline phases when present in physiological concentrations. At higher concentrations it also has a stabilizing effect on the amorphous phase. Thus local and/or systemic variations in citrate concentration may be important in determining the physical and chemical characteristics of the inorganic component of hard tissues. The inhibition of apatite formation caused by magnesium ions occurs irrespective of the mode of induction of crystallization, i.e., in reactions proceeding by intermediates, the formation and hydrolysis of the OCP phase is retarded, while in reactions where the solution parameters rule out intermediate formation, magnesium apparently inhibits the growth of new apatite directly on the seed surface.

Spectroscopic studies of mineral structure continued to focus on the development of quantitative procedures for hydroxyl ion content in apatite. A variety of parameters related to crystal size and orientation, purity, laser heating and resonance Raman effects are being systematically examined in order to improve the accuracy of the methods. Raman microprobe studies had previously suggested that the mineral huntite, $Mg_3Ca(CO_3)_4$, may be present at initial sites of mineralization. An extension of this work has now shown that huntite can serve as a seed for the formation of apatite from metastable calcium phosphate solutions. It is hoped that further experiments will shed additional light on the possible significance of huntite in the early stages of mineralization of skeletal and dental tissues.

A new project has been initiated this year to examine the physicochemical characteristics of intracellular mineral deposits, such as occur in mitochondria and platelet dense bodies. Synthetic analogues prepared in vitro closely mimic the properties of the platelet dense bodies. The initial results are providing information on the mode of serotonin storage in these bodies, the permeability requirements of their membranes and the properties of their matrix.

Experimental Morphology

Studies of secretory cell structure and function have continued to focus primarily on the Golgi apparatus, and on lysosomal function related to endocytosis and plasma membrane internalization. Studies of stimulated secretory cells have revealed interesting changes in the morphology and cytochemistry of the Golgi apparatus and GERL. In both parotid acinar cells and neurosecretory neurons, cytochemical activity characteristic of the Golgi saccules, i.e., thiamine pyrophosphatase, is present in GERL-like structures and immature secretory granules, and immature granules are frequently observed forming from the Golgi saccules. These results indicate that modulation in enzymatic and structural properties of the Golgi apparatus and GERL may occur with changes in functional activity. Further, they suggest that the Golgi saccules and GERL are closely related, forming a functional and possibly structural continuum. Despite these similarities, specific differences between the responses of these two cell types to stimulation have also been observed. For

example, stimulation of the neurosecretory neurons leads to a marked reduction in acid phosphatase activity in GERL, while the opposite appears to occur in parotid acinar cells.

The endocytosis of macromolecular tracers and their intracellular fate has been followed in exocrine acinar cells and in the endocrine cells of the anterior pituitary gland. In exocrine cells, intravenously injected tracer is endocytosed by coated vesicles and initially accumulates in a basally-located lysosomal system. Secretagogue stimulation enhances the uptake of tracer, suggesting that this process may be related to the internalization of cell surface receptors. This basolateral endocytosis contrasts with that occurring at the luminal surface, which appears to involve smooth "c" or ring-shaped vesicles and the sequestration of tracer in multivesicular and dense bodies. Double labeling experiments, however, have shown that eventual mixing of tracers endocytosed from either surface occurs within secondary lysosomes. In the anterior pituitary, endocytosed tracer also accumulates in secondary lysosomes. In the somatotrophic cells, which secrete growth hormone, tracer was also found in GERL. While the functional significance of this localization is presently unclear, it is apparent that different cell types are capable of handling the same tracer in different ways.

In a related study, the effects of the retrograde ductal injection of radiographic contrast medium in the rat submandibular gland are being examined. This procedure, known as sialography, is used clinically for diagnostic purposes, but neither the short nor long term effects on the salivary glands have been studied. Our initial results with water soluble contrast medium indicate a variable pattern of cellular damage, and extensive leakage of medium into the intercellular and connective tissue spaces. Future studies will examine the effects of lipid-soluble contrast medium, and attempt to correlate the observed morphological changes with the radiographic appearance and intra-ductal pressure.

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Broadwell, R.D.: Cytochemical localization of acid hydrolases in neurons of the mammalian central nervous system. J. Histochem. Cytochem. 28: 87-89, 1980.

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Eanes, E.D.: The influence of fluoride on the seeded growth of apatite from stable supersaturated solutions at pH 7.4. J. Dental Res. 59: 144-150, 1980.

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Hand, A.R.: Cytochemical differentiation of the Golgi Apparatus from GERL. J. Histochem. Cytochem. 28:82-86, 1980.

Hascall, Gretchen K.: Cartilage proteoglycans: comparison of sectioned and spread whole molecules. J. Ultrastruct. Res. 70:369-375, 1980.

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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 00028-13 LBS

PERIOD COVERED

October 1, 1979 to September 30, 1980

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

Hand, Arthur R.	Chief, LBS	LBS NIDR
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Ho, Betty	Biologist	LBS NIDR
Lenk, Elaine V.	Visiting Associate	LBS NIDR
Qwarnstrom, Eva E.	Visiting Fellow	LBS NIDR
Doine, Aurora I.	Guest Worker	LBS NIDR
Waters, Judith F.	Biologist	LBS NIDR
Mednieks, Maija	Staff Fellow	LBS NIDR
Weiss, Roy E.	Postdoctoral Fellow	LBS NIDR
Youmans, Patricia A.	Secretary (steno)	LBS NIDR
DeGraff, Barbara A.	Purchasing-agent	LBS NIDR
Floyd, Steven W.	Photographer (laboratory)	LBS NIDR
Frear, Cindy	Student Trainee	LBS NIDR

COOPERATING UNITS (if any)

Dr. Lois Tice, LEP, NIAMDD; Dr. Bruce J. Baum, GRC, NIA

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Experimental Morphology Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.04	3.25	2.79

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)

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 basic biochemical procedures. Major areas of investigation are: (1) the structure and function of the Golgi apparatus and GERL; (2) experimental pathology and lysosome function in salivary glands; (3) salivary gland duct cell structure and function, and (4) the effects of sialographic procedures on the structure of the rat submandibular gland.

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, radioautoraphic 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 structure and function of the Golgi apparatus and GERL; (2) experimental pathology and lysosome function in salivary glands; (3) salivary gland duct cell structure and function, and (4) the effects of sialographic procedures on the structure of the rat submandibular gland.

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 labeled tissue are prepared by the method of Kopriwa (1973), and EM radioautographs are analyzed by the procedure of Nadler (1973). Biochemical determinations of protein, DNA, amylase, peroxidase, and other enzyme activities follow standard procedures.

Major Findings:

Golgi Apparatus Structure and Function: Substantial evidence exists implicating the Golgi apparatus as an important site of secretory protein transport, modification, and packaging. Considerable heterogeneity, both structural and chemical, occurs within the Golgi apparatus. Our interests lie in relating this heterogeneity to the specific functions of the Golgi apparatus. Our previous studies suggest that GERL, a smooth membrane system located at the trans aspect of the stack of Golgi saccules and cytochemically reactive for acid phosphatase, is involved in secretory granule formation. Immature secretory granules exhibit acid phosphatase activity, and show direct membrane continuities with GERL, but we have been unable to convincingly demonstrate secretory proteins within GERL. These results raise questions concerning the route of secretory protein transport through the various components of the Golgi region, the origin of GERL and its precise relationship to other organelles, and the function of acid hydrolases in GERL and secretory granules.

Stimulation of parotid and lacrimal acinar cells by *in vivo* administration of secretagogue, in addition to causing granule discharge, induces structural and cytochemical alterations in the Golgi apparatus and GERL. GERL initially was increased in extent and acid phosphatase activity, returning to its original size and activity by 16 hours after secretory stimulation. The trans Golgi saccule, which contained thiamine pyrophosphatase activity, frequently resembled GERL in that it was narrowed and separated from the remainder of the Golgi saccules. Small immature granules containing thiamine pyrophosphatase reaction product were in continuity with the trans saccule, while larger immature granules were in continuity with GERL and contained acid

phosphatase activity. These findings indicate, as did our previous studies on the recovery of parotid acinar cells from ethionine intoxication, that modulation of enzyme activities in the Golgi apparatus and GERL may occur during changes in physiological activity. Further, they suggest that secretory granule formation may be initiated by the trans Golgi saccule but completed by GERL, and that the trans Golgi saccule, by alteration of its structural and cytochemical properties, may give rise to GERL.

Studies on parotid acinar cells of animals from 5 days old to young adult have also demonstrated changes in the structure and cytochemistry of the Golgi apparatus and GERL. During differentiation of the acinar cells, which at these ages was primarily manifested by synthesis and accumulation of secretory material, the Golgi apparatus became very extensive and the saccules became broadened and highly fenestrated. GERL, which consisted primarily of a few short cisternal segments lying adjacent to the trans saccule at 5 days of age, increased in extent with increasing age. Immature secretory granules initially were reactive for thiamine pyrophosphatase, but by 15 days of age they contained reaction product for both acid phosphatase and thiamine pyrophosphatase activity. By 30 days of age the structural and cytochemical properties of the Golgi apparatus and GERL were similar to those of adult animals.

Experimental Pathology and Lysosome Function: The morphological alterations of parotid acinar cells induced by a number of experimental procedures are being studied, with particular reference to the role of lysosomes in the production and resolution of the observed changes. Diabetes induced by streptozotocin administration has been found to markedly affect the structure of the parotid acinar and duct cells. The acinar cells accumulated large amounts of lipid, initially as small basal droplets, which apparently coalesced to form a large inclusion, giving the cell an overall appearance similar to an adipocyte. Many cells also accumulated crystalloids, similar to those observed during ethionine treatment. Lysosomes increased in number and size, but interestingly, the crystalloids were usually unreactive for lysosomal hydrolases in cytochemical preparations. Despite these extensive alterations, the cells apparently continued to synthesize and package secretory material. The cells of the striated ducts contained cytoplasmic vacuoles filled with dense amorphous material and crystalloids similar to those found in the acinar cells. As demonstrated for other tissues in humans and experimental animals, an increase occurred in the thickness of epithelial and vascular basement membranes. These morphological changes were slow to develop, compared to the rapid changes ($\sim$ 24 hr) observed in serum insulin, glucose and triglyceride levels. The peak cellular alterations occurred between 2-4 months after induction of diabetes, after which a partial recovery was noted, paralleling a return of the serum chemistry values towards normal.

Changes occurring in the parotid glands of aging animals (up to 27 months) were similar in some respects to those seen in diabetes. Lysosomes were very large, containing considerable lipid-like material. These large lysosomes were usually poorly reactive for acid hydrolases. Thickening of the basement membranes was also frequently observed. No major differences

from younger animals were observed in the organelles involved with secretory protein processing and transport, however.

Duct Cell Structure and Function: The striated or intralobular portion of the duct system of salivary glands is known to participate in salivary electrolyte resorption and secretion. Less is known about the ability of the ductal cells to modify the organic content of saliva. Radioautographic studies employing ^{3}H-fucose indicate that the striated duct cells actively synthesize both secretory and plasma membrane glycoproteins. Resorption of the organic constituents of the saliva has also been considered a property of the duct cells, but experimental evidence for this function is limited. In a previous study involving retrograde infusion of horseradish peroxidase into the main excretory duct of the parotid gland, reaction product was observed in apical vesicles of the striated duct cells. These tissues are being reexamined to characterize the nature of the resorption process and the fate of the internalized tracer. The observation of large cytoplasmic inclusions in the striated duct cells of animals with streptozotocin-induced diabetes provides additional evidence for the resorptive function of these cells.

Effects of Sialographic Procedures on the Structure of the Rat Submandibular Gland.

Our studies have thus far concentrated on the effects of infused water soluble contrast medium on the submandibular gland, and the distribution of the medium within the glandular parenchyma. Since the contrast medium is not retained within the tissues during routine processing, horseradish peroxidase (HRP) is added to the medium as a light and electron microscopic tracer. The results suggested that: (1) initial morphological changes were caused by the saliva present within the ductal system at the start of the infusion; (2) the intercalated, granular and striated ducts exhibited the greatest changes, while the acini and the excretory ducts appeared unaffected; (3) the distribution of the contrast medium throughout the gland was very uneven; (4) considerable leakage of medium from the lumen to the intercellular and connective tissue spaces occurred, probably through the junctional complexes joining adjacent cells; and (5) contrast medium was removed from the gland via the vascular and lymphatic systems.

Infusion of isotonic saline containing HRP was utilized as a control for the contrast medium. Although differences were apparent in the pressure response of the gland, the morphological effects and the distribution of the saline-HRP were very similar to those of the contrast medium. One minor difference appeared to be related to differences in pH and thus the net charge of the HRP in the two infusion solutions: binding of HRP to the luminal membrane of the duct cells was observed with saline but not with the contrast medium.

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. Although sialography is used as a clinical diagnostic procedure, little is currently known of the

effects of sialography on the salivary glands. Our studies should broaden the scientific basis of this procedure, potentially increasing its diagnostic utility and decreasing the possible damaging effects on the tissue.

Proposed Course:

Studies on the structural and cytochemical alterations of the Golgi apparatus and GERL following secretory stimulation will be continued, and immunocytochemical studies of secretory protein and lysosomal enzyme localization within the Golgi region will be initiated. A preliminary study to assess the feasibility of employing serial thin sections and computer graphics to reconstruct the three dimensional structure of the Golgi apparatus will be undertaken. Work will continue on a recently initiated morphological study of the parotid gland hypertrophy caused by chronic isoproterenol injection, and the regression of the gland after cessation of drug administration. A freeze-fracture study of junctional complex structure in resting and stimulated parotid glands has been initiated in collaboration with Dr. Lois Tice, NIAMDD. Emphasis will be placed on possible changes in junctional structure related to increased epithelial permeability. Additional experiments will be undertaken to evaluate junctional structure in hypertensive animals. The sialographic experiments will focus on the effects and distribution of lipid-soluble contrast medium, and on the recovery of the glands following the sialographic procedure. Finally, we plan to begin studies on the localization of peptide hormone and neurotransmitter receptor sites in salivary glands and other tissues.

2. Publications:

Hand, A.R.: Synthesis of secretory and plasma membrane glycoproteins by striated duct cells of rat salivary glands as visualized by radioautography after ^{3}H-fucose injection. Anat. Rec. 195:317-340, 1979.

Hand, A.R.: Cytochemical detection of peroxisomal oxidases. J. Histochem. Cytochem. 27:1367-1370, 1979.

Hand, A.R.: Salivary Glands. In Bhaskar, S.N. (ed.): Orban's Oral Histology and Embryology, 9th Edition. St. Louis, C.V. Mosby Co., pp. 336-370, 1980.

Hand, A.R.: Cytochemical differentiation of the Golgi Apparatus from GERL. J. Histochem. Cytochem. 28:82-86, 1980.

Oliver, C., Auth, R.E., and Hand, A.R.: Morphological and cytochemical alterations of the Golgi apparatus and GERL in rat parotid acinar cells during ethionine intoxication and recovery. Am. J. Anat., (in press).

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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 00163-04 LBS

PERIOD COVERED

October 1, 1979 to August 12, 1980

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

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Youmans, Patricia A.	Secretary (steno)	LBS	NIDR
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Floyd, Steven W.	Photographer (laboratory)	LBS	NIDR
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Experimental Morphology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.01	0.56	0.45

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 to study the biosynthesis of cartilage proteoglycans and their relationship to collagen in cartilage matrix. Current studies, using the Swarm rat chondrosarcoma and cells cultured from it, involve the use of tracers to detect sites of uptake in the chondrocytes, cytochemistry of intracellular channels, and the Kleinschmidt technique to study isolated proteoglycans.

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.

The experimental system for this study is the Swarm rat chondrosarcoma. This transplantable tumor 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 00134-05). 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 shows 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. The cells show two unusual features: frequent close approaches of the RER to the cell surface, and short, wispy channels near the RER. Tracer experiments using ruthenium red as a cell surface and extracellular matrix granule label, and ferritin and colloidal thorium as extracellular space markers, are in progress to determine if these are pathways for uptake of materials from the matrix. Cytochemical experiments are also underway to look at similarities between the wispy channels and the RER, Golgi apparatus, and lysosomal systems.

Proteoglycans exist in cartilage matrix in the form of large aggregates bound to hyaluronic acid. These aggregates can be extracted intact from the tumor, due to its greater water content, and their organization studied in the electron microscope after spreading by the Kleinschmidt technique. Comparison of the spread proteoglycans with the matrix granules present in cartilage after standard EM fixation and embedding techniques suggests that each matrix granule represents a monomer portion of the aggregates. It also suggests an explanation for the condensation of proteoglycans during EM processing, and provides an image of the space-filling properties of proteoglycans in fresh tissue.

Proteoglycans probably play significant roles in controlling collagen fibril formation, mineral ion transport, and cartilage mineralization. These studies have been part of a broader program aimed at elucidating mechanisms of matrix formation, composition and mineralization. Due to the recent departure of the principal investigator, this project will not be continued in the coming year.

2. Publications:

Hascall, Gretchen K.: Cartilage proteoglycans: comparison of sectioned and spread whole molecules. J. Ultrastruct. Res. 70:369-375, 1980.

Hascall, Gretchen K.: Ultrastructure of the chondrocytes and extracellular matrix of the Swarm rat chondrosarcoma. Anat. Rec. 198: (in press), 1980.

Hascall, Gretchen K.: Electron microscopy of cartilage proteoglycans. Alabama Journal of Medical Sciences, Scientific supplement (in press), 1980.

Hascall, Vincent C., and Hascall, Gretchen K.: Proteoglycans. In: Cell Biology of Extracellular Matrix. Ed. E. Hay. (in press), 1980.

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 00199-04 LBS

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

In Vitro Studies of Secretory Cell Structure and Function.

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

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Waters, Judith F.	Biologist	LBS	NIDR
Smallwood, Veronica	Biologist	LBS	NIDR
Youmans, Patricia A.	Secretary (steno)	LBS	NIDR
DeGraff, Barbara A.	Purchasing-agent	LBS	NIDR
Floyd, Steven W.	Photographer (laboratory)	LBS	NIDR
Frear, Cindy	Photographer (trainee)	LBS	NIDR
Coriell, Steve	Photographer	LBS	NIDR
Worrell, Dorrette	Biologist	LBS	NIDR

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Laboratory of Biological Structure

SECTION
Experimental Morphology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Md. 20014

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.19	0.60	1.59

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Secretory processes in several cell types are currently under investigation. 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. Uptake and fate of both soluble phase and membrane bound markers by exocrine acinar cells is also being examined in vivo and in vitro.

1. Project Description

The primary objectives of this study 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:

The isolation of the acinar cells involves sequential treatment of the glands with EDTA and collagenase-hyaluronidase solutions. The dissociated cells are filtered through 500 μ and 25 μ Nytex filters and the acinar cells separated on a Ficoll gradient. The cells are cultured as suspension cultures at 35¼C in a humidified atmosphere of 5% CO_2 in air in Ham's nutrient mixture F-12 supplemented with antibiotics and the appropriate secretagogue (exorbital lacrimal, 10^{-6}M carbamyl choline; pancreas 10^{-5}M carbamyl choline; parotid 10^{-6}M isoproterenol). Protein synthesis is assessed by determining the amount of ^{3}H-leucine or ^{3}H-proline incorporated into TCA precipitable protein.

Membrane reutilization was studied both in vivo and in vitro. For in vivo studies, horseradish peroxidase (HRP) (1 mg/gm body weight) was injected into the saphenous vein of adult, male NIH Swiss mice or Wistar-Furth rats, or native Ferritin was instilled retrogradely into the pancreas and parotid gland via the main excretory ducts. Some animals received secretagogue (isoproterenol, 20 mg/kg body weight; pilocarpine 40 mg/kg body weight) prior to or concomitant with the HRP injection. Animals were sacrificed by vascular perfusion of fixative, at varying times following HRP administration. The glands were excised, and sections incubated for peroxidase activity, or for demonstration of various marker enzymes, i.e. thiamine pyrophosphatase for Golgi saccules and trimetaphosphatase and acid phosphatase for lysosomes. For in vitro studies of membrane reutilization isolated acini were prepared by collagenase-hyaluronidase digestion, filtered through Nytex filters and separated on a Ficoll gradient. Other acini were cultured in medium containing 1% HRP as a soluble phase marker.

Major Findings:

Dissociation of rat exorbital lacrimal and parotid glands and rat pancreas 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. Addition of the appropriate secretagogue to the culture medium allowed maintenance of the cells for up to 1 month in vitro. 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. These findings indicate that exocrine gland acinar cells may be maintained in vitro and ultilized as a model system for the study of secretory processes.

Initial in vitro studies on membrane reutilization in isolated pancreatic acinar cells showed that HRP was taken up from the cell surface in small endocytic vesicles and transported to lysosomes. No HRP reaction product could be found in Golgi saccules, GERL, or immature or mature secretory granules.

On closer examination, some of the HRP was sequestered in organelles not previously noted in in vivo studies on the uptake of HRP from the apical surface of parotid acinar cells. In the in vitro studies, since the HRP was in the medium and had access to all surfaces of the cell, it was not always possible to distinguish endocytic structures which originated from the apical cell surface from those which originated from the lateral and basal surfaces. In vivo, junctional complexes at the apices of the cells restrict the passage of large molecules, such as HRP, between cells. Therefore, intravenously (IV) administered tracers normally cannot gain access to the acinar lumen and are endocytosed only by the lateral and basal cell surfaces. In resting and stimulated mouse and rat parotid and pancreatic acinar cells, IV administered HRP was localized primarily in endocytic vesicles, basal tubular lysosomes and secondary lysosomes. However, much more HRP was internalized following secretagogue administration. Characterization of the basal lysosomes showed that they were a previously unrecognized component of the lysosomal system located adjacent to the basal and lateral cell surfaces. They appeared to form an anastomosing network in the basal portion of the acinar cells. Enzyme cytochemical methods have demonstrated the presence of trimetaphosphatase, but not of acid phosphatase, arylsulfatase or nonspecific esterase in these lysosomes. Further investigation revealed that much of the material internalized at the lateral and basal cell surfaces was endocytosed in coated vesicles which form from areas of plasma membrane infolding. Double labeling experiments, employing native ferritin administered retrogradely and HRP injected intravenously, have shown that material brought in from the apical cell surface, i.e., ferritin, and material brought in from the lateral and basal cell surfaces, i.e. HRP, were initially sequestered in separate compartments. Eventual mixing of the endocytosed material occurred in secondary lysosomes adjacent to the Golgi apparatus. The endocytosis of tracer in coated vesicles as well as the stimulation of uptake by secretagogues suggest that the internalization of tracer by the lateral and basal cell surfaces may be related to receptor mediated endocytosis. It thus appears that exogenous material and cell membrane from the lateral and basal cell surfaces are directed, at least initially, to a separate component of the lysosomal system, which may play some role in receptor mediated endocytosis.

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 essential for maintainance of 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 secretagogues. Additional studies will examine the function of the basal tubular lysosomes, the distribution of membrane receptors, the fate of internalized receptors and ligands, membrane reutilization, and the role of microtubules and microfilaments in the secretory process.

2. Publications:

Oliver, C.: Isolation and maintenance of differentiated exocrine gland acinar cells in vitro. In Vitro 16:297-305, 1980.

Oliver, C.: Cytochemical localization of acid phosphatase and trimetaphosphatase activities in exocrine acinar cells. J. Histochem. Cytochem. 28:78-81, 1980.

Greenberg, J.H., and C. Oliver.: Dimethyl sulfoxide reversibibly inhibits the pigmentation of cultured neural crest cells. Arch. Biochem. Biophys. (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 00283-01

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The relationship between matrix and mineral in tooth enamel

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

Yanagisawa, Takaaki	Visiting Fellow	LBS	NIDR
Nylen, Marie U.	Director, IRP	IRP	NIDR
Termine, John D.	Research Chemist	LBS	NIDR
Lyaruu, Donacian M.	Visiting Fellow	LBS	NIDR
Lenk, Elaine V.	Visiting Associate	LBS	NIDR
Youmans, Patricia A.	Secretary (steno)	LBS	NIDR
DeGraff, Barbara A.	Purchasing-agent	LBS	NIDR
Floyd, Steven W.	Photographer (laboratory)	LBS	NIDR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Experimental Morphology Section

INSTITUTE AND LOCATION

NIH, NIDR, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.42	1.12	0.30

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)

Previous work has shown that the organic material related to the enamel crystals can be visualized by electron microscopy in thin sections of enamel demineralized by floating on phosphotungstic acid. Whether this enamel material is a preformed structure involved in crystal nucleation and orientation or represents organic material adsorbed to the surface of the crystals is unresolved. The purpose of the present study is to further characterize the structural relationship between enamel matrix material and the enamel crystals, and to obtain basic information on the nature of the organic material.

1. Project Description:

In previous work, it has been suggested that the organic structures observed in decalcified sections of dental enamel represent organic coats adsorbed to the surface of the enamel crystals and not preformed macromolecular structures. This suggestion was based in part on random observations which indicated that the shape and dimension of the organic compartments changed in parallel with changes in crystal size during enamel maturation. The purpose of this project is to characterize in a systematic fashion the structural relationship between enamel matrix and enamel crystals, and to obtain basic information on the nature of the organic matrix.

Rat lower incisors were dissected out, freeze-dried or chemically fixed, embedded in plastic, and sectioned on a Gillings-Hamco microtome. Following microradiography, selected areas of enamel were isolated, reembedded, thin-sectioned, and observed in a transmission electron microscope with or without 1% phosphotungstic acid demineralization. Similar procedures were applied to four to six day old hamster molars.

Observations of untreated thin sections established a series of stages representative of normal maturation, with the crystals ranging from thin flattened ribbons to elongated hexagonal rods. Spaces between the crystals appeared devoid of content. Comparable sections decalcified in 1% phosphotungstic acid revealed the presence of an organic material delineating hollow compartment-like structures, the orientation, shape, and size of which closely matched those of the crystals and changed in parallel with the changes of the crystals during maturation. This was particularly evident during the later stages of maturation when the crystals exhibited their characteristic hexagonal shape. At the very early stage when the crystals were extremely thin, the matrix as a rule appeared as single strands or broad sheets. Tilting of the specimen relative to the electron beam frequently made it possible to resolve the single strand into two parallel structures separated by a narrow slit-like space similar in size to the thin crystal ribbon. Partial demineralization of the sections, which was accomplished by dipping half of the grid in 1% phosphotungstic acid and studying the boundary areas, revealed intimate association between the organic compartment walls and the crystal surface indicating that each crystal is surrounded by an organic envelope closely apposed to the crystal surface. The size of the compartments generally exceeded the size of the corresponding crystals by 20 to 70 A, which may indicate the thickness of the organic envelope. The envelope appeared somewhat thicker during the earliest stage of enamel formation. The observation that the same relationship exists in enamel specimens exposed to a minimum of solvents (freeze-dried) suggests that it is probably not an artefact.

Evidence provided by recent biochemical studies shows that one class of proteins present in dental enamel, the so-called enamelins, is tightly bound to enamel crystals suggesting that the material observed in our sections is the enamelin protein. Since no organic material was observed inside or between compartments, the location of the other major class of enamel proteins, the amelogenins, remains obscure.

In an effort to answer this question, studies have been initiated taking advantage of the differential solubility of amelogenins and enamelins in 4 M guanidine. Frozen sections of hamster molars were attached to glass slides and extracted with 4 M guanidine, then fixed, embedded in epoxy resin, thin sectioned, and observed under the electron microscope with or without phosphotungstic acid demineralization as previously described. Preliminary observations indicate the presence of compartment-like structures similar to those seen in untreated enamel. In areas of newly secreted enamel matrix, which are rich in amelogenins, the wall structures appeared more distinct following guanidine extraction suggesting loss of material from the areas between the compartment-like structures or from the wall structures themselves. Work is underway to resolve this question.

Current work will be continued and completed. In addition, new studies will be initiated for the purpose of examining in more detail the relationship between apatite and enamel protein using synthetic apatite reacted in vitro with amelogenins and/or enamelins which have been isolated from bovine enamel.

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 00284-01 LBS

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Ultrastructure and Cytochemistry of Endocrine Cells

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

Broadwell, Richard D.	Research Biologist	LBS	NIDR
Oliver, Constance	Research Biologist	LBS	NIDR
Waters, Judith F.	Biologist	LBS	NIDR
Youmans, Patricia A.	Secretary (steno)	LBS	NIDR
DeGraff, Barbara A.	Purchasing-agent	LBS	NIDR
Floyd, Steven W.	Photographer (laboratory)	LBS	NIDR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Experimental Morphology Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.99	.73	.26

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 secretory process is studied in endocrine cells of the rodent neurosecretory system, anterior pituitary gland, and pancreas. Techniques employed include light and electron microscopy, enzyme cytochemistry and immunocytochemistry. Predominant areas of interest are: (1) the role of the Golgi apparatus and GERL in secretory granule formation; (2) the structural and functional association of GERL to the Golgi apparatus; (3) the role of the lysosomal system in the intracellular degradation of secretory material and internalized cell surface membrane.

1. Project Description:

Objectives:

The basic objective of this project is to further clarify the structure and function of endocrine secretory cells and the organelles involved in the secretory process. Employing light and electron microscopic, enzyme, and immunocytochemical techniques, cell ultrastructure is correlated with localization of enzyme activity and antigenicity of specific proteins or hormones. Our efforts have focused on: (1) the production and packaging of secretory material by specific cellular organelles; (2) the functional and structural interrelationships between the Golgi apparatus and GERL; and (3) the role of the lysosomal system in the degradation of secretory material and internalized cell surface membrane.

Methods Employed:

Brain, anterior pituitary and pancreas tissues for morphological examination are fixed by vascular perfusion and prepared by standard techniques. Cytochemical incubations for acid hydrolase and thiamine pyrophosphatase activities and for localization of antigenicity using antibodies against specific hormones are performed on 75 μm slices of fixed tissues.

Major Findings:

A particularly excellent model system for our studies of the secretory process and the lysosomal system of organelles has been the neurosecretory neuron of the hypothalamo-neurohypophysial system which produces and secretes vasopressin and oxytoxin hormones along with their associated carrier proteins, the neurophysins. The neurosecretory neurons can be stimulated experimentally to increase the production and secretion of the hormones by giving the animals 2% salt water to drink.

The pre-embedding staining approach to the unlabeled antibody-enzyme technique has been used to localize neurophysin immunocytochemically within organelles associated with the synthesis and packaging of the neurophysin-peptide hormone complex. Immunoreactive organelles include the nuclear envelope, rough endoplasmic reticulum, Golgi apparatus, secretory granules, and secondary lysosomes. Observable immunoreactivity in the Golgi apparatus indicates that this organelle is involved in the protein packaging scheme. No labeling of GERL was noted. The absence of immunoreactivity in GERL may be due, in part, to the inability of the large molecular weight antibodies to penetrate the GERL membrane. Similar methodology is currently being employed to localize antigenicity for insulin and glucugon hormones within the beta and alpha cells, respectively, of the rat endocrine pancreas.

In the "resting" neurosecretory neurons, secretory granule production occurs predominantly from GERL with minimal involvement of the Golgi saccules. The possible functional and structural interrelations between GERL and the Golgi saccules for the packaging of neurosecretory material and the formation of neurosecretory granules have been studied in normally hydrated, salt-stimulated

and rehydrated mice by employing enzyme cytochemical markers for acid phosphatase activity in GERL and thiamine pyrophosphatase activity in the Golgi saccules. Our results suggest that GERL and the Golgi apparatus are functionally and perhaps structurally interrelated. With salt stimulation and rehydration, the saccules of the Golgi apparatus and GERL were hypertrophied. The production of secretory granules was elevated from GERL and from all Golgi saccules as well. Thiamine pyrophosphatase activity, normally present in the innermost one or two Golgi saccules, appeared in all Golgi saccules and in GERL-like cisternae which normally contain only acid phosphatase activity. In the thiamine pyrophosphatase preparations, anastomosing channels appeared to interconnect GERL and the innermost Golgi saccule, suggesting that GERL may be either derived from the Golgi apparatus or structurally related to it.

The retrieval of membrane from the cell surface following exocytosis of neurosecretory granule contents from the neurosecretory axon terminals was studied by vascular injection of horseradish peroxidase. In normal and salt-stimulated mice, peroxidase-labeled vacuoles were channeled by retrograde axoplasmic transport directly to perikaryal secondary lysosomes for eventual enzymatic degradation. Reaction product was never observed in GERL or Golgi saccules. Most recently we have obtained similar results in the different cell types of the anterior pituitary gland. Within the somatotrophs of the anterior pituitary, however, peroxidase did appear in GERL. These findings suggest that considerable variability may occur in the way different cell types recycle or degrade internalized plasma membrane.

Proposed Course:

Since the principal investigator is leaving NIH, this project will not be continued on a formal basis. However, the studies of tracer uptake by anterior pituitary cells, and the immunocytochemical localization of insulin and glucagon, will be completed through a collaborative arrangement.

2. Publications:

Broadwell, R.D., Oliver, C., and Brightman, M.W.: Localization of neurophysin within organelles associated with protein synthesis and packaging in the hypothalamo-neurohypophysial system: An immunocytochemical study. Proc. Nat. Acad. Sci. 76:5999-6003, 1979.

Broadwell, R.D.: Cytochemical localization of acid hydrolases in neurons of the mammalian central nervous system. J. Histochem. Cytochem. 28: 87-89, 1980.

Broadwell, R.D., Oliver, C., and Brightman, M.W.: Neuronal transport of acid hydrolases and peroxidase in the lysosomal system of organelles: Involvement of agranular reticulum-like cisternae. J. Comp. Neurol. 19: 514-532, 1980.

Broadwell, R.D., and Oliver, C.: Morphological basis for the synthesis and packaging of neuronal peptides. In: Neuronal Peptides and Their Function. (in press), Marcel Dekher, publisher, N.Y.

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 00285-01 LBS

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of Protein Secretion in Salivary Glands

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

Mednieks, Maija	Staff Fellow	LBS NIDR
Hand, Arthur R.	Chief, LBS	LBS NIDR
Hyunh, Kim-Chi	Student-trainee	LBS NIDR
Ho, Betty	Biologist	LBS NIDR
Youmans, Patricia A.	Secretary (steno)	LBS NIDR
DeGraff, Barbara A.	Purchasing-agent	LBS NIDR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Experimental Morphology Section

INSTITUTE AND LOCATION

NIH, NIDR, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.05	0.70	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)

Combined biochemical and immunocytochemical approaches are undertaken to study specific stimulus responses in parotid secretory cells. Cyclic nucleotide - mediated protein phosphorylation is investigated to determine specific intracellular sites/loci and mechanisms of regulatory action in the signal processing of salivary cells during protein secretion. Accordingly, cyclic-3', 5'-adenosine monophosphate (cyclic AMP) - dependent protein kinase activity and its intracellular compartmentalization is measured in rat parotid tissue stimulated in vitro by the norephinephrine analogue isoproterenol and compared to that of unstimulated controls. Endogenous phosphoproteins and specific protein substrates which are phosphorylatively modified after stimulation are identified by employing electrophoretic separation and autoradiographic techniques. Morphological tissue changes are followed using both light and electron microscopy. Secretory product and lysosomal enzyme localization and transport will be accessed by immunological and immunocytochemical methods employing antibodies produced either conventionally in rabbits or by monoclonal methods by murine hybridomas.

1. Project Description:

The purpose of this study is to undertake an integrated approach in investigating the coupling of signal processing with intracellular events in protein secretion, employing combined morphological, biochemical and immunological methodology. This project is recently initiated and is designed to interface with ongoing studies and to employ the expertise of investigators in various disciplines to yield a comprehensive view of the events governing secretory processes in general and salivary protein export in particular.

Methods Employed:

The rat parotid gland is utilized as our model system because extensive ultrastructural studies have provided information on morphological changes in stimulated glands and parotid tissue responses have been well characterized previously and afford a basis for comparisons. The tissue is dissected in a uniform manner: freed of lymph nodes, fat and connective tissue so as to yield a reproducible wet weight from one preparation to the next. Minced tissue is incubated without or with catecholamine secretagogues, appropriate blocking agents or antagonists as well as stabilizing agents (isoproterenol, propranolol, ascorbate and mercaptoethanol, respectively) in micromolar concentrations. After incubation the washed tissue fragments are homogenized (Dounce) under controlled, isotonic conditions and subfractionated by centrifugation. Several particulate fractions and a supernatant fraction thus obtained are then studied. Standard techniques as well as those developed by this investigator and associates are employed in enzyme assays of protein-phosphotransferase, acid phosphatase, α-amylase and RNA-polymerase. Isotopic labelling of precursors and their incorporation into macromolecules (^{3}H-leucine, ^{3}H-uridine and thymidine, ^{32}P-ATP and ^{3}H or ^{32}P-cyclic nucleotides and their analogues) is used as a means to define the kinetics of secretory cell responses to stimulation. Electrophoretic and chromatographic separation techniques are employed both in characterization studies as well as preparatively in antigen purification.

Computer assisted processing and graphic display will be employed in data analysis of the biochemical results as well as in quantitation of ultrastructural perturbations of stimulated secretory cells. Antisera to proteins specifically associated with marker events in secretion (α-amylase, acid phosphatase) will be produced in either rabbits or mice and eventually by cell hybridization techniques. Immunocytochemical studies will utilize a variety of procedures (membrane permeabilization, *in situ* application of antibody reagents, pre- vs postembedding techniques, etc.) to reliably localize intracellular antigens.

Major Findings:

Overall findings show that stimulation of parotid tissue by isoproterenol is a concentration dependent event which is reflected in changes in compartmentalization and specific activity of cyclic AMP-dependent protein kinase using tissue weight, protein content of subcellular particulate fractions and distribution of acid phosphatase activity as comparative criteria. The first

particulate fraction, sedimented at low speeds (< 750 xg) from the homogenate, appeared enriched in nuclei when observed with either LM or TEM and had > 90% of RNA polymerase activity as measured by ^{3}H-uridine incorporation, but the protein kinase activity did not vary significantly (in preliminary experiments) as a consequence of isoproterenol stimulation of parotid cells. The second particulate fraction collected over a short time interval (10') at 5,000 xg, when examined microscopically appeared to consist mainly of granules, and contained 50-60% of the acid phosphatase activity, the majority of which was measurable only after addition of a nonionic detergent. This granule fraction registered a response to both low (10^{-7} - 10^{-6}M) and high (10^{-5} - 10^{-4}M) isoproterenol concentrations by a significant increase and decrease, respectively, in specific activity of cyclic AMP dependent protein kinase. When a third particulate fraction was collected (approximately 20,000 x g, 20'), which was enriched in identifiable mitochondria and lysosomes, the effect of 10^{-6} M isoproterenol was to diminish protein kinase activity. When the contents of these cell fractions were examined electrophoretically each had a characteristic banding pattern. These protein banding patterns were not significantly modified after stimulation but preliminary autoradiography results indicate that phosphorylation patterns were varied as a result of tissue exposure to exogenous catecholamines. The biochemistry of the soluble cell fraction was altered after tissue stimulation by 50 to 100% increases in cyclic AMP-dependent protein kinase activity. As in other cell fractions a pharmacological stimulatory dose (> 10^{-5}M) of isoproterenol resulted in a marked decrease of protein kinase activity. Additionally, protein kinase activity ratios were < 0.5 in the optimally stimulated tissue cytosols indicating that the enzyme was present in holoenzyme form, whereas the activity ratios in cytosols of control parotid cells were > 1.0 indicating that the protein kinase was fully dissociated into subunits in the basal state. Overall, our results are similar to those found in other stimulated cell systems whose signal amplification processes involve mediation by cyclic nucleotides: both activity and holoenzyme form are altered as a consequence of cell stimulation. The new information (activity, changes in the particulate factions) appears to support a hypothesis of intracellular compartmentalization and stimulus-related redistribution of the postranslational phosphorylation modification processes of intracellular proteins. These processes have been previously shown to play a regulatory role in cell differentiation. Additionally, it appears that several granule proteins are modified by phosphorylation and may be dependent on this process for specific function.

Significance to Dental Research:

An understanding of salivary secretory processes on the cellular and molecular level has numerous applications to dental research ranging from neurological aspects such as taste and phagic (hypo/hyper) responses and oral hygiene to influences on nutrition. Pharmacologic manipulation employing hormones, their analogues combined with cyclic nucleotides and their analogues as potentiators has state-of-the-art applications in other areas of biomedicine (notably in proliferative disorders ranging from eczema to various tumors) and may prove to be useful in control of dental disorders.

Proposed Course:

From the biochemical standpoint the next steps are first to determine whether protein kinase enzyme patterns are altered after stimulation which would offer additional evidence for a regulatory involvement of cyclic nucleotide-mediated events. Secondly, secretory protein transport, packaging and exocytosis will be followed by means of immunocytochemical and immunobiochemical 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 00012-18 LBS

PERIOD COVERED

October 1, 1979 to September 30, 1980

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

Fowler, Bruce O.	Research Chemist	LBS NIDR
Youmans, Patricia A.	Secretary (steno)	LBS NIDR
DeGraff, Barbara A.	Purchasing-agent	LBS NIDR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Molecular Structure Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20014

TOTAL MANYEARS: 1.1	PROFESSIONAL: 1.0	OTHER: 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.

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 development and evaluation of infrared and Raman spectroscopic methods for quantitation of hydroxide in stoichiometric and nonstoichiometric calcium hydroxyapatites was continued. The spectroscopic methods basically involve deducing hydroxide contents of apatites by comparison with 100% hydroxylated, essentially pure apatite standards. Assessment of variation in spectroscopic hydroxide content measurements for pure apatite standards differing only in physical properties and assessment of spectroscopic sampling technique variables on hydroxide content measurements are critical to these methods. The infrared method developed was capable of semi-quantitative hydroxide determination ($\pm$ 15% [absolute] deviation from chemically determined hydroxide values); however, the Raman method showed deviation as high as 30-40% (absolute) from the chemical values. Efforts are underway to understand the cause(s) for this deviation and perfect, if possible, the Raman method.

Raman Measurements:

The major experimental problems and factors to overcome and/or evaluate in obtaining Raman spectra of apatite powders for quantitative measurements are: (1) sample changes, decomposition and spectral band broadening caused by laser beam heating, (2) high spectral background arising from fluorescence, (3) effects of crystal orientation on spectral bands, (4) resonance Raman effects on spectral bands, (5) effects of structurally incorporated impurities on spectral bands, (6) effects of polymorphic apatite forms on spectral bands, (7) effects of structural disorder of apatite constituent ions on spectral bands, and (8) effects of crystal size and shape on spectral bands. Two small size ($\sim$ 60 m^2/g surface area) and two large size ($\sim$ 1 m^2/g) hexagonal apatite standards and two large size ($\sim$ 1 m^2/g) monoclinic apatite standards were studied. Factors (1 to 5) and (2 to 5) for the hexagonal and monoclinic apatites, respectively, were generally excluded as significantly affecting the spectroscopic hydroxide content measurements under the techniques and conditions used. Monoclinic hydroxyapatite was sensitive to factor (1), localized sample heating. Monoclinic hydroxyapatite, which converts to the hexagonal form at and above 215°C, required sampling and laser power conditions to prevent conversion to the hexagonal form. Factor (6), polymorphism, had marked effects on Raman spectra. The normalized Raman hydroxide stretching band intensity of the monoclinic form was about 2.2 times that of the hexagonal form whereas the corresponding hydroxide band area and half-band width were about 1.2 and 0.5 times, respectively, those of the hexagonal form. The narrower, higher intensity monoclinic apatite hydroxide stretching band apparently arises from the ordered hydroxide ion arrangement in the monoclinic form; the hydroxide ions are disordered in the hexagonal form. Monoclinic hydroxyapatite can be used as an approximate standard for certain hexagonal apatites based on band area measurements only. Factor (7), the degree of hydroxide order and/or size of ordered hydroxide domains in hexagonal hydroxyapatite, also had an appreciable effect on Raman hydroxide content measurements. This was experimentally inferred by comparing an apatite standard heated at 60° and 250°C. Hydroxide contents by chemical analyses and crystal size were the same for the 60° and 250°C samples; however, the spectroscopically deduced hydroxide contents were 61 and 107 % from intensity measurements, and 86 and 100 % from area measurements, respectively, for the 60° and 250°C samples. The hydroxide stretching half-band width of the 250°C sample was about 0.5 that of the 60°C sample. The increase in hydroxide band intensity and decrease in half-band width are consistent with a greater extent of hydroxide ordering in the 250°C treated sample. Thus, the degree of hydroxide ordering can contribute to uncertainty in spectroscopic hydroxide quantitation; this uncertainty, as expected, was greater for hydroxide contents derived from band intensity measurements. It appears, based on Raman hydroxide stretching half-band width comparisons between 0.1 μm and 10 μm size hexagonal apatite crystals, that the larger crystals may have a high degree of hydroxide disorder. The effects of factor (8), crystal size, have not been clearly established. Overall, consistent spectroscopic hydroxide measurements were obtained for the large size apatite standards, but not for the small size apatite standards. The two small size apatite standards ($\sim$ 60 m^2/g surface area), with essentially the same hydroxide contents by chemical analyses, showed considerable differences in Raman deduced hydroxide contents. Attempts are underway to prepare additional small size apatite standards in order to evaluate the effects of small crystal size on Raman hydroxide measurements. The high deviation of the Raman

derived hydroxide values from the chemical values is believed to be primarily caused by surface mode effects indigenous to small size crystals. Additional small crystal size spectroscopic data are required to confirm this and establish consistent spectroscopic reference values for the Raman method.

Infrared Mesurements:

The infrared method developed was capable of semi-quantitative determination of hydroxide in apatites. The spectroscopically determined hydroxide contents of 24 apatites, employing KBr pellets and band area ratio measurements, agreed within $\pm$ 15 % (absolute) of the chemically determined hydroxide contents. The effect of differences in apatite crystal size (60 m^2/g and 2 m^2/g surface-area apatites) on the hydroxide measurements using band area ratios was small, about 10%. Apatite particle size of about 2 to 4 μm for both single crystals and clusters of smaller crystals was found suitable for infrared hydroxide quantitative measurements. Prolonged grinding to produce apatite particle sizes of 1 μm and less caused decreases in infrared deduced hydroxide contents. This decrease may be real due to crystal damage, but chemical assay has not been made. The effect of differences in hydroxide order in monoclinic and hexagonal hydroxyapatite on infrared hydroxide measurements was small as compared to Raman results. The monoclinic form displayed hydroxide stretching absorbance about 1.2 times that of the hexagonal form whereas the corresponding band areas for both forms agreed within $\pm$ 5%. Monoclinic hydroxyapatite can be used as a good hydroxide standard based on band area measurements and an approximate standard based on band intensity measurements. Uncertainty in hydroxide quantitation was caused by degree of hydroxide order. The infrared deduced hydroxide content changes, observed for the hexagonal apatite heated at 60° and 250°C described under Raman Measurements above, closely paralled the Raman results.

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 infrared and Raman spectroscopic study for semi-quantitative determinations of hydroxide ions in apatites will be completed and prepared for publication.

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.

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 00074-08 LBS

PERIOD COVERED

October 1, 1979 to September 30, 1980

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

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Kaplan, Keith A.	Biological Aid (Biochem.)	LBS	NIDR
Keith, Dana-Dean	Biological Aid (Biochem.)	LBS	NIDR
Dejter, Stephen W., Jr.	Biological Aid	LBS	NIDR
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Miyamoto, Maureen S.	Staff Fellow	LBS	NIDR
Youmans, Patricia A.	Secretary (steno)	LBS	NIDR
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LAB/BRANCH

Laboratory of Biological Structure

SECTION

Molecular Structure Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.64	4.49	2.15

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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 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 and glycoprotein biochemistry in these hard tissue matrix studies.

1. Project Description:

The biochemical and biophysical properties of the extracellular matrix proteins in the bones and teeth are key elements in the molecular architecture, biological function and metabolic activities of these hard tissues. For example, mineralization in all skeletal tissue is matrix-mediated. Thus, the rate, extent and geometrical arrangement of mineral deposition in enamel, dentin and bone center about the exact distribution and unique character of their respective extracellular matrix proteins. The overall goal of this program is to study the biochemical and biophysical properties of the matrix proteins specific to each mineralizing skeletal tissue in order to better understand their structure and function.

Enamel is unique among the mammalian hard tissues because throughout its development, it is more heavily mineralized and its apatite crystallites more highly organized, larger in size and different in shape than is the case for either dentin or bone. The enamel extracellular matrix is totally noncollagenous and is almost completely lost from the tissue with increasing mineralization. In addition, the average composition of this poorly understood matrix is vastly different for developing as opposed to more fully mineralized enamel. Our experimental approach to the problems and difficulties posed by this particular tissue then became a model for protocols subsequently utilized to study the noncollagenous proteins of dentin and bone. Briefly, we employ tissue preparation and extraction procedures designed to minimize potential artefacts due to proteolysis and/or aggregation of individual matrix constituents. Further, we devised an extraction scheme that differentiates hard tissue matrix constituents on their selective affinity for apatite crystallites in dissociative solvents such as 4M guanidine hydrochloride. We try to study fetal or neonatal tissue at progressive stages of development in order to sort out the sequence of biochemical events that occur during ongoing bone and/or tooth formation. We have also constructed a number of novel electrophoretic and chromatographic systems to better assess the unusual protein chemistry encountered in studying the noncollagenous skeletal matrix constitutents.

This approach has enabled us to differentiate the developing enamel matrix into two biochemically distinct protein types, one a proline-rich class previously studied by others (the amelogenins) and the second, a newly identified, crystal-bound, acidic glycoprotein class similar in composition to that reported for fully mineralized enamel (the enamelins). Both classes display maturation-linked biochemical changes. We have purified the fetal bovine dentin phosphoprotein using dissociative fractionation methods and found it to be considerably larger in size than any other dentin phosphoprotein fraction isolated previously. This protein was found to modulate apatite crystallization and collagen fibrillogenesis in vitro, both in a calcium-dependent fashion. Details of these efforts may be found in prior years' annual reports.

In recent studies, we have initiated a comparative investigation of the enamelin species in fetal and adult human enamel tissue. Preliminary data on the enamelins of carefully dissected, normal, adult human enamel reveal that they are separable into guanidine-EDTA soluble and insoluble fractions. The guanidine-HCl-EDTA soluble adult enamelins are acidic glycoproteins of small

molecular size. Their composition is similar to the fetal bovine enamelin proteins. The guanidine-HCl-EDTA insoluble adult human enamel fraction is a composite of enamel apatite and enamelin-like protein that accounts for ∿ 3 - 5% of the total enamel dry weight. We are now actively pursuing this fraction in the hope of unraveling the cause of the enamel apatite insolubility observed for this material. We hope to compare the molecular characteristics of these adult enamelin proteins with those of comparable purified fractions isolated from fetal bovine and human enamel tissue.

In other studies now in progress, we have isolated several fetal calf, pig and sheep amelogenin components ranging in apparent molecular size from ∿ 32,000 - 6,000 daltons. These components are being extensively investigated by biochemical and immunological methods to sort out the molecular interrelationships existing between the individual members of this enamel protein class. For example, we have preliminary evidence that three of these species from bovine tissue have identical amino terminal residues and that the largest (∿ 32,000 daltons) and smallest (∿ 6,000 daltons) of these share common cyanogen bromide fragments. We will continue along these lines in an attempt to map out a working structural model for the amelogenin molecule, such as already has been done for collagen and the proteoglycans. As purified amelogenin components (and antibodies) become available, we will also study their localization within developing enamel and their general biophysical properties, with an eye towards reconstruction of the mineralizing enamel matrix.

Because of (1) its rapid developmental profile and (2) its suitability for organ culture experiments, we recently began to explore the neonatal hamster molar as a model for future studies. Shortly before birth, the hamster molar tooth germ is at a presecretory stage where cytodifferentiation of ameloblasts and odontoblasts is still in progress and matrix production has not yet begun. Within the first day after birth, early dentin matrix production and mineralization is initiated. This is followed by early enamel formation and mineralization by at least the second day of neonatal hamster life. At day 4, the ameloblasts are almost entirely secretory and enamel formation and mineralization is most active. At day 6, the ameloblasts start to reach the maturation stage. By day 8, eruption has been initiated and at days 10 to 11, the hamster molar is fully functional.

After mechanical removal of pulp and outer epithelia, 4, 6 and 7 day hamster molars were gently sonicated in phosphate-buffered saline to remove adhering cell layers. The sonicate from this step was isolated and its banding pattern on SDS-electrophoresis resembled that described for cell proteins in other systems. This was followed by guanidine extraction to remove amelogenins, leaving a crystal-rich tissue remnant when very gentle stirring was used. With more vigorous stirring, the enamel crystallites flaked off at the dentino-enamel junction, forming a milky suspension. The remaining dentin solid pieces were then removed mechanically and extracted with 4M guanidine - 0.5M EDTA to isolate dentin matrix proteins. The enamel crystallites were harvested from the guanidine suspension by centrifugation (the supernatant contained the hamster molar amelogenins) and also extracted with guanidine-EDTA to isolate the hamster molar enamelins. Examination of the free enamel crystallites by electron microscopy suggested that prior to EDTA extraction, they contained an enamelin protein coat over their mineral surfaces. Thus, it was possible to separate

cell protein, matrix amelogenin, enamelin and dentin protein fractions from these small tissues. These fractions were then examined by the electrophoretic, chromatographic and analytical methods developed earlier for fetal bovine molar tissue.

Although these studies are still in progress, it is noteworthy that the results obtained to date strictly parallel our fetal bovine molar results. The neonatal hamster amelogenins resemble the fetal bovine amelogenins in electrophoretic and chromatographic profiles and shift from high to low molecular weight size populations in going from secretory to maturing enamel stages. The neonatal hamster molar enamelin fraction has staining characteristics on electrophoresis similar to the fetal bovine variety, but contains some components considerably larger in size, ∿ 150,000 - 200,000 daltons. On the other hand, the hamster molar dentin phosphoprotein appears to be somewhat smaller (∿ 75,000 - 80,000 daltons) than its fetal bovine counterpart (∿ 100,000 daltons).

Upon completion of these biochemical studies, organ culture experiments will be initiated. Preliminary experiments suggest that the actively mineralizing 4 day hamster molar can be cultured for short periods (∿ 24 hrs.). Suitable pulse-chase experiments will be conducted to examine the biosynthesis and secretion of the amelogenin and enamelin constituents described above. In this manner, we hope to examine the number of gene products produced during amelogenesis and their ultimate fate following mineralization in the extracellular matrix.

We have now begun to isolate and characterize the noncollagenous proteins and proteoglycans from fetal bovine subperiosteal bone. The initial results appear quite promising and we intend to pursue this project actively in view of the potential long range benefits of such a study to our basic understanding of bone tissue. For example, when we applied our sequential dissociative extraction procedure to fetal bovine subperiosteal bone tissue, we were able to identify 4-5 apatite crystal-specific proteins ranging in apparent molecular size from 20,000 - 60,000 daltons (SDS electrophoresis and dissociative gel filtration). Several of these species appear to be sialated and/or phosphorylated glycoproteins based on their staining characteristics on polyacrylamide gels. We have now begun to fractionate these proteins by gel filtration chromatography in 4M guanidine-HCl and should shortly be able to assess their compositions. We hope to then employ immunochemical methods to assure the bone specificity and origin of these apatite-bound glycoproteins and then attempt to localize them in developing bone matrix by immunocytochemical methods, if warranted. Further biochemical, biophysical and biosynthetic studies will, no doubt, be in order should these early efforts at characterizing the developing bone extracellular matrix be fully successful.

Perturbances in mineral metabolism and other calcification diseases have always presented perplexing clinical difficulties in the various branches of dentistry and medicine. On a basic science level, it is almost impossible to cope with these pathologies in the absence of certitude as to the biochemical properties and biological functions of the extracellular matrix proteins, the principal gene products differentiating each skeletal tissue type. Consequently, this research program will focus its future efforts on (1) identifying the individual noncollagenous matrix proteins specific to enamel, dentin and

bone, (2) mapping out their internal molecular structure, and (3) unraveling their biogenesis, matrix localization and biological function within their parent tissues.

2. Publications:

Termine, J.D. and Torchia, D.A.: ^{13}C-^{1}H magnetic double-resonance study of fetal enamel matrix proteins. Biopolymers 19:741-750, 1980.

Fincham, A.G.: Changing amino acid profiles of developing dental enamel in individual human teeth and the comparison of the protein matrix of developing human and bovine enamel. Arch. Oral Biol. (in press), 1980.

Gelman, R.A., Conn, K.M. and Termine, J.D.: The effects of phosphoproteins on collagen self-assembly. Biochim. Biophys. Acta (in press), 1980.

Termine, J.D., Miyamoto, M.S. and Kuettner, K.E.: Lysozyme, protease and protease inhibitor proteins in fetal bovine enamel matrix extracts. J. Dent. Res. (in press), 1980.

Termine, J.D., Eanes, E.D. and Conn, K.M.: Phosphoprotein modulation of apatite crystallization. Calcif. Tiss. Intl. (in press), 1980.

Termine, J.D.: Bone and tooth mineralization: matrix effects and crystal development. Progress in Crystal Growth and Characterization (Biological Crystal Growth Special Issue), Pergamon Press, Oxford (in press), 1980.

Termine, J.D., Belcourt, A.B., Christner, P.J., Conn, K.M. and Nylen, M.U.: Properties of dissociatively extracted fetal tooth matrix proteins. I. Principal molecular species in developing bovine enamel. J. Biol. Chem. (in press), 1980.

Termine, J.D., Belcourt, A.B., Miyamoto, M.S. and Conn, K.M.: Properties of dissociatively extracted fetal tooth matrix proteins. II. Separation and purification of fetal bovine dentin phosphoprotein. J. Biol. Chem. (in press), 1980.

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 00088-07 LBS

PERIOD COVERED

October 1, 1979 to September 30, 1980

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

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Floyd, Steven W.	Photographer (laboratory)	LBS	NIDR

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Dr. Jonathan L. Costa, NIMH, NIH

LAB/BRANCH

Laboratory of Biological Structure

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Molecular Structure Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.25	1.0	2.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The properties of synthetically prepared calcium phosphates of biological interest are being studied with a variety of ultrastructural and physical-chemical techniques such as electron microscopy, x-ray diffraction, B-E-T surface area methods, and standard analytical chemistry procedures. Topics under current investigation include (1) the effect of fluoride, magnesium, and carbonate on the formation, growth and physical properties of apatite and of non-apatitic precursor phases such as octacalcium phosphate, and (2) the preparation and characterization of synthetic analogues to intracellular mineral deposits such as occur in mitochondria and in subcellular storage organelles.

1. Project Description:

Objectives:

The purpose of this project is to study the physicochemical and ultrastructural properties of synthetically prepared calcium phosphate compounds of biological interest. Of particular interest are those properties whose expression in vivo are often obscured by overlying cellular and metabolic processes. Until recently, most work done under this project was on the formation and maturation of inorganic calcium phosphate salts similar in composition and texture to extracellular mineral deposits. However, a major effort is currently underway to develop appropriate synthetic models of intracellular mineral deposits such as occur in mitochondria and in subcellular storage organelles.

The principal undertaking in the area of extracellular mineral modelling during the year covered by this report was to complete a study on the effect of Mg on the formation, growth and physical properties of apatite. Particular emphasis was placed upon determining whether the effect Mg has on the kinetics of apatite formation may in large part result from the action this ion exerts on precursor phase development. Studies on intracellular modelling concentrated on preparing and characterizing synthetic analogues to the calcium-adenosine phosphate-pyrophosphate complexes that are found in the dense bodies of blood platelets.

Methods Employed:

Synthetic calcium phosphate suspensions used in the Mg study were prepared by inoculating supersaturated solutions metastable at pH 7.4 with crystals of apatite. The suspensions were then aged at 25°C and the reaction kinetics followed by monitoring solution calcium, phosphate, and the amount of base required to keep the pH constant. Mg was added either before initiating precipitation or at various stages during the aging process. Solid reaction products were examined chemically and by various physical methods such as x-ray diffraction and transmission electron microscopy.

Synthetic dense body analogues were prepared at 37°C by spontaneous precipitation initiated through rapid addition of a 0.40 M calcium solution to an equal volume of a basic solution containing 0.10 M adenosine triphosphate (ATP), 0.11 M adenosine diphosphate (ADP), 0.04 M pyrophosphate. After the initial precipitation was completed, the pH was adjusted to and maintained at 7.4 with a pH-stat. Samples collected at regular intervals were filtered and both filtrates and solids were analyzed for all relevant chemical constituents. Dissolution properties were determined by dispersing samples of undiluted slurry into measured volumes of neutral NaCl solutions.

Major Findings:

Work done under this project in previous years has shown that the effect of growth modifiers on apatite formation in solutions at physiological pH frequently depended upon whether initial conditions of supersaturation were conducive for the formation of transient precursor phases such as amorphous

calcium phosphate (ACP) and octacalcium phosphate (OCP). As an example, when the degree of supersaturation favored OCP formation in seeded reactions, fluoride initially inhibited then later enhanced reaction development, whereas in seeded reactions where OCP formation was not evident, fluoride accelerated growth from the beginning. On the other hand, studies done during this past year showed that Mg, unlike fluoride, had the same inhibiting effect on seeded apatite growth regardless of the degree of solution supersaturation. In reactions where OCP formed, the Mg induced a delay in apatite development in part by slowing down the formation of the OCP precursor and in part by retarding its hydrolysis to apatite. It was also observed, however, that Mg could slow down seeded reactions under conditions that ruled out the possibility of precursors forming. In these latter reactions it appeared that the delay was due to Mg inhibiting directly the growth of new apatite on the seed surfaces. These findings suggest that the effect of Mg on the kinetics of seeded calcium phosphate formation at pH 7.4 and 25°C may be quite general in nature. Because Mg does not readily incorporate into the lattices of calcium phosphate salts, the adsorption of Mg at active surface sites may temporarily block these sites from participation in whatever reaction is going on at that moment - whether it be apatite growth, OCP or other precursor phase formation, or hydrolysis of OCP to apatite.

Studies conducted during the past year showed that synthetic Ca-adenosine phosphate-pyrophosphate solids which resemble quite closely the dense bodies of human blood platelets in several important properties can be prepared from aqueous solutions. Like the dense bodies themselves, these synthetic preparations were amorphous in structure and had a P/Ca ratio of 1.5. The relative proportion of ATP, ADP, and P_2O_7 in these solids (0.7, 1.0, 0.4, respectively) also matched quite well the values found for dense bodies. The synthetic analogues appear to mimic human platelet dense bodies in one other important aspect as well in that they dissolve rapidly and completely when diluted 100 or more fold with neutral saline solution.

The examination of the behavior of the synthetic analogues under controlled conditions has provided useful insights into certain properties of dense bodies not accessible by more direct means. The synthetic precipitates, for example, can be formed equally well and with similar compositions over a range of pH's (5.7 to 7.4). In all cases, the PO_4-components, although containing fewer protons than the corresponding species in free solution, have a sufficiently high proton content to suggest that cationization via proton transfer is one means by which serotonin, a biogenic amine commonly associated with platelet dense bodies, can be stored in these bodies. Also, when freshly precipitated, the synthetic solids can achieve a stoichiometry characteristic of dense bodies only when in equilibrium with solution concentrations of calcium and adenine nucleotides considerably higher than those encountered in the cytoplasm of the platelets. This observation suggest that the membrane enclosing the dense bodies does not permit equilibration with the cytoplasm. Finally, the pyrophosphate species in the synthetic solids appears to be associated with at least a single proton. This is in distinct contrast to inorganic $Ca_2P_2O_7$ salts prepared at physiological pH where the $P_2O_7^{4-}$ anion is essentially unprotonated. Thus it seems likely that the $HP_2O_7^{3-}$ in the dense bodies does not exist as a distinct Ca phase separate from the Ca nucleotides but that all species are present as components of a single phase matrix.

Significance to Dental Research:

The deposition of calcium phosphate salts in skeletal tissues occurs in a physiological environment relatively rich in ionic magnesium. As an example, the Mg level in extracellular calcifiable cartilage fluid is comparable to that found in blood plasma (0.8 mM). Although the role of Mg in mineral dynamics in vivo is not well understood, the data obtained in this project to date suggest that Mg may have a significant inhibitory effect on apatite development in bone and tooth structures. It is not known, however, whether this effect has any controlling influence on the overall biomineralization process in these structures.

Intracellular calcium precipitation is a promising yet relatively unexplored area of mineralization research. Some evidence has been reported which indicates that the storage and release of calcium and phosphate contained in intramitochondrial granules may be connected in some way with the initial stages of extracellular calcification in bone and cartilage. Although the present studies have been directed toward preparing and characterizing model compounds to platelet dense bodies and in answering questions concerning the role these bodies have in platelet function, the information obtained in these studies should prove invaluable in developing synthetic analogues to intramitochondrial granules.

Proposed Course of Project:

The current studies on the effect of inorganic anions on apatite formation, maturation, and physical properties will continue. Future emphasis will be directed toward examining how various combinations of fluoride, magnesium, and carbonate can effect apatite texture; in particular the size, shape, and surface area of individual crystals. The intracellular calcification studies will continue with emphasis on preparing and characterizing synthetic models of mitochondrial granules.

2. Publications:

Eanes, E.D.: The influence of fluoride on the seeded growth of apatite from stable supersaturated solutions at pH 7.4. J. Dental Res. 59: 144-150, 1980.

Eanes, E.D., Reddi, A.H.: The effect of fluoride on bone mineral apatite. Metab. Bone Dis. and Rel. Res. 2:3-10, 1979.

Eanes, E.D.: Crystal growth of mineral phases in skeletal tissues. Prog. Crystal Growth & Characterization (in press).

Termine, J.D., Eanes, E.D., and Conn, K.M.: Phosphoprotein modulation of apatite crystallization. Calcif. Tissue Int. (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 00162-04 LBS

PERIOD COVERED

October 1, 1979 to September 30, 1980

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

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Youmans, Patricia A.	Secretary (steno)	LBS	NIDR
DeGraff, Barbara A.	Purchasing-agent	LBS	NIDR

COOPERATING UNITS (if any)

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SECTION

Molecular Structure Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.97	1.0	.97

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☐ (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.

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 kinetic and thermodynamic information for the correlation between events that occur in solution versus those that occur in the solid state. The solid material is isolated from the well-characterized solutions by Millipore filtration and lyophilized. The calcium phosphate precipitates are analyzed for other possible lattice constituents (i.e. acid phosphate, carbonate, and hydroxide) as well as the calcium and phosphate contents.

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. This amorphous calcium phosphate (ACP) phase, if left in contact with solution, transforms into a crystalline material with an apatitic-like x-ray diffraction pattern but with the thermodynamic properties of another well-defined calcium phosphate phase, octacalcium phosphate, $Ca_8H_2(PO_4)_6$ (OCP). After a reproducible period of time, which is greatly dependent upon solution conditions, this intermediate crystalline phase transforms into a more basic calcium phosphate phase with a tricalcium phosphate, $Ca_3(PO_4)_2$ (TCP), stoichiometry. This latter phase gradually matures to hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, the thermodynamically stable phase under physiological conditions.

Since previous experimental results suggested that the first formed crystalline phases were deficient in hydroxyl ion (i.e. approximating the composition of OCP or TCP), a titration method was developed to directly analyze the precipitates for their hydroxyl content. As predicted by the thermodynamic results, the first formed crystalline phases were found to have little lattice hydroxyl ion. Surprisingly, it was also found that even the mature apatitic materials contained less hydroxyl than expected for stoichiometric HA with an apparent maximum of about one-half of the apatite hydroxyl

lattice positions filled with hydroxyl ion. A number of defect hydroxyapatites have very recently been analyzed by chemical methods with the result that a general compositional formula can be used to describe the whole range of non-stoichiometric crystalline calcium phosphates.

$$Ca_{10-X-Y}(HPO_4)_X(PO_4)_{6-X}(OH)_{2-X-2Y}$$

This model describes two types of calcium and hydroxyl vacancies. The first X-type) couples the loss of a hydroxyl and the addition of a hydrogen ion with a calcium vacancy and the second (Y-type) provides for electrical composition by two vacant hydroxyl positions for each missing calcium.

Citrate ion was shown to have an effect on the phase transitions which accompany the spontaneous precipitation of calcium phosphate at 25°C and constant pH's of 7.40 and 7.80. Citrate had a minimal effect on the amorphous-crystalline transformation at near-physiological concentrations, but had a small, measurable stabilizing effect on the amorphous phase at higher concentrations ($\geq 2 \times 10^{-4}$M). Citrate, at all concentrations studied, greatly decreased the rate of conversion of the first formed OCP-like to the more apatite-like secondary crystalline phase. The solubilities of the calcium phosphate phases were not affected, however. These results suggest that the observed specific interactions of citrate with crystalline calcium phosphates may play a causal role in determining the physical and chemical characteristics of the inorganic component of hard tissue.

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:

Future research efforts will further characterize the entire course of the precipitation of calcium phosphate from the initial formation of ACP to its eventual transformation to crystalline HA. Emphasis will be placed on determining how the solution environment affects the kinetics of precipitation processes and the final composition of the inorganic phases. Particular attention will be placed on the role that calcium phosphate crystal growth inhibitors play in influencing the final composition of the mineral phase. Initially, work will center on known physiological and pharmacological inhibitors of calcification and their mechanisms of action at the surfaces of the calcium phosphate precipitates. Since calcification inhibitors are generally proposed as regulators of normal biological mineralization and since they have often been implicated in the initiation and progression of pathological calcification conditions in the body, it would be desirable to extend the fundamental mechanistic studies to a more biologically relevant

setting. To this end, application has been made to spend a year in residence, in fiscal 1981, at the laboratory of Dr. Herbert Fleisch at the Pathophysiology Institute of the University of Berne, Switzerland, in order to receive training in new methods to better assess the role and function of physiologically active inhibitors of calcification.

2. Publications:

Meyer, J.L. and Selinger, A.H.: The effect of citrate on calcium phosphate phase transitions. Mineral Electrolyte Metab., 3:207-216, 1980.

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 00204-04 LBS

PERIOD COVERED

October 1, 1979 to September 30, 1980

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

Reddi, Akepati H.	Research Biologist	LBS	NIDR
Kuberasampath, Thangavel	Visiting Fellow	LBS	NIDR
Weiss, Roy E.	Post-doctoral Fellow	LBS	NIDR
Lenk, Elaine V.	Visiting Associate	LBS	NIDR
Sullivan, Noreen	Physical Science Technician	LBS	NIDR
Oldach, David	Biological Aid	LBS	NIDR
Mulraine, Lubin	Student-trainee, Bio. Aid	LBS	NIDR
Rattley, Charles	Student-trainee, Bio. Aid	LBS	NIDR
McDonald, Lorene	Biological Aid	LBS	NIDR
Youmans, Patricia A.	Secretary (steno)	LBS	NIDR
DeGraff, Barbara A.	Purchasing-agent	LBS	NIDR
Floyd, Steven W.	Photographer (laboratory)	LBS	NIDR

COOPERATING UNITS (if any)

LDBA, NIDR, and Dr. P.V. Hauschka, Childrens Hospital, Harvard Medical School, Boston, Mass.

LAB/BRANCH

Laboratory of Biological Structure

SECTION

Molecular Structure Section

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.46	2.11	2.35

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 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) role of fibronectin during collagenous matrix-mesenchymal cell interaction; (2) immunofluorescent localization of fibronectin during matrix-induced cartilage, bone and bone marrow development; (3) changes in types I and III collagen synthesis during matrix-induced endochondral bone development; (4) appearance of γ-carboxyglutamic acid during onset of mineralization during matrix-induced endochondral bone development; (5) synergistic action of growth hormone and thyroid stimulating hormone on endochondral bone differentiation; and (6) local influence of insulin on endochondral bone development.

1. Project Description

Introduction and Background:

The origin and evolution of multicellular organisms were marked by the appearance 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.

Role of Fibronectin During Collagenous Matrix-Mesenchymal Cell Interaction:

It has been well documented that anchorage to a substratum is a fundamental condition for *in vitro* growth of normal fibroblasts. It has been shown for many cell types that without the addition of serum to culture medium, cell attachment does not occur and subsequent growth does not ensue. Fibronectin is a serum glycoprotein that has been shown to function *in vitro* as an adhesive component for cell-substratum (usually collagen) or cell-cell interaction. Extrapolating from the *in vitro* data to the *in vivo* situation, similar conditions for growth and differentiation may be required, i.e. anchorage of cells to a suitable substratum prior to cell proliferation. The biosynthesis of fibronectin during the *in vivo* development of matrix-induced endochondral bone was investigated in rats using [^{35}S]-methionine. Fibronectin was identified in tissue extracts by its (1) comigration on electrophoretic SDS gels with human and rat fibronectin; (2) affinity for denatured collagen; (3)

cross-reactivity with purified antibody to rat plasma fibronectin: and (4) insensitivity to collagenase digestion. The implanted demineralized bone matrix bound circulating fibronectin. This may be an important initial requirement for cell attachment to the matrix. Fibronectin was present throughout the development of bone but accounted for the largest percentage of total noncollagenous protein synthesized during mesenchymal cell proliferation and hematopoiesis.

The importance of fibronectin in in vivo collagenous matrix-mesenchymal cell interaction was investigated using purified antibodies to rat plasma fibronectin. Local injections of the purified antibodies inhibited collagenous matrix-mesenchymal cell interaction, reducing proliferation and differentiation of chondroprogenitor cells. These results indicate the physiological importance of fibronectin in the initial extracellular matrix-cell interactions involved in this morphogenesis.

Immunofluorescent Localization of Fibronectin During Matrix-induced Cartilage, Bone and Bone Marrow Development:

Fibronectin was localized by indirect immunofluorescence during the various phases of endochondral bone formation in response to subcutaneously implanted demineralized bone matrix. Its histologic appearance was correlated with results of biosynthetic experiments. (1) The implanted collagenous bone matrix was coated with fibronectin prior to and during mesenchymal cell proliferation. (2) During proliferation of mesenchymal precursor cells the newly synthesized extracellular matrix exhibited a fibrillar network of fibronectin. (3) During cartilage differentiation the fibronectin in the extracellular matrix was apparently masked by proteoglycans as judged by hyaluronidase treatment. (4) Differentiating chondrocytes exhibited a uniform intracellular distribution of fibronectin. (5) Fibronectin was present in a cottony array around osteoblasts during osteogenesis. (6) The developing hematopoietic colonies revealed fibronectin associated with them. Therefore it appears that fibronectin is ubiquitous throughout the development of endochondral bone and bone marrow.

Changes in Types I and III Collagen Synthesis During Matrix-induced Endochondral Bone Development:

The changes in rates of hydroxyproline formation and biosynthesis of type I and III collagen during bone matrix-induced sequential differentiation of cartilage, bone and bone marrow in rat were investigated. Biosynthesis of types I and III collagen at different stages of this sequence was studied by labelling in vivo and in vitro with [2,3-^{3}H]proline. Pepsin-solubilized collagens were separated by sodium dodecyl sulphate/polyacrylamide slab gel electrophoresis. The results revealed that maximal amounts of type III collagen were synthesized on day 3 during mesenchymal-cell proliferation. Thereafter, there was a gradual decline in type III collagen synthesis. On day 9-20 during bone formation predominantly type I collagen was synthesized. Similar results were obtained by the use of labelling techniques both in vivo and in vitro.

Appearance of γ-Carboxyglutamic Acid During Onset of Mineralization During Matrix-induced Endochondral Bone Development:

γ-Carboxyglutamic acid (Gla) is a constituent of the non-collagenous bone protein osteocalcin. The appearance of γ-carboxyglutamic acid during de novo differentiation and development of endochondral bone has been correlated with the onset of mineralization. Residual Gla in acid-demineralized bone matrix was lost rapidly on implantation. Gla levels were basal during mesenchymal cell proliferation (day 3) and chondrogenesis (days 5-7). Gla and calcium levels began to increase during cartilage mineralization (day 9) and continuously increased after day 10 concomitant with bone differentiation. This experimental system is now amenable to further studies on the biosynthesis and function of Gla-containing proteins in developing bone. Further, this experimental model may be informative in studies on vitamin K deficient rats, and such work is currently in progress.

Influence of Growth Hormone and Thyroid Stimulating Hormone on Endochondral Bone Development:

The influence of hypophysectomy, growth hormone and thyroid stimulating hormone on the discrete phases of matrix-induced endochondral bone differentiation was investigated. ^{3}H-thymidine incorporation by proliferating mesenchymal cells on day 3 was inhibited by hypophysectomy (hypox), but not corrected by growth hormone administration. On day 7, $^{35}SO_4$ incorporation into cartilage proteoglycans was reduced by hypox but was restored to values higher than controls by growth hormone. Calcification of cartilage and bone was monitored by alkaline phosphatase activity, ^{45}Ca incorporation into bone mineral and total calcium. Alkaline phosphatase levels were maximal on day 11 in the controls and declined thereafter; however, the activity of alkaline phosphatase remained elevated in hypox rats. Hypophysectomy reduced and delayed the rate and extent of calcification as reflected by ^{45}Ca incorporation and total calcium respectively. Administration of GH and TSH alone and in combination restored the ^{45}Ca incorporation to control values in tibial metaphyses but not in the matrix-induced osteogenic plaques on day 10. These findings imply that the hormonal requirements for initiation of de novo mineralization of bone may be different from the maintenance of mineralization that was initiated in early fetal life as in the case of metaphyses. Hypophysectomy resulted in a delayed and reduced bone formation due to (1) inhibition of mesenchymal cell proliferation, (2) decreased and delayed chondrogenesis, (3) delayed and reduced vascular invasion, and (4) impaired bone formation.

Local Influence of Insulin on Endochondral Bone Development:

Previous studies from our laboratory have shown that matrix-induced bone development is impaired in diabetic rats. In further experiments we demonstrated that in streptozotocin-induced diabetes mesenchymal cell proliferation as assessed by ornithine decarboxylase activity and ^{3}H-thymidine incorporation was inhibited and was corrected by exogenous systemic insulin. More recently we have administered insulin locally at the implant site and found that it corrected the mesenchymal cell proliferation. Further, local injection of anti-insulin antibody into normal non-diabetic rats inhibited cell proliferation. These results implicate insulin directly in skeletal tissue development and metabolism.

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.

Proposed Course of Project:

The current studies on the role of fibronectin during collagenous matrix-mesenchymal cell interaction will continue. We will examine the role of fibronectin by monospecific antibodies to fibronectin. Future studies on the biochemistry of endochondral bone formation will focus on biosynthesis of glycoproteins by a combined radioautographic and biochemical approach. The role of vitamins D and K in mineralization will be examined. Additional studies will be initiated on the role of early mesenchymal cells in chondrogenesis by tissue culture techniques.

2. Publications:

Foidart, J.M. and Reddi, A.H. Immunofluorescent localization of type IV collagen and laminin during endochondral bone differentiation and regulation by pituitary growth hormone. Develop. Biol. 75:130-136, 1980.

Schwartz, R. and Reddi, A.H.: Influence of magnesium depletion on matrix-induced endochondral bone formation. Calcified Tissue International 29:445-448, 1979.

Reddi, A.H. and Sullivan, N.E. Inhibition of mineralization by experimental lathyrism during matrix-induced endochondral bone differentiation. Proc. Soc. Exp. Biol. & Med. 162:445-448, 1979.

Rath, N.C. and Reddi, A.H.: Evaluation of anti-inflammatory drugs based upon the inhibition of matrix-induced ornithine decarboxylase activity during connective tissue proliferation. Proc. Soc. Exp. Biol. & Med. 162:320-323, 1979.

Steinmann, B. and Reddi, A.H. Changes in types I and III collagen synthesis during matrix-induced endochondral bone differentiation. Biochem. J., 186:919-924, 1980.

Eanes, E.D. and Reddi, A.H. The effect of fluoride on bone mineral apatite. Metabolic Bone Disease and Related Research 2:3-10, 1979.

Weiss, R.E. and Reddi, A.H. Influence of experimental diabetes and insulin on matrix-induced cartilage and bone differentiation. Amer. J. Physiol. 238: E200-E207, 1980.

Weiss, R. E. and Reddi, A.H. Synthesis and localization of fibronectin during collagenous matrix mesenchymal cell interaction and differentiation of cartilage and bone in vivo. Proc. Nat. Acad. Sci. USA 77: 2074-2078, 1980.

Hauschka, P.V. and Reddi, A.H. Correlation of the appearance of γ-carboxyglutamic acid with the onset of mineralization in developing endochondral bone. Biochem. Biophys. Res. Comm., 92:1037-1041, 1980.

Reddi, A.H. and Sullivan, N.E. Matrix-induced endochondral bone differentiation: Influence of hypophysectomy, growth hormone and thyroid stimulating hormone. Endocrinology (in press). 1980

Reddi, A.H. Matrix-induced endochondral bone development: A model for regenerative growth. In: "Mechanisms of Growth Control" ed. R.O. Becker, C.C. Thomas (in press). 1980.

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 00251-03 LBS

PERIOD COVERED
October 1, 1979 to September 30, 1980 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

Casciani, Francis S.	Staff Fellow	LBS	NIDR
Lenk, Elaine V.	Visiting Associate	LBS	NIDR
Youmans, Patricia A.	Secretary (steno)	LBS	NIDR
DeGraff, Barbara A.	Purchasing-agent	LBS	NIDR
Floyd, Steven W.	Photographer (laboratory)	LBS	NIDR

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 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.16	0.96	0.2

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 distribution of inorganic species such as calcium, magnesium, phosphate, and carbonate as well as organically bound phosphate is being studied in a number of hard tissues using Raman and ion microprobes as well as x-ray micro-analytical techniques. Topics of particular interest include (1) correlating changes in organic matrix phosphate with mineral accumulation and (2) mapping distribution patterns of inorganic ions at mineralizing sites in developing hard tissue.

1. Project Description

Objectives:

The purpose of this project is to apply microanalytical techniques to the histological study of early mineralization events in skeletal tissue. One objective is to correlate changes in inorganic ion distribution, especially calcium, magnesium, phosphate, and carbonate, with mineralization patterns as seen in developing hard tissue structures. Another objective is to determine changes in the organic matrix, especially changes in organically bound phosphate, which may be associated with the investment and accumulation of mineral phases.

Methods Employed:

Several types of hard tissue were investigated in this study. These included the rat incisor, the epiphyses of embryonic tibia, and matrix-induced bone plaques grown in both normal rats and in rats made rachitic by dietary phosphate deficiency. The tissues chosen allowed for the investigation of the mineralization processes which occur in amelogenesis, dentinogenesis, chondrogenesis, osteogenesis, and endochondral bone formation. As detailed in last year's report, 5-30 μm thick sections for microanalytical analysis were cut from frozen unfixed tissue, mounted on an appropriate substrate (LiF or sapphire) and air-dried. Alternate sections were prepared for conventional histological examination. Photomicrographs were taken of all sections to facilitate correlation of microanalytical and histological data.

The principal microanalytical tools used in this study were the Raman, electron, and ion microprobes. The laser Raman microprobe allows one to record the molecular vibrational spectra of regions as small as 5 μm in diameter or 10^{-9} gms in mass. The electron and ion microprobes provide elemental data on the same regions. Together they are especially suited for obtaining compositional and structural information which can be directly correlated with histological features.

A new sample preparation technique was investigated which may be valuable for Raman spectroscopic analysis of biological tissue. Because the technique of Raman spectroscopy depends on the incidence of an intense beam of visible light, the sample is susceptible to localized heating and burning resulting in increased fluorescence and sample decomposition. However, by attaching the tissue sections with a thin layer of Coe Ortho Resin (see Stain Technology 54(4):229, 1979) intimate thermal contact between the tissue section and sample substrate have been obtained. The method has the advantage of resin polymerization while the tissue section is still frozen. Spectra may be obtained at greater power levels with no deleterious effects, therefore resulting in greater spectral resolution. Interference from the Raman spectrum of the embedding resin is at a minimum because of the small quantity used and the amorphous nature of the medium.

Major Findings:

The Raman spectrum of mineralizing enamel closest to the ameloblastic border suggests the presence of the carbonate mineral Huntite, $Mg_3Ca(CO_3)_4$. Phosphate bands were either very weak or absent from the spectrum. With increasing mineralization, the earliest phosphate mineral phase detected had a ν_1 P-O stretching mode at 960 cm^{-1} which indicates an apatitic structure. Spectral evidence for precursor phosphate phases such as dicalcium or octacalcium phosphate was absent. Preliminary in vitro studies indicate that Huntite can serve as an effective substrate for apatite formation. A close unit cell similarity between the two crystal structures would suggest an epitaxial growth explanation for the in vitro findings.

Taken together, these results suggest that the initial mineral phase to appear in amelogenesis is a carbonate salt and that this phase, in turn, may be a primary locus for the subsequent nucleation of enamel apatite. Electron microprobe analyses were not inconsistent with the Raman findings and interpretation, although magnesium levels were at the limit of sensitivity for the method. Ion microprobe, which has a greater Mg sensitivity, should prove to be the method of choice for this analysis. Findings similar to those for enamel were also obtained from sections of embryonic bone and mineralizing dentin.

The Amide III region of the Raman spectrum of enamel matrix indicates that the majority of the protein in this tissue has an antiparallel β-pleated sheet configuration. Similarly, another portion of the Raman spectrum was identified with the presence in this tissue of proteins having a high glutamic acid content. Most interestingly, Raman bands were identified as arising from the ring stretching mode of either adenosine or guanosine. This suggests that part of the organic phosphate in enamel matrix ascribed in last year's report as belonging to phosphoprotein, may be associated instead with nucleotide structures. In bone and dentin, the Raman spectra were typical of the triple helix of collagen. A preliminary spectral comparison with normal cartilage indicates that rachitic tissue has a lower organic phosphate concentration.

Significance to Dental Research:

The tentative identification of a mineral phase in enamel rich in magnesium and carbonate together with the reported finding that both of these ions are preferentially removed during carious attack suggest that this phase may be an important factor to consider in the development of a comprehensive theory explaining tooth mineralization.

Proposed Course of Project:

Due to the fact that the principal investigator on this project is leaving NIDR shortly and that the final year of the contract with NBS for use of the Raman microprobe will end with the current fiscal year, the project in its present form will be discontinued. It is hoped that certain aspects of this project can be pursued further if suitable personnel can be identified and made available for this purpose.

2. Publications:

Casciani, F.S. and Etz, E.S.: Raman Microprobe Study of Biological Mineralization in Situ: Enamel of the Rat Incisor. Proceedings of 14th Annual Conference of Microbeam Analysis Society, San Antonio, Texas. pp 169-172, 1979.

Summary Statement
Laboratory of Microbiology and Immunology
National Institute of Dental Research

The major program areas in this Laboratory have remained unchanged since last years report. Our research efforts continue to delineate fundamental principles which will serve as a knowledge base for formulating rational approaches to the prevention and/or treatment of oral diseases which result from complex interactions between the resident microbial flora and its host. Based upon this premise, the Laboratory of Microbiology and Immunology has contributed consistently and meaningfully during the past decade. The following is a brief summary of our progress during FY 1980.

The Microbiology Section is studying the role of plasmids in the pathogenesis, ecology and taxonomic status of oral streptococci. Of particular interest this year are the results from studies directed toward examining plasmid-mediated antibiotic resistance in an animal isolate of Streptococcus mutans and the possibility of its transfer to streptococci of human origin. S. mutans strain DL5 was isolated from a pig raised on feeds supplemented with subtherapeutic levels of tetracycline and penicillin. This organism was resistant to high levels of tetracycline (Tc), lincomycin (Lm), erythromycin (Em), and streptomycin (Sm). Significantly, the porcine isolate was shown to transfer its Tc resistance to human strains of S. faecalis and S. mutans by conjugation. Preliminary evidence indicates that the transfer of Tc resistance is mediated by a plasmid. A 12 megadalton plasmid has been isolated from the Tc resistant transconjugants of both S. faecalis and S. mutans. These isolated plasmids are currently being examined by DNA-DNA hybridization techniques and restriction endonuclease digestion to determine if they are identical. This study has important implications regarding the way in which antibiotics are used both in the treatment of infectious diseases and as animal feed supplements.

Another interesting development in the plasmid program was the demonstration that a second type of conjugative plasmid ($pAMB_1$), which mediates resistance to Em and Lm, readily underwent extensive deletions following transfer to new streptococcal host strains. Examination of five such deleted molecules indicated that several functions such as transmissibility, replication and resistance may be altered by the deletions. These deleted molecules are now being used to construct a genetic map of the resistance, replication, and transfer functions of the $pAMB_1$ plasmid.

Our inquiries into mechanisms of carbohydrate transport and metabolism by oral lactic acid bacteria have been expanded. It is now clear that the great majority of mono-and disaccharides metabolized by oral streptococci and lactobacilli are initially transported into the cell by a phosphoenol-pyruvate-dependent phosphotransferase system (PEP-PT). Transport of xylitol and ribitol by L. casei and S. avium is also effected by specific inducible PEP-PT systems. The extensive use of mutants has provided a rewarding approach to our critical analysis of these transport systems. For example, glucose is transported into S. mutans by a PEP-PT system. Using a mutant that was missing this system, it was possible to show that the glucose PEP-PT system is also responsible for the transport of

mannose and glucosamine. Significantly, the glucose PEP-PT-negative mutant was still able to grow on glucose. This clearly indicates that a second, and as yet uncharacterized glucose transport system exists in S. mutans.

The PEP-PT systems responsible for the transport of sucrose and lactose catalyze the formation of sucrose-6-phosphate (S6P) and lactose-6-phosphate (L6P) respectively. A major obstacle to studying the further metabolism of these important disaccharides is that they are not commercially available. A new procedure was developed, therefore, for their chemical synthesis. Sucrose-6-phosphate has also been prepared biologically using a mutant of S. mutans 6715 that is missing the enzyme responsible for hydrolyzing S6P. The availability of these phosphorylated substrates has now made it possible to isolate a S6P hydrolase from S. mutans. This enzyme has been purified to homogeneity and a number of its kinetic and physical properties have been characterized.

We previously described a highly specific form of cell-cell recognition between certain laboratory strains of Actinomyces and Streptococcus sanguis that resulted in the formation of large macroscopic cellular aggregates. Last year we initiated a large survey of actinomycetes and streptococci freshly isolated from human subjects to determine whether they behaved in the same manner as the laboratory strains. That survey involving over 400 new isolates has been completed. The results show that the human isolates exhibit intergeneric coaggregation patterns identical to those found for the laboratory strains. This lends strong support to the notion that the cell surface components (receptors) which mediate these specific cell-cell interactions are highly stable structures and that they are probably responsible for the selective distribution of these bacteria within the oral cavity and in the complex melieu of dental plaque. This ecological study will now focus on the complete characterization of the cell surface components responsible for the coaggregation interactions. As will be indicated subsequently, the preparation of monoclonal antibodies to these receptors should be a powerful tool in this undertaking.

The Cellular Immunology Section is investigating various mechanisms by which cell-mediated host defense responses to microbial or other inimical stimulants result in inflammation. A number of the investigators are studying immunoregulatory mediators that are produced by the activated inflammatory cells. Members of the section are intensively studying the biological activities, regulation of production and biochemical characteristics of several human and murine mediators that amplify immunological reactions. These mediators are polypeptides that are active at 10^{-10} to 10^{-15} M concentrations. It has been determined that colony-stimulating factor (CSF), in addition to its growth promoting effects, also activates macrophages to produce Interleukin-1 (IL-1). This monocyte-derived mediator is an essential second signal that augments the proliferation and differentiation of thymocytes, and promotes the immunological functions of mature lymphocytes. Recently IL-1 or very similar monocyte-derived mediators have also been found to have endogenous pyrogenic activity, to stimulate synovial cells to produce collagenase and prostaglandins and

to stimulate hepatocytes to produce acute phase proteins such as serum amyloid A (SAA). Thus, IL-1 appears to be involved in lymphocyte as well as non-lymphocyte mediated acute systemic inflammatory reactions. Using a hyperstimulated murine cell line, sufficient IL-1 activity has been obtained to permit its purification to homogeneity.

One of the macrophage dependent lymphokines that requires IL-1 to be produced by the "helper" lymphocyte subpopulation is termed Interleukin-2 (IL-2). The IL-2 is being purified and means of stimulating thymic cell (EL-4) lines to produce IL-2 have been found. IL-2 in turn promotes the proliferation and differentiation of normal cytotoxic T lymphocyte lines. IL-2 indirectly also promotes antibody production by B cells, and immune interferon production by another lymphocyte subpopulation. Furthermore, as yet unidentified lymphokines promote macrophage expression of Ia and Fc receptor membrane determinants. Thus a complex sequence of stimulant, cell and mediator interactions appears to be necessary to mount immunologically mediated inflammatory reactions to deleterious agents. It is only through an understanding of macrophage and lymphocyte functions at these molecular levels that we can learn to manipulate the inflammatory response in a therapeutic manner.

It has long been suspected that inflammation serves not only as a protective mechanism, but may if excessive, actually be destructive to the host. Indeed it has been found that activated macrophages can have immunosuppressive effects in vivo as well as in in vitro tissue culture models. Several members of the section have found that this deleterious behavior of macrophages can be reversed by indomethacin together with catalase. This implicates prostaglandins and oxygen radicals as the cause of the suppression by macrophages activated by bacterial products and/or endogenous mediators such as CSF or lymphokines. Application of this hypothesis has led to the conclusions that reduced glutathione in serum, 2-mercaptoethanol, other thiol compounds, catalase and vitamin E all have beneficial effects because they promote the elimination of deleterious oxygen radicals from in vitro cultures. The vitamin E in conjunction with indomethacin have improved the in vitro reactions of lymphocytes from anergic patients with Hodgkin's Disease. Current studies suggest that the synergistic effects of these two drugs are due to the bidirectional negative feedback effects prostaglandins and oxygen radicals have on each other. The effect of various inhibitors of prostaglandin synthetase together with radical scavengers on injurious in vitro inflammatory reactions needs to be ascertained.

Finally several investigators are studying the role of exogenous stimulants such as lipopolysaccharide endotoxins (LPS) derived from gram negative bacteria on cellular and humoral immune responses. The C3H/HeJ LPS unresponsive mutant strain of mice has been used for these studies. The C3H/HeJ mice become responsive to LPS if given normal syngeneic bone marrow cells indicating that lymphoreticular cells are responsible for LPS sensitivity. Administration of BCG vaccine or other adjuvants to the C3H/HeJ mouse normalizes its LPS reactions just as germ-free mice lose their resistance to LPS when "conventionalized". These observations led to experiments which indicated that the macrophage of the C3H/HeJ was hyporeactive to LPS and that this could be reversed by lymphokine-

containing spleen cell supernatants or cAMP agonists. The latter agents improved the Fc receptor expression and phagocytic capabilities of these macrophages as well as their reactivity to LPS. Thus, the same gene that controls LPS sensitivity appears to regulate differentiation of Fc receptor expression on membranes. Conversely, these findings suggest that normal differentiation of lymphoreticular cells depends in part on stimulation by exogenous agents such as LPS. These findings once again emphasize the crucial role of exogenous stimulants in activating and mobilizing inflammatory cells, and that they also play a vital role in promoting the development, differentiation and signal (mediator) emission by macrophages and lymphocytes.

The Humoral Immunity Section is studying the modulation of connective tissue metabolism by both cellular and humoral mechanisms. The cells of the immune system have been implicated in the production of fibrotic lesions since supernatants of activated lymphocyte and macrophage cultures contain soluble mediators which stimulate proliferation of and collagen synthesis by fibroblasts. Partial characterization of these products has revealed that lymphocyte supernatants contain two chromatographically separable factors which possess these activities. Of major interest is the recent finding that macrophages may be instrumental in the initiation of fibrosis since they also produce a factor which is chemotactic for fibroblasts. Preliminary evidence indicates that this chemotactic factor is distinguishable from that which stimulates fibroblast proliferation. The glycoprotein, fibronectin, is also produced by macrophages and its possible relationship to the other macrophage-derived biological activities is under investigation. The findings described have been obtained using peritoneal exudate cells but the demonstration that a resident macrophage population (the Kupffer cells of the liver) also produces these mediators implicates their involvement in local fibrosis.

Studies concerning the immunological abnormalities which exist in osteopetrosis have been extended to include additional animal models. The most recently acquired strain is the osteopetrotic (op) rat. The spleen cells from these animals respond minimally to both T and B cell mitogens as compared to those from normal littermates. This effect is apparently attributable to macrophage-lymphocyte intractions since the proliferative responses of lymphocytes from affected rats are restored by the removal of the macrophage population. The mechanism(s) by which macrophages modulate lymphocyte function in this and other animal strains exhibiting defective bone resorption is currently being examined.

Important new insights into the mechanisms involved in coaggregation of oral microorganisms have been obtained by the utilization of immunochemical techniques. Two immunoelectrophoretically distinct surface antigens have been demonstrated on Actinomyces viscosus T14V, a strain which expresses specific lectin activity for a surface carbohydrate on several strains of streptococci. These two high molecular weight antigens have been separated by physicochemical methods and are both fibrillar structures by electron microscopic examination. The production of monoclonal antibodies reactive with A. viscosus T14V has provided reagents which identify the presence of the lectin responsible for adherence of

this microorganism on only one of the two types of surface fibrils. These monoclonal antibodies are currently being used in the purification of the lectin-containing fibrils and the further characterization of their specificities will define the structural components of the actinomycetes which bind to other bacteria and mammalian cells. These antibodies will also serve as valuable aids for investigating the function of immunoglobulins in the intervention of the accumulation of oral bacterial coaggregates.

Immunoglobulins might be of therapeutic value when used as carriers to specifically direct biologically active agents to a local tissue site where they might influence several parameters of host defense including the destruction of tumors. Inflammatory cells possess tumoricidal activity and their concentration at the tumor site might be enhanced by the administration of tumor-specific antibodies complexed to a factor which is chemotactic for these cells. Antibodies specific for a guinea pig hepatoma have been produced and covalently coupled to the synthetic chemotactic peptide, formyl-methionyl-leucyl-phenylalanine. The antibody-peptide complexes are chemotactic for macrophages and bind specifically to tumor cells. These findings provide a basis for the current and proposed studies dealing with the in vivo evaluation of the effect of antibody-peptide complexes on local macrophage accumulation, tumor growth and mortality.

The Clinical Immunology Section continues its studies to understand the mechanisms by which cells secrete inflammatory mediators (e.g. histamine or serotonin). A fundamental question is how a signal is generated at the cell surface by a stimulus and then transmitted across the membrane. Cell triggering is due to the cross-linking of a small number of surface receptors. The formation of receptor dimers are effective for the activation of the cell. One of the early steps in cell activation appears to be phospholipid methylation. Current studies have demonstrated that inhibitors of methylation reactions inhibit the IgE-mediated histamine release from basophils. These inhibitors modulate the levels of S-adenosyl-L-methionine in the cell. However, basophils and mast cells can be activated by other inflammatory agents (e.g. C5a, f-Met-Leu-Phe, the ionophore A23187, compound 48/80) which do not require the methylation pathway. Another step which appears to be involved in secretion is phospholipase A-2 activation with the release of arachidonic acid. Further studies of these pathways will utilize mutants of basophilic cell lines which are defective in their cell secretory pathway.

Interactions between lymphocytes and basophils or mast cells may play an important role in modulating immunological reactions. Immune interferon released from lymphocytes by antigen results in an alteration of the histamine releasing capability of basophils. Therefore, immune interferon can modulate the level of histamine secretion from these cells. Other studies have shown the capacity of mast cells to mediate delayed hypersensitivity reactions. These studies therefore shed light on the modulation of both immediate and delayed hypersensitivity reactions.

A major development in immunology has been the use of hybridomas for the

production of monoclonal antibodies. With this technique spleen cells from immunized mice are fused to a cell line and clones are selected which synthesize antibody to the specific antigen. The method has great promise as a tool to prepare antibodies to single determinants on a cell surface or monoclonal antibodies of different immunoglobulin classes. The usual techniques for the preparation of these antibodies are cumbersome and require the screening of a large number of clones. Two techniques were developed in our laboratory which dramatically simplify the procedure. The techniques depend upon the expansion of the antigen-specific spleen cells by either culturing with antigen or by spleen cell transfer into irradiated animals and subsequent antigenic challenge. Utilizing these techniques we have selected monoclonal lines producing anti-hapten antibodies of different immunoglobulin classes as well as an IgE-producing hybridoma. At present we are attempting to prepare monoclonal antibodies to lymphokines and cell-surface receptors (e.g. Fc receptors). Several different hybridoma antibodies directed against the fimbriae (lectins) from Actinomyces viscosus have been prepared. As indicated earlier the monoclonal antibodies will be useful for studies of the mechanism of these coaggregation phenomena.

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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-00007-20 LMI

PERIOD COVERED
October 1, 1979 - September 30, 1981

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

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The mechanism by which various pathways of carbohydrate metabolism are regulated in oral bacteria continue to be under investigation. Special emphasis is currently placed on resolving 1) the mechanism by which Streptococcus salivarius exports the enzyme glucosyltransferase (GT) into the extracellular medium and 2) characterizing a factor (s) involved in the inactivation of a cell-associated fructosyltransferase. We have employed several techniques for altering the unsaturated: saturated fatty acid ratio in the cell membrane and then examined such modified cells for their ability to secrete GT. When the normal unsaturated:saturated fatty acid ratio of the lipid bilayer was increased 2-fold by growing the organism in the presence of Tween 80, the differential rate of extracellular GT production was very high. If the same membrane-unsaturated: saturated fatty acid ratio was imposed on cells by shifts in the growth temperature, however, the differential rate of GT production was very low. We conclude that the physical state of the bacterial membrane is not the sole factor involved in regulating GT secretion. Preliminary studies on the S. salivarius cell-associated FT indicate that this enzyme undergoes proteolytic digestion under conditions of amino acid limitation.

PHS-6040

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) resolving the mechanism by which Streptococcus salivarius exports the enzyme glucosyltransferase into the extracellular medium and 2) characterization of a factor(s) involved in the inactivation of an S. salivarius cell-associated fructosyltransferase.

METHODS EMPLOYED:

All are standard techniques routine to the type of studies herein described.

MAJOR FINDINGS:

Glucosyltransferase secretion. Our prior studies established a close link between lipid synthesis and the export of glucosyltransferase (GT) by Streptococcus salivarius. The antibiotic cerulenin, a specific inhibitor of β-keto-acyl thioester synthase, severely inhibited the incorporation of acetate into cellular lipids and completely blocked the production of extracellular GT by both growing cultures and resting cell suspensions. Cerulenin had no appreciable effect on the biosynthesis of other macro-molecules such as DNA, RNA or protein. It has been of interest to determine the nature of this requirement for de novo long chain fatty acid synthesis as it relates to the mechanism of GT export from the cell. We have considered that fatty acid synthesis may be necessary for maintaining the cell membrane in a proper physical state for the insertion and transfer of GT across the lipid bilayer.

S. salivarius has a requirement for Tween 80 for maximun production of extracellular GT when grown in a chemically defined medium. This surfactant contains a single 18 carbon monounsaturated fatty acid (oleic acid) in ester linkage with sorbitol anhydrides. Other members of the Tween series (Tween 20, 40 and 60) contain saturated fatty acids in place of oleate and are relatively ineffective in stimulating GT production. We have analyzed cells grown in the presence and absence of Tween 80 for the fatty acid content of the membrane lipids and find that cells grown with Tween 80 have membrane lipids that are heavily enriched with oleic acid. Oleic acid represented 29% of the total fatty acid content of cells grown without Tween 80 whereas this value increased to over 46% in those cells grown with the surfactant. The overall unsaturated: saturated fatty acid ratio of Tween 80 grown cells was 2.04 compared to 1.01 for

cell grown without Tween 80. This large shift in the unsaturated: saturated fatty acid ratio would greatly affect membrane fluidity and the results suggested that alterations in the physical state of the cell membrane could be a controlling factor in the transport of GT outside the cell. To further test this possibility we used shifts in growth temperature. When the organism was grown at 30°C in the absence of Tween 80, the membrane lipids were also enriched with oleic acid and had overall unsaturated: saturated fatty acid ratio indistinguishable from cells grown at 37°C with Tween 80. If the fatty acid composition of the cell membrane were the sole controlling factor for GT secretion, cells growing at 30°C in the absence of Tween 80 should be able to produce the exoenzyme as well as cells growing at 37°C with the surfactant. What was observed, however, was that the differential rate of extracellular GT production by cells growing at 30°C without Tween 80 was less than one-tenth of that observed with cells grown at either 30°C or 37°C with Tween 80. This was not due to a temperature effect on protein synthesis, because the differential rate of general protein synthesis was unaffected by temperature shifts or by Tween 80. Although the physical state of the cell membrane may be involved in GT secretion, these results show that stimulation of GT production by Tween 80 involves something more than simply altering the unsaturated; saturated fatty acid ratio of the lipid bilayer.

Cell-associated fructosyltransferase. Fructosyltransferase (FT) catalyzes the synthesis of a high molecular weight fructan polymer from sucrose. It is produced by a number of different oral bacteria and is generally found extracellularly in the culture medium. Unexpectedly, the S. salivarius FT was found to be almost exclusively cell-associated. Moreover, when cell suspensions were incubated at 37°C, the cell-associated FT activity was lost in a time dependent manner. This was not due to a release of the enzyme to the extracellular medium. Significantly, the loss of cell-associated FT activity could be prevented by the addition of a mixture of amino acids to the incubation medium. Recently, the enzyme has been released from cells before and after inactivation by treatment of the cells with mutanolysin, an N-acetylmuramidase. The cell-free enzyme was then analyzed by polyacrylamide gel electrophoresis. Both the FT activity and corresponding protein bands had disappeared in the preparation obtained from cells after inactivation. The results indicate that the loss of cell-associated FT activity is due to proteolytic hydrolysis of the protein. The fact that this activity is prevented by free amino acids suggests that the proteolysis may be regulated by the available amino acid supply in the growth medium.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

These studies on the mechanism of GTase secretion and its regulation 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, certain of 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 bacterial successfully colonize and subsist in the complex oral ecosystem. Results from our studies on glucosyltransferase secretion by oral streptococci may also provide insight into possible means of artifically controlling its production.

PROPOSED COURSE:

In general, our studies on exoenzyme secretion by members of the oral microflora will be continued. Special emphasis will be placed on resolving the nature of the requirement for de novo long chain fatty acid synthesis as it relates to the export of glucosyltransferase outside the cell. Our studies on the proteolytic digestion of the S. salivarius cell-associated fructosyltransferase will be expanded. Attempts will be made to isolate and characterize the protease involved and to determine its substrate specificity. We will also investigate the apparent role of free amino acids in regulating the activity of the protease.

PUBLICATIONS:

1. St. Martin, E.J. and C.L. Wittenberger. 1979. Regulation and function of sucrose 6-phosphate hydrolase in Streptococcus mutans. Infect. Immun. 26: 487-491.

2. St. Martin, E.J. and C.L. Wittenberger. 1980. Regulation and function of ammonia-assimilating enzymes in Streptococcus mutans. Infect. Immun. 28: 220-224.

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 00022-14 LMI B

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Comparative physiology of lactic acid bacteria and other oral microbes

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)

While the dissimilation of pentitols by Streptococcus avium and certain strains of Lactobacillus casei is catalyzed by similar enzymatic mechanisms, the manner in which the two groups of bacteria regulate and induce pentitol metabolizing enzymes appears to be different. The pentitol: PEP phosphotransferase system and pentitol dehydrogenases are gratuitously induced when L. casei is grown on gluconate or ribose; this does not occur when S. avium is grown on the same substrates. The transport systems for ribitol and xylitol differ from the sugar and sugar alcohol transport systems studied in enteric bacteria and staphylococci in their requirement for specific soluble factors other than enzyme I and histidine-containing phosphorylating protein. The Cytophaga species isolated from the gingival pockets of patients with periodontitis have been further characterized. Transition from anaerobic to aerobic growth is accompanied by the appearance of a completely functional tricarboxylic acid cycle; a compete alteration of metabolic end products form glucose and a drastic change in final cell yield and culture pH. Adsorption of these gram negative gliding bacteria to hydroxyapatite conforms to the Langmuir isotherm equation indicating that a receptor-specific interation between bacterium and substrate takes place.

OBJECTIVES:

A. A study describing the sequence of induction, regulation and mechanism of action of the enzymes participating in pentitol metabolism is being continued. Currently, emphasis has been placed on the generation of a set of mutants of Lactobacillus casei and Streptococcus avium to achieve the following goals:

1. A complete description of the PEP-dependent transport systems responsible for the uptake of xylitol/D-arabitol and ribitol.

2. Determine the mechanism by which glucose regulates pentitol metabolism and biosynthesis of pentitol-dissimilating enzymes.

3. Determine the induction sequence for pentitol transport systems and pentitol phosphate dehydrogenases.

B. A descriptive physiological and ecological study of the oral, Gram negative Cytophaga species commonly found in the pockets of patients with periodontitis is also well underway. The following areas are of primary concern:

1. A biochemical characterization of the changes in these cells upon transition from anaerobic to aerobic growth.

2. A complete taxonomic description of the existing culture collection.

3. Determine the nature of the cell wall (outer membrane) re ceptor sites responsible for the organism's attachment to tooth surfaces.

4. Estimate numbers of the organism in periodontal pockets to establish significance of microorganisms to periodontal disease.

METHODS EMPLOYED:

Conventional immunological, biochemical and bacteriological methods were employed in the studies reported here.

MAJOR FINDINGS:

A. Pentitol dissimilation by lactic acid bacteria. While strains of L. casei and S. avium metabolize ribitol, xylitol and D-arabitol by

similar enzymatic mechanisms, a significant difference exists in the mode of enzyme induction between the two groups of organisms. When grown or ribose or gluconate, strains of L. casei coinduce the ribitol and xylitol/ D-arabitol transport systems as well as the ribitol-and xylitol phosphate dehydiogenases; S. avium synthesizes none of these enzymes systems under identical growth conditions. Synthesis of these enzymes by the latter organism occurs only in the presence of specific inducers, namely, ribitol, xylitol or D-arabitol. Only two enzyme systems appear to be responsible for the catabolism of the three pentitols. One is specific for ribitol while the second metabolizes xylitol and D-arabitol. The conclusion that only a single enzyme system dissimilates both xylitol and D-arabitol is based on the following evidence; (i) genetic studies revealed that a mutation rendering one of the structural gene products of the xylitol transport system inoperative affects the D-arabitol system in an identical fashion, (ii) reciprocal induction occurs with either substrate, (iii) D-arabitol is an effective competitive inhibitor for the xylitol transport system while ribitol is not.

PEP-dependent transport systems in most gram-positive bacteria are composed of two soluble (cytosol) components, i.e. HPr and enzyme I, and two membrane-bound components, enzyme IIa/IIb. Reconstruction experiments with the xylitol and ribitol PEP: phosphotransferase system revealed that transport of these substrates involves a more complex organization of enzymes than that required for carbohydrates. In addition to the four components mentioned above, a third soluble factor (factor III) is required for the transport of ribitol and xylitol. This factor is highly specific for its substrate, therefore, the ribitol factor III will not substitute for the xylitol factor III; the same is true for reciprocal exchanges. However, the cytosol components for the xylitol transport system from L. casei will catalyze xylitol transport when combined with the membrane-bound components from the S. avium xylitol transport system. Reciprocal interchanges also transported xylitol.

B. Biochemical and attachment properties of oral Cytophaga species. As previously reported, oral Cytophaga sp. are capable of growing aerobically or anaerobically in the absence of CO_2. However, it has recently been shown that a transition from anaerobic conditions to air is accompanied by several drastic metabolic changes. In the absence of air, the pH of the medium falls from 7.1 to 5.4 during growth of the organism due to the conversion of glucose to succinic acid and trace amounts of acetic acid. Transferring such cultures to aerobic conditions of growth, succinic acid is no longer produced from glucose and the pH of the culture rises to 7.8. A comparison of the enzyme composition of aerobically vs. anaerobically grown cells reveals that both possess the enzymes of the tricarboxylic acid cycle, namely, isocitrate dihydrogenase, succinic dehydrogenase, malic enzyme and malic dehydrogenase. Only extracts of aerobically grown cells contain detectible levels of oxo-ketoglutarate dehydrogenase. Hence, aerobic growth results in the induction of this enzyme which permits the organism to ixidize

a marked increase in cell yield per mole glucose. A variety of physical and enzymatic treatments of resting cells of two strains of Cytophaga reveals that attachment to hydroxyapatite surfaces involved outer membrane components that are both protein or lipid in nature. Treatment with certain proteinases and phospholipases specific for polar head group cleavage significantly reduced the bacteriums ability to bind to hydroxyapatite beads. These bacteria adhere to hydroxyapatite to the same extent as Streptococcus sanguis; however, in contrast to the latter, pretreating the substrate with saliva drastically reduced attachment.

"SIGNIFICANCE TO BIOMEDICAL RESEARCH"

The utilization of pentitols by lactic acid bacteria appears to be a rare trait that is presently limited to one species of Streptococcus, S. avium and twenty percent of all Lactobacillus casei strains tested thus far. However, of the transfer of genetic material among lactic acid bacteria, especially interspecific transfers, occurs with any frequency, the current use of pentital sugar substitutes will eventually select far populations of oral streptococci and lactobacilli capable of using these substrates for the production of lactic and acetic acid. It is important, therefore, to determine how these organisms regulate and metabolize pentitols.

It is not yet known whether cytophaga sp. actively participates in the onset or chronic native of periodontal disease. However, their ability to glide about and subsequently colonize the tooth root surface assures their presence in the periodontal pocket. The endotoxin present in their outer membrane may contribute to the massive immunological response observed in cases of chronic periodontitis.

PROPOSED COURSE OF RESEARCH:

A. An attempt will be made to biochemically define the newly identified soluble transport components that participate in ribitol and xylitol transport. The site of glucose repression of pentitol transport can be defined specifically by determining whether synthesis of enzyme II or factor III is repressed. The collection of transport mutants will also be used to identify the inducers for the various steps of pentitol metabolism.

B. The outer membrane of the Cytophaga sp. will be analyzed with the aim of identifying the protein and lipid components involved in attachment to hydroxyapatite. Antisera against whole cells is being prepared for the purpose of estimating numbers of gliding bacteria in periodontal lesions with the ultimate goal of establishing their ecological importance. Finally, the relationship between oral and soil Cytophaga will be determined to establish the reservoir for this organisms.

PUBLICATIONS:

1. Celesk, R.A., R. McCabe and J. London, Colonization of the cementum surface of teeth by oral Gram-negative bacteria. Infect. Immun. 26: 15-18, 1979.

2. London, J. and N.M. Chace. Pentitol metabolism in Lactobacillus casei. J. Bacteriol. 140: 949-954, 1979.

3. Johnson, J.L., C.F. Phelps., C.S. Cummins, J. London and F. Gasser. Taxonomy of the Lactobacillus acidophilus group. Intern. J. Sys. Bacteriol. 30: 53-68, 1980.

4. Celesk, R.A. and J. London. Attachment of oral Cytophage species to hydroxapatite containing surfaces. 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-00042-10 LMI

PERIOD COVERED

October 1, 1979 - September 30, 1980

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

Bruce M. Chassy	Research Chemist	LMI NIDR
Porter, E. V.	Chemist	LMI NIDR
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Laboratory of Microbiology

INSTITUTE AND LOCATION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.50	2.50	.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)

The metabolism of sucrose and lactose by Streptococcus mutans and Lactobacillus casei were studied. Intracellular invertase was shown to be a sucrose-6-phosphate hydrolase in strains of S. mutans representative of the seven serotypes. The purified S6P hydrolases appear similar in these strains and differed in properties from the extracellular invertase. An ATP dependent mannofructokinase was further characterized; high yields of a stable preparation are now available to study the role of the enzyme in intracellular phosphorylation of fructose. β-galactoside metabolism (eg. lactose, lactulose, lactobionate, methyl-β-galactoside, arabinosyl-β-galactoside) was mediated by plasmid-determined lactose-PTS an P-β-galactosidase activity in L. casei. Restriction enzyme analysis of several of the lactose plasmids indicated that they share functional, but not structural homology. Using mutanolysin, methods were developed for the generation of viable spheroplasts of L. casei. These spheroplasts are being used to study transformation and cell-cell fusion in order to facilitate genetic transfer among Lactobacilli. Other data indicate that L. casei possess a number of distinct PEP-dependent sugar-specific PTS activities which are currently being characterized.

OBJECTIVES:

The oral streptococci and lactobacilli comprise the majority of bacteria 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 proably been a major selective pressure on these 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 or lactose, are required for this process.

1) One objective of this study is to understand how carbohydrates are dissimilated by various oral streptococci and lactobacilli. Primary attention has been devoted to the pathways of sucrose and lactose dissimilation, but ultimately the mechanisms underlying the fermentation of all carbohydrates will be of interest. It is anticipated that such knowledge will contribute to our understanding of both the evolution of these oral microbes and the identification of specific factors which contribute to their 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 found in some species of oral streptococci and lactobacilli is necessary in order to reach conclusions about the adaptive role hypothesized for these plasmids. While no direct role of plasmids in encoding pathogenic potential has been found, the properties elucidated to date provide powerful tools for genetic and biochemical analysis of metabolic pathways, regulation of gene expression, and the physiology of growth.

MAJOR FINDINGS:

A. Synthesis of unusual sugar phosphates.

Privious work established a synthetic route for the preparation of sucrose-6-phosphate (S6P). Since this key intermediate for studying sucrose metabolism in bacteria is not commercially available, a new and improved synthetic procedure has been developed. The initial preparation of 6-O-monotritylsucrose was the key step in the synthesis. The monotritylsucrose was acetylated and the trityl blocking group removed from the heptaacetate. The heptaacetylsucrose was phosphorylated with cyanoethylphosphate in the presence of dicyclohexylcarbodiimide. After removal of the cyanoethyl- and acetyl- blocking groups a single homogeneous isomer of sucrose phosphate, S6P, was obtained. The product was shown to be identical to the S6P prepared by the previous route which relied on the separation of a mixture of sucrose phosphates to obtain the desired isomer. New purification technology using an ascending gradient of triethylammonium borate on a Dowex-1-X8 borate ion-exchange column has been implemented to facilitate the purification and resolution of most sugar phosphates.

The product of transport of lactose into L. casei by the action of a PEP-dependent PTS has been shown to be lactose phosphate, but this compound had not been previously synthesized. By phosphorylation of lactose with hydroxyphosphorousoxydichloride in trimethylphosphate, a mixture of lactose phosphates was formed. Lactose-1-phosphate, the major sideproduct, was removed by mild acid hydrolysis and the resulting mixture purified by borate anion-exchange chromatography to give lactose-6-phosphate. This compound will be evaluated as the natural physiological substrate of phospho-P-galactosidase.

B. Characterization of S6P hydrolase and comparasion with extracellular invertase.

S6P hydrolase was previously shown to be responsible for the hydrolysis of S6P resulting from the PTS-dependent entry of sucrose into S. mutans 6715-10. These studies also demonstrated that the enzyme is identical to the intracellular invertase found in this strain of S. mutans. Seven strains of S. mutans each representing a distinct serotype, possess a similar activity. S6P hydrolase appears to be a constitutive enzyme in glucose or fructose cultured cells, but levels 2 to 4 fold higher were observed in sucrose cultured cells of the S. mutans strains studied. S6P hydrolase was purified from these strains by cellulose anion exchange and gel filltration chromatography. The preparations appear quite similar in their physical and biochemical properties. In all strains studied no separate intracellular invertase activity was present; however, the purified S6P hydrolase preparations catalyzed the hydrolysis of sucrose, as was observed with the preparation isolated from S. mutans 6715-10. A comparasion of the extracellular invertase of S. mutans LM-7 with the intracellular S6P hydrolase isolated from that strain suggested that they are two distinct proteins differing in substrate specificity, kinetic properties, isoelectric points and molecular weight. Immunological data from other laboratories support this hypothesis. Absolute proof of this point awaits a comparasion of homogenous proteins by physical means and/or the isolation of mutants lacking one, but not both, activities.

C. Characterization of mannofructokinase from S. mutans

A constitutive ATP-dependent mannofructokinase activity has been demostrated to be present in cell-free extracts of oral streptococci. The enzyme is postulated to function in the phosphorylation of intracellular fructose or mannose. Either free sugar might enter the cell under conditions were the PEP-dependent PTS in nonfunctional. In addition, the entry of sucrose into the cell, as free sucrose or its phosphate ester, and its subsequent hydrolysis would liberate free intracellular fructose. The kinase was highly purified and, although not homogeneous, strong evidence was provided that a single protein is responsible for the phosphorylation

of mannose and fructose at high efficiency. The yields of enzyme obtained were low and the catalytic activity of the final preparation was unstable. This prevented the unequivocal demonstration of the identity of the two phosphorylating activitiies. The introduction of a new cell-disruption procedure (bead mill in place of sonication) and a change to HEPES buffer during purification resulted in almost ten-fold higher yields of a stable kinase preparation. This enzyme is now being purified to homogeneity for completion of the physical and biochemical characterization.

D. Plasmid-determined β-galactoside metabolism in L. casei.

β-Galactoside (lactose, lactulose, methyl-β-galactoside, lactobionate and arabinose-β-galactoside) metabolism in several strains of L. casei subsp. casei is dependent on the presence of a specific plasmid. The metabolism of lactose in all strains of L. casei studied is initiated by the action of a PEP-dependent lactose specific PTS that transports lactose into the cell with the concomminant production of intracellular lactose-6-phosphate. The lactose-6-phosphate is cleaved to galactose-6-phosphate and glucose by the action of phospho-β-galactosidase. The galactose-6-phosphate thus formed is metabolized further through the tagatose-6-phosphate pathway. An ATP-dependent glucokinase that produces glucose-6-phosphate has also been demonstrated in cell-free extracts of L. casei. The resulting glucose-6-phosphate is probably metabolized through the glycolytic pathway, since lactose metabolism in this bacterium is homofermentative. It has been found that lactose-cultured cells of L. casei 64H can co-metabolize lactose and glucose. Glucose repression and a regulatory role for cAMP do not appear to operate in this strain. Glucose is capable of exerting a partial inhibitory effect on the induction of the lactose metabolizing enzymes that can probably be attributed to catabolite inhibition or inducer exclusion. The lactose-PTS and phospho-β-galactosidase, as well as a number of other sugar-specific PTS and activities, are not constitutive and are only expressed during growth on a specific inducer. L. casei strains cured of their specific lactose plasmid resemble L. casei subsp. alactosis strains in possessing a functional galactose-PTS and the enzymes of the tagatose phosphate pathway.

The presence of a galactose-PTS in apparently unique, since in Staphylococcus aureus and Streptococcus lactis, galactose is transported into the cell by the lactose-PTS. A further difference in the specificity of the lac-PTS activities between these species is evidenced by the observation that L. casei does not transport, or phosphorylate in cell-free systems, analogs such as thio-methyl-β-galactoside, isopropylthio-β-galactoside, or orthonitrophenyl-β-galactoside at appreciable rates as has been reported for the lac-PTS in S. aureus and S. lactis. The novel galactose-PTS was examined in a cell-free system. Membranes as well as supernatants from galactose-cultured cells are both required for in vitro phosphorylating

activity. It may be that the system requires an inducible membrane-bound Enzyme II-gal and a soluble cytoplasmic Factor-III-gal in addition to the universally required Enzyme I and Hpr. Current experiments are directed a fractionating these components. A constitutive galactokinase was also present in L. casei and its role relative to the gal-PTS remains to be evaluated. An analogous situation exists for the lactose-PTS components isolated from cells able to metabolize lactose; both induction by growth on a -galactoside and a combination of membranes and a supernatant factor are required for the demonstration of in vitro activity. These in vitro systems will allow a biochemical assessment of which components of the lactose-PTS are directly plasmid determined.

A strain of L. casei subsp. psuedoplantarum was found to have a lactose plasmid that can be eliminated by conventional plasmid curing techniques. On the other hand, strains of L. casei subsp. rhamnosis, while able to metabolize lactose by the same pathways, cannot be cured of lactose metabolism. Several of these strains do not appear to harbor plasmids. The results indicate that the metabolism of lactose in these strains may be encoded on chromosomal genes and that considerable diversity with respect to lactose metabolism exists within these members of the same specie. When three of the specific lactose plasmids (17.5 mdal plasmid isolated from ATCC 393, 23 mdal from 64H and 36 mdal from ATCC 4646) isolated from L. casei subsp. casei strains were compared by restriction endonuclease fragment analysis there was no apparent homology among them. No homology was observed between these plasmids and the 25 mdal L. casei phage genome as had been suggested by other investigators. Current efforts are directed at determining if a small common lac sequence is present in these otherwise dissimilar plasmids.

No natural system for genetic exchange has been demonstrated among Lactobacilli. Several experimental approaches aimed at the introduction of lactose metabolism into non-fermenting strains were attempted. Transformation by plasmid DNA, conjugation, and phage-mediated transduction have all proved unsucessful. Recently, mutanolysin, a lytic enzyme isolated for the lysis of S. mutans, has been used to prepare spheroplasts of Lactobacilli for the first time. Mutanolysin contaminating proteases by chromatography on carboxymethyl-cellulose, and stable spheroplasts in essentially 100% yield. A medium for their regeneration and growth was developed. Transformation and cell-cell fusion experiments are being conducted with these spheroplasts in an attempt to promote genetic exchange among various Lactobacillus strains.

E. Characterization of sugar-specific PTS activities in L. casei.

In addition to the PTS-mediated transport of glucose, galactose and lactose already described, evidence was obtained with permeabilized whole cells that maltose, fructose, tagatose, trehalose, melizitose, sucrose, cellobiose and gentiobiose can enter the cell via PTS-like

mechanisms. These systems are currently under investigation, but their independent inducibility seems to indicate a diversity of sugar-specific PTS mechanisms in L. casei. Furthermore, the existence of previously undescribed PTS activities for unsusual di- and trisaccharides suggests the presence of phosphohydrolases similar to the S6P and phospho-β-galactosidase activities already described. Efforts are underway to synthesize substrates for these enzymes by the methods described in section A. so that the enzymes might be identified and characterized. Concurrent studies are directed at elucidating the structure of the di- and trisaccharide phosphates produced by the action of these PTS activities. To date, maltose-6-phosphate, sucrose-6-phosphate and trehalose-6-phosphate have been identified. No attempt was made to examine the nature of the permeation systems that exist for substrates such as L-sorbose, D-gluconate and D-ribose that support growth, but do not appear to enter the cell by a PTS-like mechanism.

SIGNIFICANCE TO BIOMEDICAL RESEARCH

The mechanism of carbohydrate utilization by oral microbes has doubtless been an important selective criterion in their evolutionary development. 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 ability of the ecosystem to support a benign rather than a pathogenic microflora.

Considering the demonstrated role of plasmids in coding for pathogenic potential, confering antibiotic resistance, as well as coding for antigen and bacteriocin production in other bacteria, it would be of great value to know if such relationships exist between plasmids and 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 in an emerging and changing ecosystem.

PROPOSED COURSE:

1. To complete the characterization and comparasion of S6P hydrolases isolated from various S. mutans serotypes and to purify and definitively characterize the mannofructokinase isolated from S. mutans.

2. To synthesize various unusual sugar phosphates for use in studying phosphohydrolases.

3. To characterize and fractionate the components of various sugar-specific PTS activities in L. casei.

4. To exploit the spheroplast formation technique in an attempt to develope a genetic exchange system among Lactobacilli. If this course is unsucessful the nature of the lactose genes that are plasmid-coded in L. casei will be evaluated by transformation into another suitable host, cloning into a suitable host, or by direct structural analysis of the plasmids.

PUBLICATIONS:

1. Chassy, Bruce M. and Giuffrida, Alfred (1980), "Method for the Lysis of Gram-Positive, Asporogenous Bacteria with Lysozyme", Appl. and Envion. Microbiol. 39, 153-158.

2. Porter, E.V., Chassy, B.M., and Holmlund, C.E. (1979) "Partial Pufification and Properties of a Specific Glucokinase from Streptococcus mutans SL-1", Biochim. Biophys. Acta 611, 289-298.

3. Porter, E.V., Chassy, B.M., and Holmlund, C.E. (1980) "Partial Purification and Properties of a Mannofructokinase from Streptococcus mutans SL-1", Infection and Immunity, October 1980, 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-00043-10 LMI

PERIOD COVERED

October 1, 1979 - September 30, 1980

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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Flatow, Ursula	Microbiologist Tech	LMI NIDR
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SECTION

Microbiology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.50	.50	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)

The possibility that extracellular dextranase contributes to the structural complexity of the adherent and insoluble glucan synthesized from sucrose by cariogenic streptococci is under investigation. For this project, a set of independent dextranase-deficient mutants has been isolated from Streptococcus mutans 6715-13. These mutants are being compared to their parent with respect to sucrose-mediated adherence to smooth surfaces, and the synthesis of soluble and insoluble glucans. The possible role of plasmids in the physiology and ecology of oral streptococci is being studied also. Currently under investigation is a tetracycline resistance trait from S. mutans. This trait is transferable to certain other streptococci via a conjugation-like mechanism, and a small plasmid has been identified in one of the recipients that had received the tetracycline resistance trait.

OBJECTIVES:

The ongoing research has two general objectives, which will be discussed sequentially.

1. One focal plane of our attention has been to increase our understanding of the enzymology of insoluble glucan synthesis by oral streptococci. The insoluble glucan synthesized by S. mutans is complex because it is highly branched and contains sequences of both α-(1,6)- and α-(1,3)-linked glucosyl residues. It has been proposed by others that the enzyme dextranase is involved in the biogenesis of this complex glucan. The objective of our present study has been to test this proposal. With this in mind, we have isolated a series of independent dextranase mutants and have begun to ascertain whether glucan synthesis is affected in these mutants.

2. The second general objective of our studies has been to characterize the structure and function of plasmids in oral streptococci. In the past, we have tested the thesis that the plasmid pAM7 controls glucan synthesis in S. mutans LM7. More recently we have studied the novel product that is obtained when pAM7 is subjected to pH 12-13 and then neutralized. Because of our continuing interest in S. mutans plasmids, a new study has been initiated in collaboration with Dr. LeBlanc. The objective is to identify and study an S. mutans plasmid with a clear-cut function. Previous studies done at NIDR and elsewhere have shown that plasmids occur in S. mutans, albeit at a low frequency (∿5-10%). However, so far it has been impossible to associate a phenotypic trait with any of these plasmids. This has made it impossible to study transmissibility and other typical plasmid encoded properties. Because a strain of S. mutans (19S) had been identified that is resistant to several antibiotics (notably erythromycin and tetracycline), and because multiple antibiotic resistance is often plasmidmediated, it seemed worthwhile to examine this isolate more in detail. Preliminary studies showed that the tetracycline resistance was transmissible, and because conjugal transfer of antibiotic resistance is often mediated by plasmids, we decided to extend these studies. Even though the early experiments did not reveal plasmid DNA in 19S, we subsequently detected plasmid DNA in the transconjugants (see major findings). Among our longer term goals, we intend to compare this plasmid with tetracycline resistance plasmids from other species.

METHODS EMPLOYED:

The methods used to isolate dextranase mutants and to study glucan synthesis by these mutants have been described in last year's report.

A new method was developed to rapidly (4 h) isolate plasmid DNA from relatively large batches of cells (100 ml and up). Instead of lysis with a detergent, lysis of the cells and denaturation of non-supercoiled DNA are affected in one step by exposure of lysozyme-treated cells to pH 12.2-12.4. After neutralization, high speed centrifugation is used to

remove virtually all chromosomal DNA. Plasmid DNA is then partially purified from the supernatant fluid ("cleared lysate") using classical methods, before being subjected to agarose electrophoresis or CsCl-ethidium bromide centrifugation. The recent progress in the physical characterization of the tetracycline resistance trait in the transconjugants (see below under Major Findings 2b) is largely due to the development of this new method.

MAJOR FINDINGS:

1. Last year two presumptive dextranase deficient mutants of S. mutans 6715-13 were identified as the result of a newly developed screening procedure. However, quantitative assays showed that only one of these (mutant 4) was markedly deficient in dextranase activity (this mutant was originally isolated by Tanzer et al. as a glucan-synthesis mutant). Because it was known that this mutant synthesized much more soluble glucan from sucrose than the parent, the possible association between these two observations was investigated. Activity stains after electrophoresis of concentrated culture fluids showed that mutant 4 synthesized glucan bands in the gel from sucrose that stained much more strongly in the periodate-Schiff reaction than the corresponding bands from the parent (this is a test for GT-S, because 1,6-linked glucosyl residues react). Quantitative assays of fractions separated by electrophoresis confirmed this observation, as well as the absence of dextranase activity in mutant 4. However, the fact that dextranase and GT-S virtually co-electrophoresed made it impossible to rule out that these observations were due to interactions between these two enzymes during the assays. To resolve the question whether the greatly increased synthesis of soluble glucan shown by mutant 4 was due to the dextranase deficiency, or whether mutant 4 is a double mutant (higher in GT-S and deficient in dextranase), other dextranase mutants were sought. If these would behave like mutant 4, then this would lend support to the hypothesis that dextranase and glucosyltransferase interact. About 10 dextranase mutants were independently isolated after relatively mild mutagenesis (in order to limit the probability of double mutations). Even though these mutants have not all been studied in detail, none of them is exactly like mutant 4 with respect to soluble glucan synthesis. Thus, it appears likely that mutant 4 is indeed a double mutant, and that it cannot be used to study the relationship between dextranase activity and glucan synthesis. It remains to be determined whether the newly isolated mutants synthesize more soluble glucan than the parent.

2a. Last year we reported the discovery of a novel DNA species, "X", in pAM7 preparations that had been purified by a new procedure. Further research showed that X originated from pAM7 during the alkali treatment that was part of the new plasmid purification procedure. X had a higher electrophoretic mobility than pAM7 and electron microscopy showed circular

molecules with an apparent size of 1.8 Mdalton (pAM7 is 3.6 Mdal). However, sucrose gradient centrifugal analysis failed to support the notion that X was a half-sized pAM7 molecule. Instead, the results suggested that X had arisen from pAM7 by translocation of the Watson and Crick strands with respect to each other during the alkali treatment. At least two properties of X appear to be novel: (1) its smooth circular appearance (other alkali-denatured plasmids are either highly twisted or circular with knob-like protrusions); (2) its lability during CsCl-ethidium bromide gradient centrifugation (other alkali-denatured plasmids are stable and co-centrifuge with supercoiled DNA).

2b. With respect to the collaborative study (with Dr. LeBlanc) on the multiple antibiotic resistant 19S strain of S. mutans, the initial findings are: (1) 19S transferred its tetracycline resistance to S. faecalis JH2-2 and to S. mutans 6715-10 during conjugal matings. No transfer of erythromycin resistance was observed. The S. mutans 6715-10 transconjugant could act as a donor of the tetracycline resistance trait to S. faecalis. (2) All S. faecalis transconjugants showed a new DNA band after electrophoresis, but an unusually high number of cells had to be processed in order to detect this band. A very recent CsCl-ethidium bromide centrifugation experiment showed that this new band represents supercoiled DNA; its molecular weight was estimated by electrophoresis to be 8 $\pm$ 2 million.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

The study on the biogenesis of insoluble glucan by S. mutans is significant because insoluble glucan synthesis is associated with the development of dental plaque on teeth as a result of dietary sucrose consumption. In addition, S. mutans is associated with the induction of caries lesions in humans consuming a "modern" diet. Further characterization of the interrelationship between the glucosyltransferases, dextranase, and insoluble glucan synthesis is also of significance because the glucosyltransferase preparations that have been used for immunization against dental caries also contain dextranase.

Whereas streptococci have been of clinical significance for a long time, the discovery of plasmids in this genus dates from only 1972. Thus, compared with the plasmids from gram-negative organisms, knowledge about streptococcal plasmids is relatively limited. Our studies on the structure and function of S. mutans plasmids are designed to advance this knowledge in a general way.

PROPOSED COURSE OF STUDIES:

1. The dextranase mutants just isolated from S. mutans 6715-13 need to be characterized. Specifically, it must be determined whether these mutants synthesize more soluble glucan than the parent. It is also important to determine if the dextranase deficiency has an effect on virulence.

2. In order to better characterize the dextranase mutants, we propose to isolate and study dextranase-positive revertants.

3. The studies on the transmissible tetracycline resistance trait present in S. mutans 19S are promising and need to be continued. Specifically, we propose to determine:

(a) Whether the tetracycline resistance of S. mutans 19S is due to a plasmid, or to a transposon which has integrated into the chromosome;

(b) if the S. mutans 6715-10 transconjugants contain a plasmid, it will be important to determine whether this plasmid is identical to the 8 Mdalton one detected in the S. faecalis transconjugants;

(c) In conjunction with these studies we will need to ascertain whether the transmissibility is due to a mobilizing plasmid;

(d) In case 19S contains a tetracycline resistance plasmid, we will determine if this plasmid underwent modification in the other hosts.

4. It would be of general relevance to compare the plasmid present in the S. faecalis transconjugants with tetracycline resistance plasmids from other streptococci, and map the tetracycline resistance gene(s).

PUBLICATIONS:

1. Donkersloot, J. A., and Harr, R.J. More sensitive test agar for detection of dextranase-producing oral streptococci and identification of two glucan synthesis-defective dextranase mutants of Streptococcus mutans 6715. J. Clin. Microbiol. 10: 919-922, 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 00096-06 LMI

PERIOD COVERED
October 1, 1979 - September 30, 1980 CT 0060103

TITLE OF PROJECT (80 characters or less)
Microbiological features of Cerivco-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

Keyes, Paul H.	Dental Director	LMI NIDR
Howard, Surya A.	Dental Health Tech.	LMI NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Microbiology Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.00	1.00	1.00

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Additional microscopic observations of circumradicular bacterial plaques removed from persons with and without periodontal lesions revealed differences in the types and number of cells, in the organization of the various forms, and in the behavior of the microbial complexes. Bacterial turbulence and a high pyogenic potential characterize bacterial adhesions associated with disease. Therefore, an important therapeutic goal becomes the elimination of turbulent pyogenic complexes on the outer surfaces of the non-motile "bacteriomats" that coat the radicular surfaces adjacent to pocket walls. After pyogenic bacterial complexes have been suppressed or eliminated, all signs of destructive periodontitis have subsided. Suppression can be attained by treating lesions by debridement with an antiseptic, home-care cleansing with mild antibacterial agents, and occasional courses of antibiotics. However, one cannot use clinical signs of improvement as a reliable indicator of the microbiological status of a case. Active fields can be found in areas that do not appear markedly hyperemic or unhealthy by visual inspection. Microscopic findings permit subclinical disclosures that enable a clinician to modulate the therapy to bring the microbial populations under control before there has been further irreparable damage to periodontal tissues.

PHS-3040

OBJECTIVES:

This project aims to determine whether any consistent differences exist between those bacterial populations that colonize tooth surfaces adjacent to healthy gingival margins and those that colonize cervico-radicular surfaces adjacent to inflammed periodontal tissues. It aims to test the value of the phase contrast microscope to assess the status of circumradicular spaces before treatment, during the stages of debridement and chemotherapy, and while patients are in the stage of long range maintenance. It follows the clinical course of teeth that have remained free of pyogenic bacterial populations, teeth with moderate control of such populations, and those in which control has not been attained.

MAJOR FINDINGS:

Additional observations over the past year in persons with periodontal health and disease support findings previously described, namely that microbiotas associated with disease differ in five respects from those associated with health: The types of bacteria differ; ipso facto there are quantitative differences; there are differences in patterns of organization; and there are behavioral differences. In addition the microbiotas differ in their pyogenic potential as reflected by the prevalence of white blood cells. Populations associated with health consist of loosely tangled networks of thread forms to which coccoidal and other small forms adhere, and the outer surfaces of these congregations have not been colonized by turbulent populations of spirochetes, rods, amoebae, etc. Furthermore, but few white blood cells locate adjacent to such bacterial mats.

Bacterial congregations associated with suppurative inflammation and destructive tissue reactions - autogenous excision of teeth - usually consist of three layers: 1) an adherent layer of dead mineralized organisms, calculus, presumably formed only by non-vital static bacteria 2) a layer of stationary, non calcified and presumably living forms, 3) an outer highly organized layer of organisms that translocate and attach themselves to the outer surface of the non-motile adherent forms. Turbulence is the outstanding feature of the outermost layer, which is adjacent to the cells that form the wall of the pocket. Numerous white blood cells congregate in crevicular spaces but these cannot migrate close to the bacterial complex because of the highly active turbulence generated by spirochetes, flexing rods, etc. It has become apparent that the hard surfaces of the roots of teeth provide an ecological niche for bacteria specifically adapted for attachment and growth upon such surfaces. Apparently in time, non-motile, static populations proliferate, invade, and gradually induce sufficent space and an appropriate surface

for motile populations that require a sheltered aquatic milieau. The periodontal spaces adjacent to inflammed tissues support a turbulent world of aquatic microlife.

The phase contrast microscope enables the clinician to monitor microbiologial changes, if any, that occur during various phases of treatment. It also provides some insight into the antibacterial potential of various agents used in the operatory by clinicians and at home by patients. Periodontal lesions can be treated as suppurating wounds infected with intruding, highly contaminated hard bodies: 1) by careful debridement with antiseptics such as Chloramine-T, 2) by careful daily cleansing with mild antiseptic agents, and 3) a course of antibiotic if microscopic findings show the presence of white blood cells in high numbers and / or bacteria conductive to turbulence, i.e. populations not associated with health. One essential objective of therapy appears to be the conversion of populations in circumradicular spaces from the turbulent pyogenic to the non-turbulent non-pyogenic.

Observations in patients under control from 3 to over 6 years show that lesions heal and teeth remain functional whenever pyogenic populations have been controlled. Clinical changes include: marked reduction in bleeding and suppuration, improvement in tissue tone and contour, reductions in pocket spaces, improvements in configurations of alveolar bone, tightening of teeth and elimination of mouth odor.

Further observations of patients with controlled "periodontitis" reveal that minor orthodontic correction of teeth can be well tolerated.

An examination of material removed from a necrotic pulp of a tooth (caries free) with a combined periodontal-periapical lesion revealed spirochetes, and motile rods. This observation, made in collaboration with Dr. W. De Rijk, is believed to be the first of its kind. Further are expected.

SIGNIFICANCE:

The findings disclosed by this investigation are beginning to change thinking with respect to the management of periodontal lesions. Unless future disclosures negate observations made over a period of seven years, methods to markedly improve the diagnosis, management, and prognosis are available. However, the phase-contrast microscope is essential equipment if clinicians are to minimize the risk of leaving patients with uncontrolled bacterial infection.

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-00174-05 LMI

PERIOD COVERED
October 1, 1979 through September 30, 1980

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

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Donkersloot, Jacob	Research Microbiologist	LMI NIDR
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COOPERATING UNITS (if any)

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) A porcine isolate of Streptococcus mutans transferred tetracycline (Tc) resistance, by conjugation, to human strains of S. faecalis and S. mutans. A 12 megadalton plasmid was isolated from the transconjugant clones but not from the donor strain. Four of 16 streptococcal isolates obtained from the oral cavities of human patients were also able to transfer Tc resistance to S. faecalis. A second type of conjugative plasmid, pAMβ_1, mediating resistance to erythromycin and lincomycin, readly undergoes extensive deletions following transfer to new streptococcal host strains. These deletions, dependent upon the host in which they occur, involve different regions of the parent molecule. Preliminary results suggest that several functions, such as transmissibility, replication and resistance, may be altered by the deletions. These data indicate that it will be possible to construct a physical and genetic map of plasmid pAMβ_1. Pentoses are fermented by very few strains of S. lactis. Strain DR1253, however, is able to utilize three pentoses, L-arabinsoe, D-xylose and D-ribose. The ability of this strain to metabolize D-xylose appears to be plasmid-mediated. The metabolic pathways by which L-arabinsoe and D-xylose are metabolized, via pentose isomerase and pentulokinase activities, are inducible in S. lactis and appear to be independently regulated.

The long-range goal of this research program continues to be the elucidation of the role of bacterial plasmids in the pathogenesis, ecology and taxonomic status of the oral microbial flora, with special emphasis on the streptococci. This report summarizes progress made on the following individual projects: 1) plasmid-mediated antibiotic resistance in an animal isolate of Streptococcus mutans and its transmissibility to streptococcal isolates of human origin; 2) assessment of the extent of plasmid-associated tetracycline resistance among oral streptococci; 3) the use of plasmid deletions to elucidate mechanisms of plasmid-determined functions; and 4) studies on the possible role of plasmids, in, and the physiological mechanisms of, pentose metabolism by Streptococcus lactis.

METHODS EMPLOYED:

Most procedures used in these studies were standard microbial, molecular and biochemical techniques with minor modifications required for application to streptococci. Genetic transfer methods and plasmid isolation techniques were those developed in this laboratory during the past five years and described in previous reports.

MAJOR FINDINGS:

Transmissible Antibiotic Resistance in an Animal Isolate of Streptococcus mutans. A strain of S. mutans (DL5), isolated from a pig raised on feeds supplemented with subtherapeutic levels of tetracycline and penicillin, was found to be resistant to high levels of at least four antibiotics. The in vitro minimal inhibitory concentrations (MIC's) for this isolate were 40 mg/ml for streptomycin (Sm), 1.5 mg/ml for erythromycin (Em) or lincomycin (Lm), and 150 µg/ml for tetracycline (Tc). These high MIC's for Sm and Tc, and the dual resistance to Em and Lm, have often been found to be plasmid-determined among natural isolates of streptococci. Preliminary results, employing several standard plasmid isolation techniques, suggested that strain DL5 was devoid of plasmid DNA. However, conjugation experiments using strain DL5 as a potential donor and a human isolate of S. faecalis (DL3) as a potential recipient, indicated that the S. faecalis strain could obtain the Tc resistance trait from DL5 at relatively high frequencies. When such matings were performed on membrane filters, under aerobic conditions, the conjugation frequencies were approximately 10^{-5} per donor colony forming unit (CFU). Conjugation frequencies of aerobic broth matings were always 2- to 5-fold lower than the aerobic filter matings, and at least two orders of magnitude lower in anaerobic matings, whether in broth or on filters. We have been unable to demonstrate transmissibility of the Sm, Em or Lm resistance traits of strain DL5. This procine isolate was able to transfer its Tc resistance to other strains of S. mutans obtained from human sources. The conjugation frequencies of these intraspecies matings were approximately 5×10^{-8} per donor CFU, and could only be affected on membrane filters under anaerobic conditions. The S. mutans transconjugants, obtained only under anaerobic conditions, were able to transfer Tc resistance to S. faecalis strain DL3 under aerobic conditions. These

results suggest that the properties of both donor and recipient strains can influence optimum conditions of conjugation among streptococci. Although initial experiments suggested that S. mutans strain DL5 was devoid of plasmid DNA, recent data suggest that the Tc resistance of this strain is plasmid-mediated. A plasmid with a molecular weight of approximately 12 x 10^6 has been isolated from Tc resistant transconjugants of both S. faecalis and S. mutans. This plasmid DNA could not be isolated from the Tc sensitive parent strains of either of these species. We are currently examining the plasmids from the S. faecalis and S. mutans transconjugants, by DNA-DNA hybridization techniques and restriction endonuclease digestion, to determine if they are identical.

Recently, when chloramphenicol was added to a culture of S. mutans strain DL5 in the exponential phase of growth, and the culture allowed to incubate until growth had stopped, DNA extracts yielded three new bands, in addition to the band of chromosomal DNA, when examined by agarose gel electrophoresis. We are currently attempting to isolate sufficient quantities of this DNA for use in transformation experiments with the Challis strain of S. sanguis, to establish whether the Sm, Em and Lm resistances are also plasmid-borne in strain DL5.

The Role of Plasmids in Resistance of the Oral Streptococci to Tetracycline. In a previous study designed to evaluate the effects of tetracycline on the oral microbial flora of periodontal pockets we obtained over 400 Tc resistant streptococcal isolates. More than 40 of these have been subjected to various plasmid curing techniques and agents and, to date, only two strains have become sensitive to Tc. In only one of these strains were we able to associate this loss with the loss of a specific plasmid. We have not been able to demonstrate the presence of plasmids in many of these isolates, only 21% of these having been shown to contain one or more plasmid species. In light of the results obtained with the S. mutans Tc resistance donor strain, we have begun testing the Tc resistant clinical isolates, regardless of their apparent possession or lack of plasmids, for an ability to transfer the Tc resistance trait in conjugation experiments. Four of 16 strains tested so far were able to serve as Tc resistance donors. These conjugation experiments, as well as transformations with plasmid preparations from those isolates from which extrachromosomal DNA can be obtained, are continuing.

Further Studies on Deletions in a Resistance Plasmid. We reported last year that a strain of S. anginosus containing pAMβ_1 (β), a 17 megadalton conjugative plasmid originally isolated from a strain of S. faecalis, and mediating resistance to Em and Lm, occasionally yielded resistant clones which were no longer capable of transferring their resistance traits in conjugation experiments. Examination of the plasmid DNA from such isolates revealed that the original plasmid had undergone a 9 megadalton deletion, resulting in the loss of transmissibility. It has since become apparent that such deletions in the β plasmid are common following transfer to a new bacterial host. We are making a collection of streptococcus strains containing these deleted plasmids and have begun to study them with regard to several plasmid-associated functions. Whereas the original intact β plasmid is generally present in the host

cell in one or only a few copies per genome equivalent, four of the deleted plasmids are present in multiple copy numbers and are much more resistant to spontaneous curing in the absence of selective pressure. Four of the five derivatives examined thus far have lost one or more gene functions required for conjugation. The fifth derivative has lost the antibiotic resistance traits but has retained conjugative functions, as determined by its ability to mobilize other resistance plasmids. Restriction endonuclease digestion patterns suggest that, somewhat dependent on the strain in which the deletions occur, these smaller molecules have lost different segments of the original β plasmid. We are currently using these deletions, as well as others appearing in different streptococcus strains, to construct a genetic map of the resistance, replication and transfer functions of the β plasmid. Precise localization of these functions on the β plasmid genome will permit the construction of unique molecular probes for determining the genetic relatedness of similar functions on other streptococcal plasmids, or even streptococcal chromosomes.

Pentose Metabolism by a Strain of Streptococcus lactis. We have previously reported that the ability of strains of S. lactis to metabolize several carbohydrates may be plasmid-associated. In most of these instances the metabolism of the sugar is initiated by a specific phosphoenolpyruvate-dependent phosphotransferase system (PTS). The loss of the sugar-specific PTS activity was accompanied by the loss of a specific plasmid molecule. In one strain of S. lactis, strain DR1253, the ability to grow at the expense of the 5-carbon sugar, D-xylose, a non-PTS substrate, was also correlated with the presence of a plasmid. The ability to ferment pentoses is rare among strains of S. lactis, yet strain DR1253 was found to metabolize, in addition to D-xylose, L-arabinose and D-ribose as well. While initial plasmid curing attempts have not been fruitful with regard to the latter two fermentative properties, we have obtained some very useful information with respect to the mechanisms of pentose metabolism in S. lactis. Diauxie experiments have revealed a pecking order in the preference of this organism for pentose utilization in that L-arabinose is metabolized before D-xylose, which is preferred over D-ribose. As expected, D-glucose is utilized in preference to any of these pentoses. D-xylose and L-arabinose are catabolized via inducible pentose isomerase and pentulokinase activities, as is common among the Enterobacteriaceae. D-xylose isomerase activity is induced only during growth on D-xylose, and there is no cross-reactivity detectable on other pentoses tested. Similarly, only L-arabinose will induce the corresponding isomearase activity, which appears to be specific for L-arabinose. Preliminary kinetic data suggest relatively low affinities of the isomerases for their specific substrates, with apparent Km values on the order of 2.5×10^{-3} M. Work is currently in progress on the kinase activities for the pentuloses D-xylulose and L-ribulose.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

Studies on the molecular biology and genetics of the streptococci and their plasmids are expected to contribute to a better understanding of

this clinically important group of bacteria, and of extrachromosomal elements in general. It is also hoped that such studies will serve to confirm basic biological principals common to gram-positive and gram-negative bacteria or, alternatively, to uncover significant differences in the evolutionary patterns of these two major bacterial groups. The proliferation of plasmidmediated bacterial antibiotic resistance due to the wide-spread use, and often abuse, of antimicrobials in human medicine has rendered certain antibiotics almost totally useless for the treatment of some bacterial diseases. A large proportion of antibiotics produced by pharmaceutical companies are currently used in subtherapeutic amounts as growth stimulants in animal feeds. That such use has lead to a proliferation of antibiotic resistant bacteria among farm animals has been well-documented. One argument contends that such increases in resistant bacteria among animal populations pose no threat to human health because animal bacterial strains do not interact with human bacterial strains. The demonstration in this laboratory that a streptococcal isolate from a pig can transfer plasmid-mediated antibiotic resistance to human streptococcal strains is, therefore, particularly significant.

PROPOSED COURSE:

A major effort during the next year will concentrate on experiments designed to assess the role of plasmids in antibiotic resistance and its dissemination among populations of oral streptococci. Plasmids reponsible for these resistance traits, whether transferred by conjugation or transformation, will be purified and analyzed by restriction endonuclease digestion, molecular hybridization or heteroduplex analysis to detect common sequences involved in resistance phenotypes and, where applicable, transmissibility. These results will help to determine common origins or evolutionary divergence of these traits.

Physical and partial genetic maps of the β plasmid and deleted derivatives should be completed during the next year. With replication and transfer regions precisely defined, these molecules will be used as molecular probes for comparing similar functions on other plasmids identified in the studies described above. In collaboration with Dr. Frank Macrina at the Medical College of Virginia, attempts will be made to clone the various functions of the β plasmid, on recombinant DNA vehicles developed in his laboratory, in order to confirm results obtained by the genetic and physical mapping techniques. We will also attempt to incorporate the transfer gene(s) from the β plasmid into the chromosome of the Challis strain of S. sanguis as an initial step in the use of conjugation to provide the first genetic map of a streptococcal chromosome.

Among the tetracycline resistance plasmids studied thus far, we have noted some intriguing differences with regard to regulation and levels of resistance. Several of these plasmids have been transferred, by conjugation, to a strain of S. faecalis to provide a common genetic background for further studies. These transconjugant isolates will be used to determine the levels of resistance conferred by these plasmids

and whether the resistance is inducible or consititutive, amplifiable, or transposable to the host chromosome or other plasmids. By comparison of these properties in the S. faecalis host with those of the original host in which the plasmid was first observed, it will be possible to determine whether some of these properties are plasmid or host dependent with regard to regulation.

PUBLICATIONS:

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2. LeBlanc, D.J., V.L. Crow and L.N. Lee. 1980. Plasmid-mediated carbohydrate catabolic enzymes among strains of Streptococcus lactis. In C. Stuttard and K.R. Rozee (eds.). Plasmids and Transposons: Environmental Effects and Maintenance Mechanisms. Academic Press, N.Y. pp. 31-41.

3. Hawley, R.J., L.N., Lee and D.J. LeBlanc. 1980. Effects of tetracycline on the streptococcal flora of periodontal pockets. Antimicrob. Agents Chemother. 17: 372-378.

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-00208-4 LMI

PERIOD COVERED
October 1, 1979 through September 30, 1980

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

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Several metabolic pathways present in Streptococcus mutans have been examined using mutant analysis techniques. Analysis of glucose phosphotransferase system (PTS) negative-mutants revealed that the glucose PTS can also be used to transport mannose, glucosamine and 2-deoxy-D-glucose (2DG). Such mutants are resistant to growth inhibition by 2DG and catabolic repression by glucose. Glucose PTS negative-mutants are still capable of rapid growth on glucose and therefore, possess a second glucose transport system. A new pathway for maltose utilization was identified. Maltose is transported into S. mutans by a phosphotransferase system. Maltose, therefore, does not enter the cell as free maltose but rather as maltose-phosphate.

OBJECTIVES: ZO1 DE-00208-4 LMI

The general objectives of this research program are to use genetic and molecular techniques to examine the regulation and function of several, key metabolic reactions present in cariogenic microorganisms. More specifically, we have undertaken the study of sucrose, glucose and maltose utilization by S. mutans.

METHODS EMPLOYED:

Biological synthesis of sucrose 6-phosphate. In order to continue our studies on the pathways of sucrose utilization by S. mutans it was necessary to obtain preparative amounts of a key metabolic intermediate, sucrose 6-phosphate (S-6-P). The purified compound was then used to examine the regulation of an intracellular S-6-P hydrolase. A mutant strain of S. mutans that can accomplish the biological synthesis of S-6-P was constructed. The mutant is constitutive for the synthesis of sucrose phosphotransferase activity and permeabilized cells of this strain can synthesize S-6-P when incubated with sucrose and phosphoenolpyruvate. Because this strain is missing S-6-P hydrolase activity, no further metabolism of S-6-P can occur and it is released into the incubation medium. A rapid column separation was employed to purify the product and the compound was isolated as a stable barium salt.

MAJOR FINDINGS:

1) Regulation and function of sucrose 6-phosphate hydrolase. Sucrose is transported into S. mutans by a phosphotransferase system. Sucrose enters not as free sucrose but rather as sucrose 6-phosphate (S-6-P). Therefore, an intracellular hydrolase is required to cleave S-6-P into glucose 6-phosphate and fructose. We have examined the regulation of the S-6-P hydrolase and found that it is synthesized constitutively unlike the sucrose phosphotransferase system that is only synthesized in the presence of sucrose. Growth on one substrate, fructose, repressed the synthesis of the hydrolase. The hydrolase has a specific activity of approximately 30 nmol/min/mg (dry wt of cells) and an apparent Km for S-6-P of 0.3 mM. We have isolated mutants of S. mutans that are missing S-6-P hydrolase activity and found that such mutants were also missing their high Km intracellular invertase activity. Other workers have since purified the S-6-P hydrolase and found it to be identical to this presumed invertase. Further analysis of these mutants revealed that, unlike the parent strain, the growth of S-6-P hydrolase negative mutants was severely inhibited by the presence of low levels of sucrose in the medium. This inhibition is most likely caused by the intracellular accumulation of S-6-P that cannot be metabolized further or excreted. This finding suggests that analogs of sucrose, that are substrates for the sucrose PTS but not cleaved by the hydrolase, would serve as very specific and effective growth inhibitors of S. mutans. This project has been completed.

2) Specificity and function of the glucose phosphotransferase system. Glucose has previously been shown to be transported into S. mutans by a phosphotransferase system (PTS). It has not been determined, however, if the glucose PTS can be used to transport other carbohydrates or if there is more than one transport system for glucose. We have found that the glucose PTS in S. mutans is synthesized constitutively but subject to repression when grown on fructose. Glucose PTS has a specific activity of approximately 35 n mole/min/mg (dry wt of cells) and a Km of approximately 25 μM for glucose. Using a mutant that is missing glucose PTS activity, we were able to demonstrate that the constitutive glucose PTS can also be used to transport mannose, glucosamine and 2-deoxy-D-glucose (2-DG). Mutants that are missing glucose PTS are thus resistant to growth inhibition by 2-DG. Because of the ease with which 2-DG resistant mutants can be isolated, it does not appear to be an effective agent for the control of oral microbes. The mutants were also resistant to catabolite repression by glucose and were still capable of inducing the enzymes of other catabolic pathways when grown in the presence of glucose. The most significant finding was that glucose PTS negative-mutants are still capable of rapid growth on glucose. These results suggest that a second transport system other than PTS exists for the transport of glucose. The nature of this second glucose transport system is currently being examined in collaboration with Dr. Ian Hamilton.

3) Characterization of a new maltose transport system. Maltose is a common dietary carbohydrate that is derived from the hydrolysis of starch and glycogen. Two general pathways by which bacteria utilize this dissacharide have been described. Maltose is either cleaved into its two component glucose molecules extracellularly or transported into the cell as free maltose prior to cleavage. S. mutans does not produce an extracellular or cell bound maltose hydrolase and maltose must be transported into the cell in order to be metabolized. We have found a maltose PTS that can be used to transport maltose. The product of this reaction was isolated and identified as a stable phosphate ester of maltose. Maltose, therefore, does not enter the cell as free maltose but rather as maltose-phosphate. Maltose PTS activity was only induced in cells that had been grown on maltose. In addition, maltose PTS was shown to be separate from glucose and sucrose PTS because mutants that are missing these PTS activities still retain maltose PTS activity. The maltose PTS has a specific activity of approximately 40 nmole/min/mg (dry wt of cells) and an apparent Km of 19 μM. The stoichiometry of the reaction was one in our assay system and no other glucose containing dissacharides were active in this system. Mutants that were selected for the inability to utilize maltose were missing maltose PTS activity. The first step in maltose utilization, therefore, is transport by a PTS that yields intracellular maltose-phosphate. This project has been completed.

4) Identificiation of an intracellular maltose cleaving activity. We have demonstrated that maltose is transported into S. mutans by a PTS system. A second enzyme that is capable of cleaving intracellular maltose-phosphate would be required to permit further dissimilation of this compound. Because substrate amounts of maltose-phosphate are not currently available, we have not been able to assay directly for this activity. However, maltose grown cells do produce low levels of an enzyme activity that is capable of cleaving maltose. We suspect that the true substrate for this intracellular enzyme might be maltose-phosphate rather than maltose. A maltose-phosphate cleaving enzyme has not been described previously. The reaction of this enzyme with maltose as substrate gave an apparent Km of 1 mM and the activity was stable at 4°C for several days. Preliminary experiments suggest that this maltose cleaving enzyme does not act by direct hydrolysis of maltose but rather that a transferase reaction is responsible for breaking the glycosidic linkage. Stoichiometric measurements revealed that only one half of a mole of glucose was released for every mole of maltose that was supplied to the reaction. In addition, a coupled reaction that produced endogenous maltose-phosphate did not release the expected yield of free glucose and glucose-phosphate. Work on this project has been postponed due to loss of technical support.

5) Alternate mechanisms for sucrose utilization. With the knowledge that sucrose is transported into S. mutans by a PTS system, it is now possible to examine directly the role played by extracellular enzymes in the metabolism of sucrose. Strain DR0001/2 of S. mutans is missing sucrose transport activity, does not produce extracellular sucrose cleaving enzymes and is thus unable to grow on sucrose. We have isolated ten independent mutants of this strain that can again grow on sucrose but have not regained their sucrose transport activity. Rather, all of the mutants now produce high levels of extracellular and cell bound sucrose cleaving enzyme activity. The highest levels of this activity were obtained when fructose was used as the growth substrate. This activity released equimolar amounts of glucose and fructose from sucrose and dextran T-10 did not stimulate the reaction. These results suggest that the mutants have regained the ability to metabolize sucrose by producing high levels of an extracellular invertase like enzyme. Preliminary results suggest that a colorometric spray test can be used to isolate secondary mutants that are missing this invertase like activity. These secondary mutants can then be used to examine the role played by glucosyl- and fructosyl-transferases in the metabolism of sucrose.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH

The pathogenic potential of oral microorganisms resides in their ability to effectively colonize and proliferate on dental enamel and heart value tissue surfaces. Once established on these sites, the production of large amounts of metabolic end-products from fermentative metabolism

can lead to localized destruction on these sites. The use of genetic and molecular analysis techniques to examine the basic physiology and metabolism of these bacteria will permit us to determine which of the many properties possessed by these microbes might be important for the expression of disease.

PROPOSED COURSE OF STUDIES:

1) Molecular characterization of Streptococcus mutants genes by genetic transfer to Streptococcus sanguis (Challis). No genetic exchange system exists for the analysis of S. mutans genes using S. mutans alone. However, genetic techniques have been developed that can be used to transfer S. mutans genes into S. sanguis. Both heterologous chromosomal and plasmid DNA can be used to transform S. sanguis. In addition, we have shown that S. mutans and S. sanguis are capable of exchanging plasmid DNA by a conjugation like mechanism. Because these two species have been shown to exchange genetic material by the natural mechanisms described above, they are also suitable for use in molecular cloning experiments using recombinatnt DNA technology. The recent development of an S. sanguis cloning vehicle by other workers will make these experiments possible.

2) Analysis of the lactose operon. In order to transfer the genes of lactose operon from S. mutans to S. sanguis a suitable recipient is required. We have isolated a stable phospho-β-galactosidase (PβG) negative mutant of S. sanguis. This mutant is not only unable to grow on lactose but inhibited by lactose. Using this stable recipient, we have transferred the chromosomal gene for PβG synthesis from S. mutans into S. sanguis by heterologous transformation. In addition, we have also transferred the presumptive lactose catabolic plasmid from S. lactis into this recipient. We are currently constructing mutants of S. sanguis that are missing other portions of the lactose operon. This collection of recipients will be used to determine if the lactose genes in S. mutans form a contiguous and coordinately regulated operon. These mutants can also be used to determine how many of the genes for lactose utilization are contained on the lactose plasmid of S. lactis. Complementation experiments using cloned lactose genes will permit us to determine if the lactose operon in S. mutans is under positive or negative regulatory control.

3) Analysis of sucrose genes. Using the knowledge we have gained regarding the mechanisms of sucrose transport and hydrolysis by S. mutans, we can now construct suitable sucrose negative mutants of S. sanguis to serve as recipients for S. mutans genes that code for sucrose metabolizing enzymes. In addition, these recipients will be used to isolate the presumptive sucrose catabolic plasmid that has been described in S. lactis. We are currently constructing mutants of S. sanguis that are missing extracellular soluble glucosyl-transferase and sucrose PTS.

4) Analysis of glucose transport genes. Both S. sanguis and S. mutans have a similar glucose PTS. Using mutants of S. sanguis that are resistant to the non-permissive glucose analog 2-DG and selecting for growth on metabolizable glucose analogs, we will attempt to transfer the glucose PTS genes from S. mutans to S. sanguis. These experiments will permit us to determine how many separate genes are required for glucose transport in S. mutans and if these genes are contiguous on the chromosome with genes that code for general PTS enzymes.

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1. St. Martin, E. J. and C. L. Wittenberger. 1979. Regulation and function of sucrose 6-phosphate hydrolase in Streptococcus mutans. Infect. Immun. 26: 487-491.

2. St. Martin, E. J. and C. L. Wittenberger. 1980. Regulation and function of ammonia-assimilating enzymes in Streptococcus mutans. Infect. Immun. 28: 220-224.

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PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Cell-Cell Interactions between Oral Actinomycetes and Other Oral Bacteria

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

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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)

More than 400 freshly isolated oral bacteria obtained from subgingival samples of human subjects were identified and analyzed for their ability to coaggregate with other previously characterized oral actinomycetes and streptococci (stock culture collection strains-some isolated more than 10 years ago). The coaggregation properties of the new and old strains were compared. Of the new isolates, the predominant participants were Actinomyces viscosus, A. naeslundii, and Streptococcus sanguis. All of the A. viscosus and more than 70% of the A. naeslundii and S. sanguis isolates coaggregated. However, none of a wide variety of other common oral bacteria, including S. mutans, coaggregated. Using the new isolates, four new patterns of coaggregation were observed; however, more than 80% of the patterns with both new and old strains were identical. These results indicate that the cell surface components which are involved in these coaggregations are very stable structures and that they mediate a highly specific set of cell-cell interactions. Further study of these interactions between oral bacteria is expected to provide a better understanding of the ecology of these bacteria in the subgingival ecological niche.

OBJECTIVES: Z01 DE-00273-02 LMI

The overall focus of this project is to identify and characterize the cell surface components on oral bacteria isolated from the subgingival site. We are especially interested in the components which mediate lectin-carbohydrate interactions between actinomycetes and streptococci. This report presents the results of our investigations dealing with:

1) specificity of cell-cell interactions (coaggregation) between oral actinomycetes and streptococci;
2) comparison of coaggregation properties exhibited by freshly isolated oral bacteria and old stock culture collection strains;
3) stability and binding properties of cell surface components on oral bacteria;
4) extent of distribution of lactose-reversible cell-cell interactions between oral bacteria.

METHODS EMPLOYED:

All techniques used are standard methods routine to the type of study conducted.

MAJOR FINDINGS:

Specificity of cell-cell interactions (coaggregation) between oral actinomycetes and streptococci. A study to determine the nature and uniqueness of the cell-cell interactions between oral actinomycetes and streptococci was initiated by isolating and identifying more than 400 fresh strains from human subgingival samples. These strains were picked randomly, purified from a population of oral bacteria, and tested for their ability to coaggregate with previously characterized reagent strains of oral actinomycetes or streptococci. One very siginificant finding was that 100% of the new Actinomyces viscosus isolates coaggregated with oral streptococci and more than 80% of the A. naeslundii isolates did likewise. None of the A. odontolyticus, Arachnia propionica, Bifidobacterium adolescentis or Bacterionema matruchotii isolates gave similar coaggregation patterns. In addition, none of the gram-negative filamentous oral bacteria coaggregated with either streptococci or actinomycetes. The only streptococci which coaggregated with actinomycetes were those which belonged to the "viridans group" and included the species S. sanguis, S. mitis, S. MG-intermedius, and S. morbillorum. Significantly, none of the S. mutans isolates coaggregated with any of the actinomycetes. Therefore, there exists a very high degree of specificity for cell-cell interactions among oral bacteria. The predominant participants were A. viscosus and A. naeslundii and certain "viridans" streptococci of which S. sanguis was most commonly found in this study.

Comparison of coaggregation properties exhibited by freshly isolated oral bacteria and old stock culture collection strains. After completing our earlier study using laboratory-maintained stock culture collection strains, it was of interest to compare those results with data obtained using fresly isolated strains. Based on the coaggregation patterns between oral actinomycetes and streptococci, two groups of actinomycetes and four groups of streptococci were delineated in the previous investigation.

In our current study using fresh isolates, more than 80% of the coaggregation reactions fell into these six previously characterized groups. The other coaggregation reactions observed describe three new groups of actinomycetes and one new streptococcal group. It should be emphasized, however, that these new groups represent only a minor part of the cell-cell interactions that take place between these bacteria. Thus, most of the coaggregations that occurred between either old stock culture collection strains or freshly isolated strains were of identical patterns.

Stability and binding properties of cell surface components on oral bacteria. The fact that both fresh isolates and old stock culture strains exhibited the same coaggregation patterns suggested that the cell surface components which mediated coaggregation were unaltered by repeated culture transfers and long term storage under a wide variety of conditions. We have also shown that bacterial suspensions stored for two years in protective buffer at $4^{\circ}C$ completely retained all coaggregation properties. Four of the five streptococcal coaggregation groups and five of the six actinomycete groups exhibited lactose-reversible binding. Members of these groups constituted 85% of the new oral isolates that coaggregated in this study. Based on this current study, it appears that the lactose-reversible site plays a key role in the binding properties between oral actinomycetes and streptococci. The mechanism of binding between the two cell types appears to be a lectin-carbohydrate interaction involving a lectin on one bacterial cell surface and a carbohydrate component on the other surface. The lectin component can be on either cell and this property is used to delineate coaggregation groups. Both cell types can possess lectin molecules that participate in lactose-reversible interactions. However, the lectins involved in coaggregations that are not reversed by lactose are found only on the streptococcal surface.

Extent of distribution of lactose-reversible cell-cell interactions among oral bacteria. The only Actinomyces species which exhibited lactose-reversible coaggregation were A. viscosus and A. naeslundii, and in each case, the coaggregations were with streptococci. All of the A. viscosus and 84% of the A. naeslundii isolates coaggregated. In total, more than 30 strains of A. israelii, A. odontolyticus, and A. meyeri have been tested and none gave lactose-reversible coaggregation. All but two strains of the streptococci that coaggregated in a lactose-reversible pattern were either S. sanguis or the closely related S. mitis: the other two strains belonged to S. morbillorum. None of the S. mutans or S. anginosus-constellatus isolates coaggregated at all and, only coaggregations not reversed by lactose were observed with S. MG-intermedius isolates. Thus, lactose-reversible coaggregations seem to be limited to those that occur between A. viscosus or A. naeslundii strains and members of S. sanguis, S. mitis, or S. morbillorum, which are "viridans" streptococci. The only exception is the observation that three strains of Propionibacterium acnes gave lactose-reversible coaggregation with only group 1 streptococci. This interaction will receive further study since it is the only example of this kind of coaggregation pattern.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESERACH:

Evidence for a highly specific set of coaggregation patterns was confirmed

in this study of freshly isolated oral actinomycetes and streptococci. Identical patterns had previously been characterized by using laboratory strains maintained in stock culture collections for several years. The fact that both older stock culture strains and freshly isolated strains exhibit identical coaggregation properties gives strong support to the idea that cell surface components on these oral bacteria are very stable structures and are likely to be involved in mediating cell-cell interactions in the oral cavity. Furthermore, these interactions are probably responsible for the selective distribution of these bacteria within the oral cavity. The actinomycetes are thought to play a role in the etiology of root surface caries and periodontal disease. The relationship of the interaction of these actinomycetes and oral sreptococci to the disease process is unclear at this time. However, further investigation of the cell surface properties of these bacteria should provide needed information about the molecular basis for cell-cell interactions and cell-solid support adherance by these bacteria.

PROPOSED COURSE:

Based on the results of this current study using fresh oral isolates, we have proposed a model which describes the range of cell-cell interactions which is limited by the highly specific nature of the interactions. To test the validity of this model will require the use of mutants that are unable to coaggregate. We have already developed a selection regimen for the enrichment of such mutants in a natural population of bacteria and havesuccessfully isolated several different types. Additional enrichments are expected to be conducted to obtain a large number of mutants. Such mutants will be defective in a single type of cell-surface component and a study of such mutants will provide an independent analysis of each component involved in coaggregation of oral actinomycetes and streptococci. A collection of mutants defective in different surface components will be invaluable in elucidating the molecular mechanisms of these cell-cell recognition systems.

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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-00045-09 LMI

PERIOD COVERED

October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Role of Macrophage and Lymphocyte Mediators in Immunity and Inflammation

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

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Dougherty, Suanne F.	Biologist	LMI NIDR
Carter, Charles	Microbiologist	LMI NIDR
Raupp, Laurie	Bio Lab Tech	LMI NIDR
Northoff, Hinnak	Guest Worker	LMI NIDR
Stadler, Beda	Visiting Fellow	LMI NIDR
Farrar, John J.	Research Microbiologist	LMI NIDR
Mizel, Steven	Senior Staff Fellow	LMI NIDR
Siraganian, Reuben	Chief, Clinical Immunology	LMI NIDR

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Bonnie Mathieson, NIAID, NIH; Kendall Smith, Dartmouth Med. Sch.; Douglas Olson, Meloy Labs, Springfield, VA.

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SECTION

Cellular Immunology Section

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6.37	3.37	3.00

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Human monocytes and lymphocytes are activated by a wide variety of antigenic or polyclonal stimulants to produce a multiplicity of immunoregulatory mediators. Activated monocytes produce interleukin-1 (IL-1) which promotes murine thymocyte proliferation but also changes the thymocyte membrane surface markers from peanut agglutinin positive (PNA^+) to negative and from $Lyl\ 2^+$ to $Lyl\ 2^-$. These phenotypic changes characterize the more mature immunocompetent medullary thymocyte and are associated with the development of IL-1 induced thymocytes to manifest helper activity. IL-1 also enables monocyte depleted T lymphocytes to be activated to produce lymphokines such as IL-2. Sufficient IL-2 activity can be obtained to identify it as a 15,000 MW polypeptide. IL-2 can be differentiated from the 15,000 MW IL-1 using bioassays and they can be separated by chromatography procedures. Helper lymphocytes production of IL-2 is negatively regulated by T suppressor lymphocytes. Furthermore, regulation of immune interferon production correlates directly with that of IL-2 production and therefore also appears to be controlled by suppressor T cells.

The regulation of production and role of mediators produced by human monocytes and lymphocytes that amplify immunological and inflammatory reactions is being investigated. These endogenous nonspecific factors are produced when these cells are activated by a wide variety of antigenic, polyclonal or even injurious stimulants. The monocyte derived mediator is now called interleukin 1 (IL-1). IL-1 has a wide variety of biological effects including promoting thymocyte proliferation, increasing lymphocyte functions such as lymphokines and immunoglobulin production and also stimulating nonlymphoid cells that contribute to systemic inflammatory reactions. Thus IL-1 or a similar factor stimulates cells in the hypothalamic fever center and it may therefore be related to endogenous pyrogen. IL-1 stimulates "reticular" fibroblasts in the synovia of inflamed joints to produce inflammatory prostaglandins and collagenase. IL-1 also appears to stimulate hepatocytes to produce acute phase proteins such as serum amyloid A (SAA). The lymphocyte mediator which is produced by the "helper-inducer" subpopulation of lymphocytes is called interleukin-2 (IL-2). It also has a variety of key biological effects on lymphocyte growth and functions including promoting the proliferation and maturation of the cytotoxic T lymphocyte (CTL) subpopulation. IL-2 can in fact support the long term growth of the normal CTL which is due to its T cell growth factor (TCGF) activity. IL-2 indirectly also promotes cell proliferation and immunoglobulin production. Thus both IL-1 and IL-2 are key endogenous amplifying signals which we are attempting to characterize biochemically and biologically. Understanding the mechanisms that control the production as well as the means by which these mediators exert their crucial effects in immunity and inflammation can potentially provide means of therapeutic manipulation of inflammatory reactions.

METHODS:

We are studying human IL-1 and IL-2 predominantly in tissue culture and infrequently assay their *in vivo* effects in animal models. The IL-1 is obtained from the buffy coat fractions of peripheral blood (purified by Ficoll-Hypaque centrifugation) kindly supplied us by the NIH Blood Bank and American Red Cross. The IL-2 is obtained either from the buffy coat of peripheral blood or from tonsillar or splenic tissues kindly supplied by the Pathology Dept. of Children's Hospital of Washington, D. C. The cells are cultured for several days in large batches with the appropriate stimulants and the supernatant activity tested, concentrated and purified. The mediators are routinely bioassayed for their mitogenic effect on murine (C3H/HeJ) thymocytes, peripheral human T and B lymphocytes and on murine CTLL. The effect of the mediators on thymocyte differentiation markers is being studied using fluorescence activated cell sorter (FACS) techniques. The capacity of these mediators to increase immunoglobulin and lymphokine production and promote nonlymphocytic cell functions is also being tested.

1) It has been observed that except for the mitogenic effect of human IL-1 on immature mouse thymocytes, IL-1 generally promotes lymphocyte differentiation and functions. We therefore investigated the effects of IL-1 on murine thymocyte differentation in collaboration with Dr. B. Mathieson of NIAID using the FACS. We stimulated peanut agglutinin (PNA) positive or negative thymocyte subpopulations with human IL-1 in the presence or absence of an exogenous polyclonal stimulant such as phytohemagglutinin (PHA). This revealed that only the more mature medullary (PNA^-) thymocytes could proliferate in response to these stimuli by themselves. However, simultaneous stimulation with both of these signals induced considerable proliferative activity by immunocompetent PNA^+ cortical as well as the medullary thymocytes. This suggested that one of these signals, namely IL-1, might have a maturational effect on the PNA^+ thymocytes. We therefore studied the effect of these stimulants on the phenotypic markers of thymocytes. We observed that the predominantly Lyl^+2^+ PNA^+ thymocytes when cultured with IL-1 w/wo PHA changed in part to Lyl^+2^- PNA^- thymocytes which is the characteristic phenotype of medullary thymocytes. We then investigated in collaboration with Dr. John Farrar whether there was a concommitant change in the immunological capabilities of these thymocytes. We observed that unfractionated thymocytes that have no significant helper capabilities after incubation with IL-1 w/wo PHA also developed the characteristically greater capacity of medullary thymocytes to function as helper cells. These findings may reflect normal in vivo thymocyte developmental processes since there are considerable numbers of macrophages which are known to secrete IL-1 present in the thymus.

2) In conjunction with Dr. Hinnak Northoff we observed that antigenic or polyclonal activation of lymphocytes to produce IL-2 requires the presence of monocytes. Therefore in conjunction with Dr. Kendal Smith (Dartmouth Medical School) we investigated whether the IL-1 product of macrophages was needed for IL-2 production. Indeed, we observed that macrophage depleted T lymphocytes could not be stimulated to produce IL-2 unless supplemented with IL-1. Thus an endogenous (IL-1) as well as exogenous (lectin mitogen or antigen) signal is required to activate T lymphocytes. Furthermore these findings support the idea that the IL-1 and IL-2 mediators may act sequentially on distinct lymphoid subpopulations to promulgate immune responses.

3) Dr. Hinnak Northoff also observed that the production of human IL-2 is regulated by T suppressor lymphocytes. This inhibition of IL-2 production appears to be cell contact dependent rather than mediated by supernatant factors. Thus any in vitro conditions that promote the function of "helper-inducer" lymphocytes at the expense of the T suppressor lymphocytes promote IL-2 production. This includes use of "aged"rather than "fresh" cells, use of serum free media, use of multiple stimulants such as phorbol myristic acetate (PMA) or mixed lymphocyte reactions (MLR) in conjunction with lectins or the addition of cimetidine which

competitively inhibits suppressor cells. Conversely theophylline and other cAMP agonists increase suppressor cell functions and decrease IL-2 production.

This observation complements those of Laurie Raupp who is finding that human Tγ lymphocytes which are enriched in suppressor cells have much greater cAMP responses when stimulated by cAMP agonists than do the Tμ subpopulations that contain more of the helper cells.

4) Dr. Northoff in collaboration with Dr. John Hooks has also observed a direct relationship between IL-2 and immune interferon (IF) production. This suggests, as have the murine studies of John and William Farrar, that human IL-2 and IF are also sequentially linked and both appear to be regulated by T suppressor cells.

5) Dr. Beda Stadler has investigated the relationship of IL-2 production to the different phases of the cell cycle. He observes that any conditions that prolong the G1 phase of the cell cycle favors the production of IL-2. Thus PMA and hydroxyurea which prolong and arrest cells in G1 respectively markedly enhance IL-2 production. These findings have the pragmatic effect of enhancing our ability to produce more IL-2 activity. Dr. Stadler is sequentially purifying IL-2 by chromatography on phenyl-sepharose, DEAE anion exchange and S200 sephacryl. This has yielded IL-2 activity in a single peak of 15,000 MW. This material is being used to immunize BalB/C mice and their spleen cells are then fused with B lymphocyte lines by Dr. R. Siraganian in order to grow hybrid clones that may produce antibody against IL-2.

6) Finally we are continuing to produce the human IL-1 in quantity and purifying it with the help of Drs. Steven Mizel and John Farrar. Human monocytes produce IL-1 best in serum containing cultures and the IL-1 is partly adsorbed by human serum proteins. We have found that IL-1 can be partly dissociated from the 60,000 MW proteins to yield the 15,000 MW IL-1 by incubation at 37°C. The IL-1 is being sequentially chromatographed on phenyl sepharose, and sephacryl 200 but 80% of the activity is lost by the former procedure. We are therefore exploring the use of isoelectric focussing (IEF) and anti HSA affinity columns as the initial purification steps. In the meantime we are also immunizing mice with partially purified gel fractions and are in the process of fusing their spleen cells to obtain antibody producing hybridoma cell lines.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

Periodontal inflammation is immunologically mediated and has been shown to involve both cellular and humoral host reactions to pathogenic microorganisms. In the case of man, the contribution of host defense mechanisms to the inflammatory state can best be analyzed using tissue culture models. We have previously demonstrated that pathogenic oral organisms such as Actinomyces viscosus are potent in inducing in vitro IL-1 and lymphokine production. Thus monocyte as well as lymphocyte derived mediators presumeably actively participate in oral inflammatory processes. Since these mediators play an important role in augmenting inflammatory responses,

and since they can be recovered from supernatants, they provide us with the opportunity of manipulating tangible regulatory factors that modulate inflammation including that which occurs in periodontal diseases.

PROPOSED COURSE:

We will continue to purify, isolate and identify the monocyte and lymphocyte derived immunoregulatory factors. Technological advances now provide us with the opportunity of obtaining monoclonal antibodies from hybridoma cell lines to partially purified factors. Such antibodies can be used to perform radioimmunoassays, to study the effect of competitively blocking the biological activities of the factors and to obtain greater purification using affinity chromatography. Microsequencing techniques are becoming available that will permit analysis of $\leq$ 10 pM quantities of proteins. Even though the mediators are biologically active an $\leq 10^{-11}$ M concentrations, it should be feasible to obtain them in sufficient homogeneity and adequate amounts for identification. Additionally, we will continue our studies of the production, interrelationships and effects of the mediators.

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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-00131-06 LMI

PERIOD COVERED
October 1, 1979 - September 30, 1980

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

Farrar, John J.	Research Microbiologist	LMI NIDR
Fuller-Farrar, Janet	Microbiologist	LMI NIDR
Hilfiker, Mary L.	Post-doctoral Fellow	LMI NIDR
Farrar, William	Post-doctoral Fellow	LMI NIDR

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SECTION
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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.25	3.25	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Macrophages and T cells interact with each other and in the process synthesize and secrete a variety of antigen-nonspecific soluble factors which serve to induce and amplify both humoral and cell-mediated immune responses. We have utilized our basic observations on the effects of phorbol esters on lymphocytes to describe a technique to induce mouse spleen cells to produce 5-20 times as much Interleukin 2 (IL2) as conventionally stimulated spleen cells. In addition, the phorbol esters have been used to stimulate the murine thymoma, EL-4 to produce IL 2 in very large quantities in vitro. The use of the thymoma culture supernatants as a source of IL 2 should facilitate the purification to homogeneity of the factor. Additionally, the large amounts of factor obtainable from the thymoma should allow in vivo studies of possible therapeutic uses for IL 2. Studies on the mechanism of action of IL 2 indicate that the factor interacts with a receptor on the target cell and induces that cell to produce immune interferon which is required for the subsequent lymphocyte activation events.

OBJECTIVES:

The principal objectives of this research project are 1) to purify and characterize the antigen nonspecific murine lymphokine Interleukin 2 (IL 2) and 2) to determine the mechanism-of-action of this soluble factor on the humoral and cell mediated immune responses.

METHODS:

Standard in vitro lymphocyte culture methods for the induction and assay of antibody and cell-mediated immune responses were utilized including the enumeration of antibody forming cells by the Jerne hemolytic plaque-forming cell (PFC) assay and the ^{51}Cr release assay for quantitation of cytotoxic lymphocytes. Standard cell separation procedures were used to prepare purified preparations of B cells, T cells, and macrophages.

MAJOR FINDINGS:

On the production of IL-2 by mouse thymoma cells. We have previously demonstrated that the addition of PMA to Con A-stimulated purified T cell populations induced the production of IL 2. Thus, PMA afforded us a mechanism by which we could activate T cells in the absence of macrophages. We therefore, attempted to activate a variety of T cell lines with PMA. A subline of the mouse thymoma EL 4 was found to produce very large quantities of IL 2 when stimulated with PMA. On a per cell basis the EL 4 was found to produce 19,000 times as much IL 2 as optimally stimulated mouse spleen cells. Preliminary characterization of the EL-4-derived IL 2 has been conducted. The factor exhibits the same molecular weight (30,000) and isoelectric point heterogeneity (3.8 to 4.4) as normal murine spleen cell-derived IL 2. Further, the thymoma-derived IL 2 exhibits the same spectrum of biological activities including the capacity to enhance humoral and cell mediated immune responses as does normal splenic IL 2.

On the production of IL-2 by human lymphocyte cell lines. We have examined several human T cell lymphomas for production of human IL 2 which is biochemically distinct from the murine derived molecule. Experiments have identified at least one human lymphoma that demonstrates soluble IL 2-like activity following stimulation with PMA and Con A. Preliminary experiments are currently being conducted to biochemically characterize and confirm identity of the lymphoma-derived activity with the IL 2 produced by normal peripheral blood lymphocytes.

On the absorption of IL 2 by CT6 cells. The factor-dependent, murine, T cell line, CT6, has been used to investigate the absorption by cells of IL 2. Absorption of biological activity of IL 2 is being studied rather than binding of radiolabelled factor because IL 2 has not as yet been purified to a sufficient degree for iodination. Partially pruified (gel filtration) IL 2 derived from murine spleen cells and from the EL4 cell line has been used with no detectable difference in the absorption properties due to the origin of the factors. Absorption of IL 2 is

determined by comparing the activity (measured by TCGF assay) of supernatants incubated with CT6 cells to a supernatant incubated without cells and is therefore expressed as percent absorbed.

The CT6 cells rapidly absorb IL 2 at 4°C and 37°C with 90% absorption occurring between 5 and 15 min at 4°C and between 2 and 3 min at 37°C. The absorption is linear with the number of cells/ml and is specific as a human T cell line, CEH-C7, does not absorb this IL 2. Although incubation of the cells with DNP (5×10^{-4} M) for 30 min prior to addition of IL 2 in glucose-free conditions, significantly decreases the absorption of IL 2 by CT6 cells, it is unclear whether metabolic energy is required for binding of IL 2 or for subsequent incorporation of IL 2 into cells. It will be difficult to definitively answer the question until a radiolabelled IL 2 is available. However, it is possible to absorb IL 2 and then elute the factor off the CT6 cells by a subsequent incubation (5 min, 4°C) with pH 2 buffer. Furthermore, since the treatment with the Ph 2 buffer is not toxic (85% viability as measured by trypan blue exclusive), it is possible to treat CT6 cells with the pH 2 buffer, not only eluting off any factor on the cell surface but also making the cells incapable of absorbing more factor. A kinetic study of the reappearance of receptors (i.e., ability to absorb factor) following pH 2 treatment is currently in progress.

These studies characterize the absorption of IL 2 by cells and provide a essential foundation for future binding studies.

On the role of interferon and IL 2 on the generation of cytotoxic T lymohocytes. We have previously shown that the activation of alloantigen-specific cytotoxic T lymphocyte (CTL) precursors is dependent upon the presence of both macrophages and helper T cells or regulatory molecules derived from these facilitative cells. Three biochemically distinct helper factors have been identified: Interleukin 1 (macrophage-derived), Interleukin 2 (T cell derived), and immune interferon. All three factors are found in the supernatants of mixed lymphocyte cultures (MLC), however the removal of macrophages from these cultures completely ablates the production of these factors as well as the induction of CTL. The addition of Interleukin 2 to these macrophage-depleted MLC restores the ability of responder T cells to: 1) bypass the requirement for macrophage soluble function, 2) produce immune interferon and 3) generate CTL. The kinetics and dose-response of immune interferon production in response to Interleukin 2 correlates with the generation of CTL. The production of immune interferon as well as the generation of CTL requires T cells, alloantigen and Interleukin 2. Furthermore, the induction of CTL by Interleukin 2 was neutralized by the addition of anti-immune interferon sera. These data suggest that: 1) the regulation of immune interferon production is based on a T to T cell interaction mediated by Interleukin 2 and 2) immune interferon production is required for IL 2 induced cytotoxic T lymphocytes. These findings are consistent with the hypothesis that the induction of CTL involves a linear cell-factor interaction in which Interleukin 1 (macrophage-derived) stimulates T cells to produce Interleukin

2 which in turn stimulates other T cells to produce immune interferon and become cytotoxic.

Significance to Biomed. Periodontal disease is characterized by an inflammatory response which is regulated by a complex network which includes antigen, antibody, complement, macrophages, helper and suppressor T cells and B cells as active components. These components serve to control the initiation, expression, maintenance and suppression of the inflammatory response and, hence, periodontal disease. Maintenance of this complex regulatory network is achieved by interactions between the participating cells; interactions which are mediated, in part, by soluble factors produced by the cells. The elucidation of the mechanism of action of the soluble factors, one of which is IL 2, is crucial to our understanding of inflammation.

Proposed Course: During the ensueing year, we shall continue our efforts to purify the EL-4-derived IL 2 and to prepare a homogenious radiolabelled product. Additionally, studies on the mechanism of action of the phorbol esters on lymphocyte activation will be conducted. Experiments on the IL 2 receptor on target cells will continue in an effort to determine the mechanism by which IL 2 activates T lymphocytes.

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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-00167-05 LMI

PERIOD COVERED

October 1, 1979 - September 30, 1980

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

☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The LPS hyporesponsive C3H/HeJ mouse strain has been utilized to help elucidate the mechanism of action of LPS. These mice possess a mutation in a single autosomal gene which results in an abnormal tolerance to the effects of LPS in vivo and is expressed in the T and B lymphocytes, macrophages(MØ) and fibroblasts. Additionally, MØ Fc-mediated phagocytosis is abnormal, suggesting a defect in MØ differentiation. In vivo, the LPS defect in C3H/HeJ mice can be partially reversed by preinfection with BCG, a potent immunostimulant. In vitro, the C3H/HeJ MØ LPS and phagocytic defects can be reversed by treatment with a lymphokine-rich, Con A-stimulated, spleen cell supernatant and the phagocytic defect by cAMP agonists. Furthermore, coculture of C3H/HeJ MØ with purified T lymphocytes derived from LPS responsive animals (including BCG-infected C3H/HeJ mice) results in the production of Interleukin-1 (IL-1) by the MØ. The systemic effects of endotoxins are mediated to a great extent by acute phase reactants such as prostaglandins, IL-1 and serum amyloid A (SAA). An LPS-stimulated monokine has been shown to stimulate hepatocytes to produce SAA. Thus the capacity to respond to LPS is required for normal macrophage differentiation, for the ability to produce monokines which in turn induce SAA production and / or other symptoms of endotoxicity.

OBJECTIVES

The major objectives of this project are: 1) to analyze the cellular mechanisms and cellular interactions underlying endotoxin-induced reactions, 2) to characterize biochemically the nature of intercellular and intracellular signals by which bacterial lipopolysaccharides and related products activate lymphocytes and macrophages and 3) to evaluate the role that oral bacterial lipopolysaccharides play in the intitiation and maintenance of the chronic inflammation associated with periodontal disease.

MAJOR FINDINGS

C3H/HeJ mice exhibit a profound genetic defect which is expressed as an inability to respond to bacterial endotoxin in vivo. Moreover, the individual cell types derived from these mice have been examined and are similarly unreactive to LPS in vitro. We have utilized the C3H/HeJ mouse strain as a genetic model to elucidate the mechanisms by which endotoxins exert their many biological effects.

We previously demonstrated that the transfer of syngeneic bone marrow cells derived form LPS responsive C3H/HeN mice into lethally irradiated C3H/HeJ mice rendered them significantly more LPS sensitive, suggesting that the cells responsible for LPS sensitivity are both radiosensitive and of lymphoreticular origin. Additionally, we demonstrated that the potent macrophage activating agent, BCG, renders C3H/HeJ mice significantly more LPS sensitive. Other agents known to act upon macrophages (i.e., carageenan, zymosan, cortisone, glucan, $ZnCl_2$, methyl palmitate and others) have also been shown to alter the in vivo LPS responses of normal mice. Finally, we have previously examined the CBA/N mouse strain, which lacks endotoxin sensitive B lymphocytes. These mice possess macrophages which are LPS sensitive in vitro and are fully sensitive to the toxic effects of LPS in vivo.

With this information in mind, we have directed our analysis of LPS sensitivity primarily toward the macrophage as the central effector cell in mediating endotoxicity. Our major findings are listed as follows:

1. If highly purified peritoneal T lymphocytes derived from LPS responsive mice (including the BCG infected C3H/HeJ mouse strain) are cocultured with LPS hyporesponsive C3H/HeJ macrophages plus and LPS stimulus, the culture supernatants obtained from these cocultures are mitogenic for thymocytes. Neither the purified C3H/HeJ macrophage cultures nor the purified T cell preparations alone responded to LPS to produce LAF. These data strengthen the role of the macrophage as the central effector cell in mediating endotoxicity and assign to other cell types, such as the T lymphocyte, an immunoregulatory role, acting to modulate the LPS sensitivity of the macrophage.

2. We previously demonstrated that serum derived from LPS stimulated C3H/HeN, but not C3H/HeJ, mice, administered to C3H/HeJ mice, led to the induction of the circulating product, serum amyloid A (SAA). We have

also previously shown that LPS-stimulated C3H/HeN, but not C3H/HeJ, macrophages produce a soluble factor capable of inducing SAA in vivo. We have since observed that the kinetics of LAF production in the LPS-stimulated macrophage culture supernatants paralleled the kinetics of "SAA inducer" production. Therefore, we sought to determine whether the SAA inducer was identical to LAF. To do this we have examined LAF-rich preparations from a number of sources and have found that in addition to crude LPS-stimulated C3H/HeN macrophage culture supernatants, partially purified LPS-induced human LAF also possessed good SAA-inducing activity. Similarly, when human monocytes stimulated with muramyldipeptide were used as the source of LAF, SAA inducing activity was observed. Two electrophorectically distinct species of rabbit endogenous pyrogen, which also contain LAF activity, were potent SAA inducers. Finally, a 10,000-fold purified preparation of LAF prepared from phorbol myristic actate-stimulated $P388D_1$ macrophage cell line cultures, was active in inducing SAA. These data data support the notion that the SAA-inducer produced by LPS responsive macrophages is probably LAF. These data also demonstrate two important points. First, the SAA inducer can clearly act across species barriers, emphasizing the retention of this molecule during phylogeny as an important immunoregulatory signal. Secondly, if the SAA inducer is indeed identical to LAF, it suggests that this molecule is able to act on nonlymphoid cell types such as hepatocytes, in addition to lymphoid cell.

3. In addition to the macrophage LPS defect, C3H/HeJ macrophages lose, upon culturing, the ability to phagocytose through the Fc receptor. This was demonstrated by measuring a decreased capacity to phagocytose ^{51}Cr-labeled oponized erythrocytes and was found to be secondary to a loss in Fc receptor binding capacity. We have also found that we can completely reverse this defect in macrophage differentiation by exposing the macrophage cultures to a lymphokine-rich, Concanavalin A-stimulated spleen cell culture supernatant. This same treatment renders the C3H/HeJ macrophage sensitive to LPS as assessed by LAF production and LPS-induced cytotoxicity. These findings support the hypothesis that a certain state of macrophage differentiation is a prerequisite for LPS sensitivity and are completely consistent with our data demonstrating T cell modulation of macrophage LPS sensitivity. The fact that a second LPS-unresponsive mouse strain, C57B1/10ScN, also echibits the same defect argues strongly that the differentiation defect is controlled by the same gene that controls LPS snesitivity.

4. In order to better understand the mechanism by which the lymphokine-rich culture supernatant (CS) corrected the C3H/HeJ macrophage defects, we have taken two approches to examine the nature of the cellular signals involved. First, there is abundant evidence in the literature to suggest that elevations in intracellular cAMP levels are associated with alterations in the expression of membrane receptors. We have found that DBcAMP, as well as 8-Br-cAMP are efficient stimulants of Fc receptor

mediated binding and phagocytosis in C3H/HeJ macrophage cultures. Additionally, other intracellular cAMP agonists, such as isoproterenol plus isobutylmethylxanthine or prostaglandin E_2 increase Fc-mediated phagocytosis in a dose dependent manner. We have found that the agent active in CS is not prostaglandin since the CS can be generated in the presence of indomethacin, a potent inhibitor of the cyclooxygenase pathway required for prostaglandin synthesis. Additionally, the lymphokine-rich culture supernatant does not enhance Fc mediated phagocytosis by inducing prostaglandins since indomethacin fails to alter its Fc receptor converting activity. We have found, however, that the CS increased macrophage intracellular cAMP levels by approximately 60%. These data suggest that the enhancement of Fc receptor capacity in C3H/HeJ macrophage cultures by CS involves a cAMP dependent step which is prostaglandin-independent.

5. We have also begun a biochemical analysis of the active components in CS. We have found that the active factors are nondialyzable and exhibit tremendous molecular weight heterogeneity when applied to molecular sieve chromatography. Additionally, our factor adsorbs to hydroxyl apatite and DEAE cellulose and can be eluted from both materials by increasing the salt concentration of the eluting buffer. This factor exhibits an isoelectric focusing point between pI 4 and 5.5, suggesting a certain degree of charge heterogeneity. The biological activity of our CS is heat stabile at 56° C for 30 minutes, but labile at 100° C for 30 minutes; it is sensitive to overnite dialysis against pH 2 and 10 buffers, and exhibits no loss in biological activity upon prolonged storage at 4° C. The cellular source, existing biochemical characterization, and the ability to elevate intracellular cAMP levels strongly suggest the possibility that the factor involved is perhaps Type II interferon. We have tested the efficacy of the more available, Type I interferon in altering the C3H/HeJ macrophage phagocytic defect and have found that this material is fully capable of altering the Fc receptor defect in C3H/HeJ macrophage cultures.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH

The ubiquitous nature of bacterial endotoxins, in conjunction with their ability to act as potent activators of B lymphocytes and macrophages, support the notion that endotoxins are important naturally occurring differentiation signals in the developing immune system. However, under certain conditions, endotoxins may induce pathological conditions within the host manifested by fever, hypoglycemia, and shock. Our research is focused upon understanding the role of endotoxin as a positive immunoregulatory substance, but also to understand the mechanisms by which it exerts its toxic effects.

Since periodontal disease is due to chronic inflammatory responses in the gingiva, and since endotoxins from oral bacterial flora are abundant in the mouth, it is important to understand how endotoxins activate the cells involved the initiation and prologation of the chronic inflammation that is associated with periodontal disease. Perhaps, with this approach, we can begin to understand the

etiology of periodontal disease and explore methods to prevent or counteract this condition.

PROPOSED COURSE

1. We plan to complete studies of the biochemical nature of the *in vitro* lymphokine that is responsible for correction of the C3H/HeJ macrophage LPS and differentiation defects. A study of the relationship between the factors correcting the two defects will be studied. To date, we have not been able to chemically separate these two activities, suggesting that the same factor corrects both defects.

2. We propose to examine the question of whether T cells from LPS responsive mice spontaneously release differentiative lymphokines or if LPS stimulation of these cells is required. We also hope to establish whether the factor active in CS is identical to the normal T cell factor.

3. We plan to pursue the characterization of the SAA inducer by testing the capacity of antisera prepared against endogenous pyrogen to abrogate the SAA-inducing capacity. Furthermore, we plan to further test the theory that the SAA inducer is LAF by treating highly purified murine LAF with phenylglyoxol, an agent which specifically inactivates arginine residues essential to LAF activity. Should these treatments render the LAF preparation unable to induce SAA, it would argue strongly that LAF is the SAA inducer. Additionally, we plan to examine the role of SAA and other closely related acute phase reactants in mediating the pathological effects of endotoxin.

4. We plan to examine the role of cAMP in inducing LPS sensitivity. Since a cAMP-dependent step appears to be an important signal in reversing the C3H/HeJ macrophage differentiation defect, we plan to examine whether cAMP can also reverse the LPS hyporesponsiveness exhibited by these cells.

5. We will try to place normal animals in an LPS-depleted environment and exammine their macrophages for a reversal of LPS-sensitivity and decreased Fc-receptor mediated phagocytic ability.

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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-00209-04 LMI

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical and Biological Characterization of Interleukin 1

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

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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 a superinduction protocol have been used to obtain relatively high levels of the macrophage immunostimulatory peptide, interleukin 1 (previously termed lymphocyte activating factor). The interleukin 1 has been purified to homogeneity with a yield of approximately 60 µg/5 l culture fluid. The purified interleukin 1 is composed of three charge species of identical molecular weight. Biologic studies have revealed that interleukin 1 may play a critical role in T cell activation as well as in rheumatoid synovial cell activation. Thus, via a single mediator, the macrophage may regulate the behavior of a number of cell types involved in immune and inflammatory responses.

The major objectives of this ongoing project have been: 1) to purify to homogeneity the macrophage-derive interleukin 1 (IL 1) (previously termed lymphocyte activating factor) which appears to play an important, if not essential role in T lymphocyte activation; 2) to biochemically characterize and sequence the interleukin 1; and 3) to characterize the spectrum of biologic activities of interleukin 1 as well as its mechanism of action.

MAJOR FINDINGS:

A. Studies on the Production of Maximal Quantities of LAF IL 1.

During the past few years, we have placed a major emphasis on the development of procedures to maximize the production of IL 1 by the $P388D_1$ murine macrophage cell line. This project has been of great importance because of the small amounts of IL 1 that are normally produced by macrophages from any source, human, mouse or mouse cell line. Our studies clearly demonstrate the value of murine macrophage cell lines as cell sources of IL 1. By evaluating a number of agents and treatments, we found that the tumor promoter and inflammatory agent, phorbol myristic acetate (PMA) was an excellent stimulant for IL 1 production. More recently, we have found that the production of cell line IL 1 can be greatly enhanced on a per cell basis by using a superinduction protocol involving a brief incubation of the cells with actinomycin D after an initial 5 hr incubation of the cells with PMA and cycloheximide. The yield of IL 1 after 24 hrs is approximately 10-20-fold greater with a 50-fold increase in specific activity than that obtained after 6 days with cells incubated with only PMA. Thus this superinduction procedure permits us to generate relatively large amounts of IL 1 without having to deal with long incubations or unwidely volumes of culture fluid and cells.

B. Purification and Chemical Characterization of IL 1.

The study of monokines and lymphokines has been severely hampered both experimentally and theoretically by the absence of well-defined and highly purified mediators. In the course of our studies on the $P388D_1$ macrophage cell line IL 1 we have not only purified this mediator, but have also defined some of its important chemical features. $P388D_1$ IL 1 is a single polypeptide chain of 12,000-16,000 m.w. that is relatively resistant to the destruction of its biologic activity by proteolytic enzymes and denaturing agents such as urea and SDS. The biologically active form of IL 1 is not dependent on disulfide linkages as evidenced by the insensitivity of IL 1 activity to incubation with 2-mercaptoethanol and iodoacetamide. However, IL 1 activity was almost completely destroyed during a 4 hr incubation with 1% phenylglyoxal, a compound which covalently attaches to arginine residues. We have also found that human IL 1 is similarly affected by phenylglyoxal. Phenylglyoxal-treated IL 1 is not only inhibited in its thymocyte mitogenic activity, but also inactive in in vitro antibody responses (S. B. Mizel and J. J. Farrar, unpublished observations) as well as in the stimulation of rheumatoid synovial cell

collagenase and prostaglandin production (S. B. Mizel, J.-M. Dayer, S. M. Krane, and S. E. Mergenhagen, submitted for publication). In contrast, histidine modification with diethylpyrocarbonate has little if any effect on IL 1 activity. Treatment of IL 1 with cyanogen bromide did not result in the appearnce of any biologically active fragments.

Using 1) culture fluid from superinduced $P388D_1$ cells and 2) a purification sequence of ammonium sulfate fractionation, phenyl Sepharose hydrophobic chromatography, Ultrodex AcA54 gel filtration chromatography, and preparative isoelectrofocusing, we have purified IL 1 to homogeneity. This material is composed of three charge species of identical molecular weight. All forms of IL 1 are biologically active in the subnanomolar range. Maximal thymocyte proliferation responses are obtained with approximately 5×10^{-10} M IL 1. From approximately 2×10^{10} cells we have obtained 64 µg of purified IL 1. Amino acid sequencing studies are presently being initiated with the purified IL 1. Our recent studies clearly demonstrate that IL 1 can be produced and purified in sufficieint quantities of biochemical and biological studies.

C. Studies on the Biologic Properties of IL 1.

In addition to our studies on the purification of IL 1 we have also examined the biologic properties of the cell line-derived peptide. The results of our recent studies clearly demonstrate that IL 1 may play a general role in macrophage-dependent T cell activation in both humoral and cell mediated immune responses.

T cells. In some situations, IL 1 can apparently function in a singular manner to enhance T cell activation. For example, we have found that IL 1 by itself markedly enhances the generation of stable E (sheep erythrocytes)-rosette (37^{o}C) forming human peripheral blood T lymphocytes (A. Ben-Zvi, S. B. Mizel, and J. J. Oppenheim, manuscript in preparation). Unstimulated normal human T lymphocytes exhibit little if any stable E-rosette forming capacity (0-2%). However, when these cells were incubated with IL 1 for 72 hrs, the percent stable E-rosette forming cells increased to approximately 60%. This value is similar to that observed with the synovial T cells from patients with rheumatoid arthritis. This similarity in E-rosette values is of some possible significance since IL 1 appears to also affect the functional activity of collagenase and prostaglandin producing synovial cells from patients with rheumatoid arthritis (see below). However, in contrast to the mitogenic effect of IL 1 on thymocytes, the stable E-rosette forming T cells generated by IL 1 are not triggered to proliferate.

In contrast to the ability of IL 1 to stimulate thymocyte proliferation and the generation of stable E-rosette forming T cells, we have found that the ability of IL 1 to enhance the proliferation of peripheral T cells, the generation of cytotoxic T cells, and the production of Interleukin 2 (IL 2) is dependent on the presence of a second signal, the priming antigen or a T cell mitogen such as PHA. This is also the case with the effect of IL 1 on in vitro antibody responses to thymic-dependent antigens. Thus, in these T cell activation processes, IL 1 does not appear to be a

polyclonal activating factor. For example, we observed that IL 1 restored the proliferative response of macrophage-depleted ovalbumin (OVA) primed mouse lymph node T cells in the presence of OVA, but not in the presence of an unrelated antigen such as bovine serum albumin. In the absence of OVA, IL 1 had no effect on lymph node T cell proliferation.

Although IL 1 restored antigen-dependent T cell responses in macrophage-depleted cultures, it did not restore responses in macrophage-free cultures. This conclusion is based on our observation that macrophage-depleted OVA-primed lymph node T cell enriched populations no longer responded to OVA and IL 1 when the cells were first treated with a specific anti-macrophage serum and complement. The responsiveness of the cells was restored, however, by the addition of 2.5% x-irradiated adherent peritoneal cells from unprimed mice. The residual macrophages in the T cell enriched population were presumably required for antigen processing and presentation, but were present in insufficient numbers to produce the required levels of IL 1.

A number of investigators have reported that the production of IL 2 is macrophage dependent. In addition, we have demonstrated that IL 1 can replace the major "non-presentation" function of the macrophage in this process. The results from several laboratories indicate that the production and secretion of IL 2 may be a key catalytic event in the clonal expansion and functional maturation of T cells. Recently, Gillis and Scheid made the important observation that two T cell lymphomas, LBRM-33 and RBL-3I, could produce relatively high levels of IL 2 in response to PHA. Interestingly, a subclone of LBRM was isolated (1A5) that did not produce IL 2 in response to PHA. In conjunction with Dr. S. Gillis, we investigated whether IL 1 could 1) augment the stimulatory effect of PHA in the parent LBRM line and 2) convert the 1A5 PHA-unresponsive clone to an IL 2 producing state in the presence of PHA. Our results (Gillis and Mizel, manuscript in preparation) clearly demonstrate that IL 1 can enhance the production of IL 2 by LBRM cells in the presence of PHA. Our results (Gillis and Mizel, manuscript in preparation) clearly demonstrate that IL 1 can enhance the production of IL 2 by LBRM cells in the presence of a suboptimal concentration of PHA and can also convert the 1A5 cell line to an IL 2 producer in the presence of PHA. Fifty percent of the maximal response in IL 2 production by both cell lines was obtained with only 0.5 units/ml IL 1 (+ 1 μg/ml PHA) as opposed to 5-10 units/ml IL 1 in the thymocyte assay. This concentration difference may have several possible explanations, but we favor the view that the difference derives from the possibility that, unlike the direct effect of IL 1 on IL 2 production, the stimulation of thymocyte proliferation by IL 1 is secondary to the induction of IL 2, a lymphokine that has clearly been shown to be directly mitogenic for T cells. Thus in the action of IL 1 on IL 2 production there is no dampening of the stimulatory signal as most likely occurs when the activating signal in thymocyte proliferation must function via more than one cell. Both LBRM and 1A5 cells absorb or bind IL 1. Of interest was our finding that on a per cell basis, the 1A5 cells appeared to absorb approximately ten times more IL 1 than the LBRM cells. Thus the 1A5 cells may prove especially useful in not only dissecting the biochemical events in IL 1-mediated T cell activation, but may also be of great value in defining and characterizing a receptor(s)

for IL 1.

Synovial Cells. In addition to its general role in T cell activation, we have also found that IL 1 can modulate the activity of a population of non-lymphoid cells. Isolated human adherent synovial cells obtained from rheumatoid synovectomy preparations produce, in primary culture, large quantities of collagenase and prostaglandins. Although the precise cellular nature of the synovial cells is not established, these cells do not possess the morphological or functional markers of macrophages or lymphocytes and are different from normal dermal fibroblasts in that they produce less collagen per cell. The ability of synovial cells in vitro to produce collagenase and prostaglandins declines with time in culture, but can be restored by addition of supernatant media from cultures of human peripheral blood mononuclear cells. The active factor in the human mononuclear cell culture supernatant has a m.w. of approximately 14,000 and has been shown to be macrophage-derived. In view of the similarities with respect to cell of origin and m.w. between this factor and IL 1, we initiated a collaborative study with Drs. J.-M. Dayer and S. M. Krane who have been responsible for the excellent work on the synovial cell activating factor from macrophages. Our results indicate that partially purified human synovial cell activating factor possesses thymocyte mitogenic activity and conversely, purified murine IL 1 markedly enhances collagenase and prostaglandin production by human rheumatoid synovial cells (S. B. Mizel, J.-M. Dayer, S. M. Krane, and S. E. Mergenhagen, submitted for publication). Recently Deshmukh-Phadke, et al. found that a 12,000-14,000 m.w. macrophage product stimulated chondrocyte collagenase production. In view of our results, it is quite possible that this factor is also related to IL 1. Thus, through the production and release of IL 1, macrophages at sites of inflammation may regulate the inflammatory activity of not only T cells, but also of the non-lymphoid populations that are involved in the destruction of connective tissue.

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 and molecular levels that we will be able to rationally manipulate the immune system for therapeutic purposes. It has become increasingly evident that the macrophage is an essential participant in the initiation, focusing, and amplification of antigen, mitogen, and pathogen-induced immune and inflammatory responses. The macrophage possesses the potential to interact with a variety of cell types that participate in immune and inflammatory reactions. IL 1 may play an important, if not essential role in many of these cellular reactions, especially with regard to T cell activation for both cellular and humoral immune responses. Our recent observation that IL 1 possesses the ability to stimulate some T cell lymphomas to produce the T cell mitogenic

peptide, Interleukin 2 (IL 2), raises the interesting, albeit speculative possibility that IL 1 may contribute both negatively (stimulation of T cell-dependent anti-tumor immunity) and positively (enhancement of IL 2 production for T cell lymphoma growth) to the in vivo propagation of T cell lymphomas. The recent finding of DeLarco et al. that virus-transformed fibroblasts produce their own growth factor (sarcoma growth factor) is certainly consistent with the idea that IL 2 derived from a T cell tumor may enhance the growth of the same tumor T cells. Thus the eventual elucidation of the biochemical mechanism(s) by which IL 1 acts may contribute to not only our basic knowledge of the immune system, but also potentially to the development of specific pharmacological agents that can modify the action of this important mediator in specific clinical situations.

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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-00238-03 LMI

PERIOD COVERED
October 1, 1979 through September 1, 1980

TITLE OF PROJECT (80 characters or less)
Regulation of Macrophage Functions in Inflammatory and Immune Responses

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

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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)

Mechanisms regulating macrophage functions in both inflammatory and immune responses are under investigation. With regard to nonspecific functions, research has concentrated upon regulation of mediator production by macrophages treated with inflammatory microbial products and soluble mediators. One class of mediators, the colony stimulating factors (CSF), were found to stimulate macrophages to produce both lymphocyte activating factor (interleukin 1) and interferon. Additionally, CSF was found to sensitize macrophages to bacterial lipopolysaccharide through an intermediary interferon priming stage and to modulate a primary immune response in vitro. Preliminary experiments performed with fibroblasts supernatants rich in CSF suggest that soluble fibroblast products can also stimulate macrophages to produce a fibroblast activating factor and toxic oxygen metabolites. With regard to specific functions, supernatants from mitogen-stimulated spleen cells were found to enhance macrophage-T lymphocyte cooperation. Active supernatants converted phenotypically Ia-antigen negative macrophages to Ia^+ cells capable of presenting antigen to T-lymphocytes. Work in progress is designed to further investigate the macrophage regulating activities of CSF, interferon, and the active splenic supernatants.

PHS-6040
(Rev. 10-76)

OBJECTIVES:

The major objective of this project is to investigate regulatory mechanisms operating in inflammatory and immune responses to modulate macrophage functions. Particular emphasis is placed on understanding interactions between macrophages and inflammatory microbial products and in interactions between macrophages and other lymphoid and non-lymphoid cells.

MAJOR FINDINGS:

During preceding years of this project several aspects of macrophage-endotoxin interactions were investigated. Basically it was found that E prostaglandins (PGE) regulate production of both colony-stimulating factors (CSF) and interferon by endotoxin-stimulated macrophages. Production of both mediators was suppressed by PGE. Preliminary evidence indicated that CSF also influences macrophage responses to endotoxin. CSF-cloned macrophages produced more interferon on a per cell basis than normal macrophages while normal macrophages preincubated with a source of CSF also responded in a hypersensitive fashion. A partially purified CSF preparation was also found to enhance LAF production by endotoxin-stimulated macrophages. Additionally crude CSF preparations by themselves were found to stimulate macrophages to produce LAF.

During the past year the roles of CSF in regulating macrophage functions have been more clearly defined. Summaries of our results with CSF are discussed as follows:

1. The factor in crude L cell (clone 929) supernatant preparations responsible for inducing LAF production by exudate macrophages was identified as CSF. Preliminary experiments using gel filtration techniques and antiserum prepared against partially purified L cell CSF had suggested that the active material was CSF. Confirmatory experiments were performed with L cell CSF that had been purified to homogeneity. Approximately 1,000 to 3,000 units of purified CSF were required to stimulate macrophages to produce LAF. Additionally preparations of partially purified lymphokine CSF also stimulated macrophages to produce LAF. Again this stimulatory capacity of lymphokine preparations required approximately 1,000 to 3,000 units of CSF activity to induce LAF production. Recently LAF has been found to be identical to endogenous pyrogen, a macrophage product that induces fever and a variety of effects caused by febrile reactions. Therefore, CSF which is produced by a variety of cells associated with inflammatory reactions, has the capacity to stimulate macrophages to produce an important inflammatory product with lymphostimulatory properties.

2. Since CSF was found to stimulate macrophages to produce LAF, the effect of CSF on a developing primary immune response was investigated. Addition of partially purified L cell CSF to murine spleen cells incubated with sheep red blood cells (SRBC) resulted in an enhanced anti-SRBC plaque forming cell response. This enhancement, however, was observed only in cultures containing suboptimal numbers of spleen cells. Addition of purified CSF to similar cultures containing optimal numbers of spleen cells resulted in a dose dependent suppression of the anti-SRBC

plaque response. This suppression was totally neutralized by addition of 2-mercaptoethanol (2-ME) to the cultures. Since 2-ME acts to reduce the toxic effects of oxygen metabolites produced in cultures, it is proposed that, in cultures with more closely associated cells, CSF stimulates production of oxygen metabolites by macrophages thereby suppressing the developing immune response. In cultures containing fewer macrophages, the enhancing effect of CSF, i.e. LAF production, overrides this suppressive effect.

3. Preliminary studies had shown that CSF-propagated macrophages become highly sensitive to endotoxin as measured by interferon production. Additonally CSF propagated macrophages were found to produce a soluble product that is toxic for tumor cells in vitro. Antigenically and physiochemically similar cytotoxic activities were found in the sera of endotoxin-hypersensitive mice (tumor necrosis factor) and in the supernatant media of macrophages from hypersensitive mice following treatment with LPS. These results suggested that CSF might sensitize macrophages to endotoxin. This was confirmed by exposing exudate macrophages to purified L cell CSF. CSF-pretreated macrophages produced significantly more interferon upon LPS challenge than control cells. This sensitizing activity required approximately 1,000 units of CSF, a dose similar to that required for CSF to induce LAF production. This effect of CSF, however, was found to be mediated by a secondary product of CSF-stimulated macrophages. Supernatants of CSF-treated macrophages contained low but significant levels of type 1 interferon. This macrophage produced interferon "primed" the cells in the cultures so that upon subsequent challenge with LPS or other interferon inducers there was an enhanced production of interferon above that of the non-CSF treated control macrophages. The enhancing effect of CSF could be replaced by substitution of type 1 interferon for CSF or neutralized by the addition to CSF-stimulated macrophage cultures of antiserum specific for type 1 interferon.

4. Additionally the effect of LPS on CSF-dependent macrophage proliferation has been investigated. Since LPS and infection with gram negative bacteria elicit enhanced production of CSF and alterations of myelopoiesis in vivo, this investigation was designed to determine if LPS might influence macrophage proliferation directly as well as indirectly by increasing CSF secretion. In the presence of optimal and supraoptimal concentrations of CSF, LPS significantly inhibited macrophage colony formation. This inhibition was not due to cytotoxicity, to the production of inhibitory E prostaglandins or to the production of "tumor necrosis factor". Preliminary experiments using anti-interferon and 2-mercaptoethanol have resulted in partial reversal of this suppressive effect of LPS suggesting that it may be related to the LPS-sensitizing effect of CSF discussed in section 3 above and also in section 2 with regard to the production of toxic oxygen metabolites. In the presence of suboptimal concentrations of CSF, LPS augmented rather than inhibited macrophage colony formation. This enhancing activity was apparent with as little as 10^{-7} µg/ml of LPS. Under certain conditions LPS apparently has the capacity to sensitize macrophages to CSF which may be important under

physiological conditions not only in enhancing the production of macrophages but also in promoting macrophage functions in an inflammatory response. A combination of elevated CSF and LPS levels, however, may both inhibit macrophage proliferation and at the same time promote destructive or suppressive activities of macrophages within an inflammatory lesion or infected site.

5. Macrophages produce a soluble mediator, fibroblast activating factor, (FAF) that stimulates fibroblasts to proliferate and secrete collagen. Since fibroblasts produce a material (CSF) that stimulates macrophages to produce active molecules, the effect of soluble fibroblast products on FAF secretion by macrophages has been investigated. Preliminary results indicate that fibroblasts do produce a soluble factor(s) that can stimulate macrophages to produce FAF. Additionally this stimulatory activity appears to be SF, although this identity has not yet been critically established. Regardless of the identity of the active fibroblast product, these results establish that a bidirectional communication exists between macrophages and fibroblasts that can potentially augment functions of both cell type.

Another portion of this project has been concerned with regulation of immunologically specific macrophage functions. In this regard, the genetically restricted antigen presenting function of macrophages has been investigated. The requirement for macrophage-T lymphocyte collaboration in induction of immune responses is regulated in part by specific glycoproteins (Ia-antigens) located on the membranes of a subpopulation of macrophages. Only Ia^+ macrophages are able to act as accessory cells in the activation of T lymphocytes. This investigation was designed to determine if T lymphocytes can influence Ia-antigen expression by macrophages and thus modulate the capacity of macrophages to participate in the induction of a specific immune response.

Supernatants from mitogen-stimulated spleen cell cultures induced a dose-dependent increase in the precentage of Ia^+ cells in cultures of peritoneal exudate macrophages. In the absence of active spleen supernatant, there was a decline in Ia^+ cells in similar cultures. The increase in Ia^+ cells in cultures treated with active supernatants was not due to adsorption of soluble Ia-antigen or to the selective outgrowth of Ia^+ cells but rather to a phenotypic conversion of Ia^- to Ia^+ cells. Macrophages treated with active splenic supernatants also stimulated the proliferation of allogeneic spleen cells signficantly better than control cells in a mixed leukocyte reaction (MLR). Pretreatment of supernatant cultured macrophages with anti-Ia and complement before addition of splenic responder cells abrogated their stimulatory capacity, indicating the Ia-dependence of the MLR. Based on these data, it is proposed that regulatory lymphokine(s) can induce both the expression of the Ia^+ phenotype by macrophages and the functional capability to stimulate activation of T lymphocytes, and that in the absence of such mediator(s) macrophages lose these capabilities.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

Macrophages occupy a central role in virtually all immune and inflammatory

responses. Besides ingesting and killing microbial organisms these cells also function to activate T lymphocytes and to secrete a variety of substance which have profound effects on surrounding tissues and other cells of the immune system. These secretory products can either augment host defenses and repair processes or under certain circumtances promote tissue destruction. The experiments described above represent attempts to ascertain regulatory mechanisms functioning to control macrophage activities. The findings can be divided into two major categories. First, the colony-stimulating factors, which are produced by a variety of cell types involved in immunological and inflammatory responses, serve to augment a variety of immunologically nonspecific macrophage functions. These factors which have classically been known as myelopoietins were found not only to enhance macrophage responses to bacterial endotoxin but also to directly stimulate secretion of active molecules by macrophages. Colony-stimulating factors, therefore, appear to play dual physiological roles functioning both to increase the macrophage/granulocyte population and to stimulate macrophage secretory functions. Under normal circumstances CSF may, therefore, function to enhance both host defense and repair processes. However, under conditions of antigen persistence or autoimmune diseases, CSF and secondary macrophage products may promote the destructive activities associated with chronic inflammation. Second, genetically restricted functions of macrophages expressed through the phenotypically Ia^+ macrophage subpopulation appear to be regulated by products of stimulated lymphocytes. These products may, therefore, be important factors in regulating the development of specific immune responses. A thorough understanding of these different molecular regulators of macrophage functions should provide a sound basis for manipulations designed to augment or depress selected aspects of the host's immune system.

PROPOSED COURSE:

The roles of CSF and interferon as regulators of macrophage functions will remain under investigation. The effect of interferon on macrophage secretory functions stimulated by LPS will be investigated with an emphasis placed on the stimulated secretion of LAF, CSF, FAF, prostaglandins, oxygen metabolites and tumor necrosis factor. This investigation is proposed to determine the role of interferon in pathological states characterized by macrophages that are hypersensitive to LPS. The role of CSF in influencing macrophage functions particularly in the primary antibody response and in fibroblast interactions will also remain under investigation. Additionally it has been found that a murine T cell line will produce large amounts of CSF upon proper stimulation. Since the CSF produced by T lymphocytes has not yet been isolated these cells may provide the large amounts of CSF required for purification of this molecule. During the upcoming year attempts will be made to characterize this T cell product biochemically and functionally and to compare it to that produced by normal T lymphocytes. The ultimate goal of this portion of the study will be the eventual isolation of the molecule.

With regard to studies involved with the phenotypic conversion of Ia^- to Ia^+ macrophages by supernatants of activated spleen cells, several lines

of investigation are proposed as follows: (i) cellular source (s) of the active molecules will be investigated, (ii) attempts will be made to characterize the molecule(s) biochemically, and (iii) the effect of the lymphokine on other Ia-dependent macrophage functions such as antigen presentation will be investigated.

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ting factor (interleukin 1) by macrophages activated with colony-stimulating factors. J. Immunol. In press.

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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-00242-03 LMI

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Role of Oxygen Radicals in Inflammation

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

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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)

We have defined some of the effects of oxygen radicals (superoxide, hydroxyl, and hydrogen peroxide) in inflammation, by using several in vitro models of inflammatory cellular interactions. We have examined the conditions which cause macrophages to become activated to produce oxygen radicals. We have shown that scavengers of oxygen radicals and other agents which protect responding lymphocytes from damage by oxygen radicals enhance both proliferative and differentiative functions of murine lymphocytes, in vitro. Similarly, agents which stimulate oxygen radical production by macrophages cause suppression of lymphocyte functions unless additional protective mechanisms are supplied. We have shown that agents present in chronic inflammation (bacterial endotoxin, cytokines) activate macrophages to release oxygen radicals. We have demonstrated an intimate relationship between the production of oxygen radicals and prostaglandins. In studies of patients with either Hodgkin's disease or chronic periodontitis, we have shown the presence of macrophages which produce oxygen radicals and prostaglandins, thereby suppressing lymphocyte responses. Definition of the role of oxygen radicals in chronic inflammation can lead to modulation of both the tissue destruction and immune response.

Histological and pathological examinations of chronic inflammatory sites, such as those present in periodontal disease, have shown not only that the cellular infiltrate deals inadequately with the noxious stimuli present (e.g., endotoxin from dental plaque bacteria), but also that the inflammatory cells may contribute to the tissue damage. Since one of the potentially immunosuppressive and tissue destructive processes which tissue macrophages are capable of expressing at inflammatory sites is the production of oxygen radicals, we are studying the role of oxygen radicals in those processes. The murine primary antibody response is an in vitro system which requires the interaction of antigens, T cells, B cells and macrophages. Any deleterious effect on any component of the system leads to suppression of the final response. We have shown that the content of the oxygen radical scavenger, reduced glutathione, in the serum used in cell cultures is critical for the support of the response of those cells. Similarly, the enhancement of the antibody response by 2-mercaptoethanol is mediated by its augmentation of the availability of reduced glutathione in cultures. This enhancement effect of 2-mercaptoethanol is mimicked by inhibitors of cell membrane lipid peroxidation and is additive with suboptimal doses of those agents.

Macrophages which have been activated in vivo by killed bacterial cells are capable of suppressing the proliferation of normal mouse spleen cells responding to the mitogen concanavalin A. We have shown that this suppression can be totally reversed by the addition of both the oxygen radical scavenger, catalase, and the prostaglandin synthesis inhibitor, indomethacin. Thus, we have implicated both oxygen radicals and prostaglandins as mediators of the observed suppression. By direct measurements, we have confirmed the observation that activated macrophages produce significant quantities of both prostaglandins and oxygen radicals. We are currently defining the apparent feedback regulation of these two mediator groups upon the production of each other.

We have found that normal macrophages which have not been activated in vivo can be treated in vitro so as to express the characteristics of activated macrophages. Treatment of macrophages with either bacterial endotoxin or lymphokine-rich supernatants can develop the potential of those cells to generate oxygen radicals. Similarly, we have shown that the presence of non-degradable, phagocytosable particles or soluble stimulators of hexose monophosphate shunt activity causes suppression of lymphocyte responses; the suppression is totally reversible by scavengers of oxygen radicals.

In Hodgkin's disease a lymphoma of humans, the proliferation of lectin-stimulated peripheral blood lymphocytes is severely depressed. In collaboration with Dr. Richard DeShazo, we have found that this suppression is attributable to macrophages in the responding cell population; furthermore, this suppression is partially reversible by the addition of both oxygen radical scavengers and indomethacin.

In collaboration with Dr. Philip Fox, we have found that in certain cases of advanced periodontal disease of humans, peripheral blood lymphocyte

proliferation is also depressed. Again, this suppression is reversed by the removal of activated macrophages or the treatment of the cultures with oxygen radical scavengers and indomethacin. Thus, the mechanisms we have been studying, in vitro, appear to have importance in pathological conditions, in vivo.

OBJECTIVES:

1. Demonstrate the deleterious effects on mammalian cells of spontaneously generated oxygen radicals.

2. Delineate the interactions of prostaglandins and oxygen radicals in inflammation.

3. Define both effective stimulants for the production of and scavengers against the effects of oxygen radicals.

4. Modulate the negative effects of oxygen radicals by the use of agents which either neutralize the activity or stimulate the production of oxygen radicals.

5. Apply these same research objectives to the study of pathological processes such as periodontal disease.

METHODS EMPLOYED:

1. In vitro model systems:
   a. Primary antibody response of murine spleen cells to erythrocyte antigens.
   b. Mitogen-induced and antigen-induced proliferation of murine spleen cells or human peripheral blood leukocytes.
   c. Monolayers of mouse peritoneal exudate cells grown in scintillation vials for chemiluminescence determination.
2. Assay for glutathione: Spectrofluorometric methods.
3. Assay for primary antibody response: Direct (IgM) plaque forming cells measured by complement-mediated, antibody-dependent hemolytic assay.
4. Assay for lymphocyte proliferation: cultured cells were pulsed with tritiated thymidine, cultured for an additional period and radioactivity was measured on a scintillation counter.
5. Assay for prostaglandins: radioimmunoassay specific for class E prostaglandins.
6. Assay for oxygen radicals: Cells were cultured in scintillation vials; when the chemiluminescence enhancer, luminol, is present, stimulated or non-stimulated oxygen radical production can be measured on a scintillation counter.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

The interactions of responding immune cells with each other or with neighboring cell types have been shown to involve a number of relatively long-lived, isolable mediators (e.g., lymphokines, monokines, stable

prostaglandins). The involvement of relatively short-lived mediators (e.g., oxygen radicals, thromboxanes, prostaglandin endoperoxides, prostacyclin) has only recently become apparent. Oxygen radicals have been implicated in such in vivo conditions as rheumatoid arthritis and hyperuricemic arthritis as well as in vitro models of killing by immune cells. These mediators not only can affect surrounding cells types, but also can modulate the activity of other responding cells. Thus, an understanding of the mechanisms whereby oxygen radicals interact with various cells holds the promise of modulation of both immune reactivity and tissue damage in chronic inflammatory conditions such as granulomata and periodontal disease.

PROPOSED COURSE:

We propose to continue these studies in several different ways:
1. Using the primary antibody response and mitogen-induced lymphocyte proliferation, we will investigate the possibility that oxygen radicals might also be short-lived positive signals of cellular interaction.

2. Using the chemiluminescence assay we will investigate the mechanism of macrophage activation and its modulation by a number of pharmacological and biological agents.

3. Using the antigen- and mitogen-induced lymphocyte proliferation assay, we will expand the base of our periodontal disease study and investigate the relationship of specific immune defects to periodontal condition.

PUBLICATIONS:

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2. Hoffeld, J.T. and Oppenheim, J.J. 1980. Enhancement of the primary antibody response by 2-mercaptoethanol is mediated by its action on glutathione in the serum. European Journal of Immunology, 10:391-395.

3. Metzger, Z., Hoffeld, J.T. and Oppenheim, J.J. 1980. Macrophage-mediated suppression: I. Evidence for participation of both hydrogen peroxide and prostaglandins in suppression of murine lymphocyte proliferation, Journal of Immunology, 124:983-988.

4. Hoffeld, J.T. and Farrar, J.J. 1980. The characteristics, functions, and interactions of macrophages, T cells and B cells in the humoral immune response. In: Dental Microbiology. J. McGhee and S. Michalek (eds.) J.B. Lippincott. New York. (in press).

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8. Moore, R.N., Hoffeld, J.T., Farrar, J.J., Mergenhagen, S.E., Oppenheim, J.J. and Shadduck, R.K. 1981. Colony-stimulating factors: myeloproliferative agents with macrophage stimulating activities. In: Lymphokine Reports, vol.3. E. Pick (ed.). Academic Press. 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-00261-02 LMI

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Signal Requirements for Lymphocyte Activation

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

Weinblatt, Anita Corman	Post Doctoral Fellow	LMI NIDR
Oppenheim, Joost J.	Medical Director	LMI NIDR
Jenkins, Mark	Bio Lab Technician	LMI NIDR

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Cellular Immunology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.12	.62	.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 induction of T-lymphocyte activation requires one signal from an exogenous agent such as an antigen plant lectin or allogeneic cell and a second signal from a macrophage. Phorbol myristic acetate (PMA) enables macrophage depleted T cells to proliferate in response to a mitogenic but not an antigenic stimulus. Since the macrophage signal is replaced by PMA, we can more readily study the other signal required for activation. We have demonstrated that culture supernatants of peritoneal exudate lymphocytes (PELS) pulsed with an antigen have mitogenic activity only in the presence of PMA. This factor has the following characteristics. There is a restriction for antigen specificity at the production end of this T-cell mitogenic factor (TMF) since supernatants of PELS have mitogenic activity only when pulsed with an antigen with which the donor guinea pig has been primed. There appears to be no restriction for antigen specificity at the effector end of this TMF activity since an active supernatant is able to induce mitogenic activity on PMA treated T cells of unimmunized animals. We plan to further characterize the nature of this factor, which resembles T cell growth factor and will begin the biochemical characterization of this factor.

To understand the molecular signals required to activate T cells.

MAJOR FINDINGS:

The induction of T-lymphocyte activation in vitro is believed to require two signals: one from the interaction of an exogenous agent, such as an antigen, plant lectin or an allogeneic cell; the second from the interaction with an accessory cell such as a macrophage. The accessory cell mediated signal can be replaced by a macrophage-derived soluble factor such as lymphocyte activating factor (LAF). In our previous progress report we described how the synthetic compound phorbol myristic acetate (PMA) was able to substitute for macrophages as a source of one of the activating signals in the mitogenic activation of highly purified guinea pig lymphocytes.

In contrast to its effects on mitogenic stimulation, PMA was unable to restore the response of T cells to DNP-OVA, the antigen to which the donor guinea pigs had been immunized. This result was not too surprising. Recent evidence has indicated that macrophages may have two functions in the activation of antigen primed T cells: (1) presentation of antigen in an immunogenic form and (2) production of a factor, LAF which somehow drives the antigen specific response. The presentation function is genetically restricted in that the macrophage and T cell must be histocompatible in the Ia region of the MHC while the actions of LAF are antigen non-specific and genetically unrestricted. Thus, although PMA might be able to replace the non-specific macrophage signal, viable macrophages must generate an additional antigen specific signal. We therefore looked in our system for some form of "macrophage processed antigen" that would induce T cell proliferation in the presence of PMA. If identified, we were eager to know whether the material would contain antigen or Ia determinants and/or if there would be genetic or antigenic restriction in its effect.

Macrophage monolayers made from mineral oil induced peritoneal exudate cells of immunized guinea pigs were pulsed with antigen, washed one time and cultured for 4 to 48 hours. In the presence of PMA, T cells treated with supernatants from antigen pulsed macrophages proliferated as well as T cells stimulated with antigen and intact macrophages. This activity was maximal at 4-24 hours of culture and was detectable for up to 48 hours.

In the next series of experiments, we changed the protocol by washing the macrophage monolayers four times before and after pulsing with antigen. We found that after this procedure very little or no activity was found in the macrophage supernatants. We postulated that this extensive washing might be eliminating a non-adherent cell (probably a T cell) needed for generating the activity. We next isolated lymphocytes from peritoneal exudate cells (PELS), pulsed them with antigen for 1 hour and looked for activity in the supernatants of these cells. We found that when these supernatants were tested on PMA treated T cells they had about five times more activity than supernatants generated on macrophage monolayers. Antigen-pulsed PEL supernatant had no activity in the absence of PMA. The time course of appearance of activity in antigen-

pulsed PEL supernatants was the same as that generated on macrophage monolayers. There is a restriction for antigen specificity at the production end of this T-cell mitogenic activity; supernatants of PELS have mitogenic activity only when pulsed with an antigen with which the donor guinea pigs have been primed. We have preliminary evidence to indicate that there is no restriction for antigen specificity at the effector end of this T cell mitogenic activity. Thus, an active supernatant was able to induce mitogenic activity on PMA treated T cells of unimmunized as well as immunized guinea pigs.

The nature of this T cell mitogenic activity remains to be established. The fact that active supernatants have T cell mitogenic activity on unimmunized guinea pig T cells would argue against the possibility that we are measuring "processed antigen", but rather that we are looking at a T cell mitogenic factor similar to the mouse T-cell growth factor, IL-2.

SIGNIFICANCE TO BIOMEDICAL RESEARCH:

The process of T cell activation is fundamental to almost all immune reactions. In order to more fully understand the immune process it is important to identify and characterize these factors. This will aid in the development of pharmacological agents which can specifically modulate immunity in certain disease states. Our results to date indicate that PMA will be extremely valuable for elucidating the biochemical alterations involved in the process of T cell activation.

PROPOSED COURSE:

Further characterization of the guinea pig T cell mitogenic factor. We plan to further characterize the nature of the T cell mitogenic activity found in the supernatants of antigen pulsed PEL preparations. First, we will confirm our preliminary observation that active supernatants are mitogenic for unimmunized guinea pig T cells. Thus, T cells which were immunologically naive of the antigen which stimulated the production of the mitogenic activity were still able to respond to the mitogenic activity. From this we can conclude that there is no restriction for antigen specificity at the effector end of the T cell mitogenic activity.

Next, we will determine if there is any genetic restriction in the system. We will test to see if the cells producing the activity must be histocompatible in the Ir region with the cells responding to the activity. If such a restriction exists, we will do blocking experiments with the appropriate anti-Ia antisera.

We also plan to test active supernatants on guinea pig thymocytes to determine if they contain guinea pig LAF. As a positive control we will generate guinea pig LAF by treating macrophages with LPS or PMA and test it on guinea pig thymocytes. In addition we will test the supernatants for T cell growth factor (TCGF) activity by measuring proliferation of a TCGF-dependent cell line.

Biochemical characterization of the guinea pig T cell mitogenic factor. We also plan to begin the biochemical characterization of the mitogenic

factor contained in active supernatants. First we will determine if we can generate the factor in the absence of serum. This is an intrinsic purification step. Then will attempt to determine the molecular weight of the factor using appropriate gel filtration columns. We also plan to utilize DEAE and hydroxylapatite column chromatography.

PUBLICATIONS:

Weinblatt, A.C., S.N. Vogel Vogel and D.L. Rosenstreich. Depletion of macrophages by nylon or rayon wool. In Manual of macrophage methodology: Collection, characteristic, and function. J.B. Herscowitz et al (eds.). Marcel Dekker Inc., 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 Z01 DE-00308-01 LMI

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Genetic Control of Resistance to HSV Infection in Mice

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

Weinblatt, Anita Corman	Post Doctoral Fellow	LMI NIDR
Oppenheim, Joost J.	Medical Director	LMI NIDR
Jenkins, Mark	Bio Lab Technician	LMI NIDR

COOPERATING UNITS (if any)
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LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Cellular Immunology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.12	.62	.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)

Resistance to herpes simplex virus-Type 1, the causative agent of acute gingivostomatitis and recurrent herpes labialis is under polygenic control. Using recombinant inbred strains, we have already identified one major gene responsible for resistance which appears to be on Chromosome 1, linked to the gene which controls expoxide hydrase. We are currently investigating the number, location and resistance mechanisms controlled by these genes. We plan to confirm this linkage in other inbred strains and by formal backcross analysis. The identification of this resistance gene will allow us to better study the role of the immune response in limiting infection by Herpes viruses. Results from these studies will aid in prevention and therapy of HSV infections in man.

OBJECTIVES:

The objective of this project is to identify and map the genes that control resistance to herpes simplex virus-Type 1, which is involved in oral disease. Once identified, we can determine the mechanisms by which these genes control immunological processes.

MAJOR FINDINGS:

Resistance to HSV-1 is believed to be under polygenic control and we have made significant progress in the identification of a major gene responsible for resistance to HSV-1 infection in mice. First, we screened a battery of inbred strains for their ability to resist fatal HSV-1 infection, including the progenitor strains of all presently available recombinant inbred lines. The lethal dose required to kill 50% of the test animals was calculated for each strain (LD_{50}). The results showed that the strains appear to distribute into three catagories: very susceptible, moderately susceptible and resistant. Thus, we confirmed that resistance to HSV-1 was under polygenic control.

We next employed the B x D set of recombinant inbred mouse strains in order to map the number and chromosomal location of resistance genes. The progenitor strains of this set are DBA/2J which is very sensitive to HSV-1 infection and C57Bl/6J which is very resistant. Each gene has a unique strain distribution pattern. We typed each strain for resistance to HSV-1 and compared the strain distribution for HSV-1 resistance to strain distribution patterns of other genes which had previously been catalogued. If a gene shows the same strain distribution pattern as a gene already known the two genes may be linked.

In a series of experiments with the B x D strains, we have established the possible association of a major gene controlling HSV-1 resistance with a number of marker genes on chromosome 1. These genes are: the gene which controls the pH optimum of the drug metabolizing enzyme, epoxide hydrase (Eph-1, proposed gene symbol); the gene which determines the presence of Ly 9; and the Mls locus, the major lymphocyte activating determinant outside the major histocompatibility complex (MHC) of the mouse. The association of the Herpes resistance gene with Eph-1 held in 17 out of 22 B x D strains; the exceptions were B x D 14, 15, and 24 which were as susceptible to HSV-1 as the DBA parent but have the C57Bl/6 form of Eph-1 and B x D 2 and 29 which were as resistant to HSV-1 as the C57Bl/6 parent but have the DBA form of Eph-1. The linkage of the Herpes resistance gene to another chromosome 1 marker, Ly 9, showed the same pattern. The exceptions were B x D 14 and 15 which have the C57Bl/6 form of Ly 9 but were sensitive to HSV-1 and B x D 2 and 29 which have the DBA form of Ly 9 but were resistant to HSV-1. Thus, B x D 14, 15, 24, 2 and 29 are recombinants in the region of chromosome 1 where these genes are located.

Individuals vary in their susceptibily to infectious diseases including those caused by oral agents such as HSV-1. The identification of a major gene which controls resistance to HSV-1 will allow us to better study the role of the immune response in limiting infection by Herpes viruses. Results from these studies will aid in prevention and therapy of HSV infections in man.

PROPOSED COURSE:

1. Genetic linkage. In order to obtain further evidence for the linkage of the Herpes Resistance Gene to chromsome 1 marker genes, we are currently testing the C x B recombinant inbred line. These strains were derived from a Balb/cBy parent which is sensitive to HSV-1 and a C57Bl/6By parent which is resistant to HSV-1 infection. We expect that the strain distribution pattern of the Herpes resistance gene in these strains will confirm the gene's association with chromosome 1 marker genes.

We also plan to test some B6.C congenic animals. B6.C-H-25^{c} line carries the H-25 histocompatibility allele of the Balb/cBy on a C57Bl/6 background. It is known that H-25 is located in the region of chromosome 1 where we believe the Herpes resistance gene is located. If the Herpes resistance gene is very close to H-25, it might have been transferred with it. Thus we would expect that this line would be sensitive to HSV-1 infection.

Finally, we will confirm the gene linkages by a formal backcross linkage analysis. F_1 hybrids from susceptible and resistant parents will be backcrossed to susceptible parents. The concordant inheritance of HSV-1 resistance and the genes believed to be linked to it will confirm the proposed linkage. The recombination strength (map distance) and gene order will also be determine.

2. Mechanism of gene action. We also plan to begin to look at the mechanism of action of the Herpes resistance gene we have identified. Although inheritance of resistance as measured by death is polygenic, a cellular expression of resistance may exist in vitro and may be controlled by the Herpes resistance gene we have identified.

First we plan to monitor changes in the serum interferon (IF) levels in response to HSV-1 using a vesicular stomatitis virus plaque reduction assay. If fruitful, an attempt will be made to identify the cell types producing interferon by in vitro cultures of lymphocytes, macrophages, or fibroblasts.

We will also investigate whether this gene controls some aspect of lymphocyte or macrophage function using a variety of in vitro techniques. In both infected and HSV-immunized mice, the action of macrophages will be determined by examining the ability of HSV to replicate the these cells and the ability of HSV to induce macrophage stimulation as measured morphologically, enzymatically and by the enhanced production of monokines. Lymphocyte function will be determined by investigating HSV-antigen induced proliferation and production of lymphokines by these cells.

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-00046-09 LMI

PERIOD COVERED

October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Role of Lymphocyte and Macrophage Mediators in Inflammation

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

Wahl, Sharon M.	Research Microbiologist	LMI NIDR
Wahl, Larry M.	Research Biologist	LMI NIDR
McCarthy, James B.	Biologist	LMI NIDR
Tsukamoto, Yoshio	Visiting Fellow	LMI NIDR
Helsel, William	Microbiologist	LMI NIDR

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Microbiology and Immunology, NIDR

SECTION

Humoral Immunity Section

INSTITUTE AND LOCATION

NIDR/NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.25	1.75	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)

The close proximity of lymphoid components and fibroblasts in inflammatory sites implicates these lymphocytes and macrophages in the regulation of fibroblast function. Our current investigations deal with how the cellular interactions between the lymphoid system and fibroblasts may regulate connective tissue metabolism in both normal tissue repair and in pathological conditions associated with inflammatory reactions. Macrophages can be activated by a variety of agents including endotoxin, muramyl dipeptide or lymphokines to produce biologically active molecules (monokines). One of these monokines can cause fibroblasts to actively migrate or chemotax. Another monokine produce by activated macrophages stimulates fibroblasts to divide. These fibroblasts also become metabolically activated, generating enhanced levels of prostaglandins, collagen and proteins. In addition to the macrophages, inflammatory lesions may contain lymphocytes which when stimulated by specific antigens also influence fibroblast functions by the release of soluble mediators (lymphokines). Lymphocyte and macrophage products may thus provide a molecular link between the inflammatory response and the subsequent fibroplasia and collagen deposition which accompany inflammation.

OBJECTIVES:

The ongoing research in this laboratory focuses on characterization of the mechanisms by which the immune system may modulate connective tissue metabolism. 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 degradation of collagen and the connective tissue matrix and whether these same cells could direct the repair of this tissue injury following the inflammatory response. The repair of tissue injury, which may be extensive in chronic inflammation, can result in irreversible replacement of the original tissue by collagen. This collagen production and fibrosis then not only serve to repair the damaged tissue, but may also cause fibrotic organ damage leading to organ dupfunction. Because of the participation of fibroblasts in the final outcome of many inflammatory lesions, it is important to understand what regulates their activities under these circumstances. To this end we have attempted to investigate whether macrophages and lymphocytes modulate the fibrotic response by the release of inhibitory or facilitatory soluble signals.

METHODS EMPLOYED:

Methods utilized to investigate the aforementioned objectives include already published procedures for macrophage chemotaxis, lymphocyte culture and proliferation, lymphokine production, prostaglandin assay, cyclic AMP and GMP determinations, macrophage culture and monokine production, fibroblast culture, fibroblast chemotaxis, analysis of collagen formation, isolation and culture of hepatic Kupffer cells, antibody production and column chromatography.

MAJOR FINDINGS:

Lymphocytes exposed to specific antigen respond by producing numerous effector molecules. One such recently described molecule, fibroblast activating factor (FAF), exerts its effect on quiescent fibroblasts by causing these cells to proliferate and to increase their synthesis of protein. T lymphocytes from dinitrophinylated ovalbumin (KNP-OA) immunized guinea pigs, when challenged with the antigen in vitro produced significant amounts of this mediator within 24-48 hr. Addition of these supernatants to subconfluent cultures of guinea pig dermal fibroblasts in serum-free medium resulted in fibroblast proliferation. This enhanced fibroblast proliferation appeared to be stimulated by a molecule of 40-60,000 daltons and by a smaller molecule (10-15,000 daltons) contained within the lymphocyte supernatants. Lymphocyte-derived mediators can also modulate other fibroblast activities, including enhanced prostaglandins synthesis and elevations in intracellular cyclic nucleotide (cAMP) levels. A significant increase in protein synthesis of both the collagen and noncollagenous types occurred after fibroblast exposure to the lymphocyte derived products.

In addition to T cells, guinea pig peritoneal macrophages stimulated with lipopolysaccharide or muramyl dipeptide produced a soluble factor (monokine) which activated fibroblasts to proliferate. This factor is nondialyzable, heat stable at 56°C and labile at 100°C and has a molecular weight of 40-50,000 daltons. The proliferative factor adsorbs to hydroxylapatite and appears to be acidic by its ability to adsorb to diethylaminoethyl cellulose columns. Additionally, activated macrophages produce a molecule which is chemotactic for fibroblasts. This larger molecular weight protein does not increase random migration but is responsible for directed migration of the fibroblasts. The production of this chemotactic factor appears to be prostaglandin regulated since indomethacin inhibits its appearance in macrophage cultures. The macrophage derived chemotactic factor is distinct from the fibroblast proliferative factor since it has a molecular weight greater than 80,000 daltons. This factor is a trypsin-sensitive protein which adsorbs to hydroxylapatite. Macrophages also produce the glycoprotein, fibronectin, which may be the molecule in the macrophage supernatants which is chemotactic for fibroblasts. Studies are in progress to determine whether a single or multiple macrophage products may be involved in attracting fibroblasts. Fibronectin appears to have other fibroblast regulatory functions which are being explored. Monoclonal antibodies against fibronectin are being prepared to characterize the role of this important connective tissue constituent in regulating both fibroblast and macrophage functions. It thus appears that both macrophages and lymphocytes when appropriately activated release soluble molecules that can regulate fibroblast function. These fibroblast activating factors may contribute to the fibrotic reactions associated with chronic inflammatory lesions.

Further investigation into the mechanisms of macrophage activation revealed that muramyl dipeptide, lipopolysaccharide and lymphocyte-derived macrophage activating factor which induced fibroblast activating factor production also activated the macrophages to produce collagenase. Collagenase production was dependent upon an increase in prostaglandin synthesis which in turn elevated intracellular levels of cAMP. Impairment of this obligatory change in cAMP inhibited enzyme synthesis, whereas facilitating the elevations in cAMP within the macrophage caused greater collagenase synthesis. These findings suggest potential regulatory mechanisms in the control of collagenase synthesis and consequently in the control of connective tissue degradation.

In light of our findings that lymphocytes and macrophages can influence fibroblast function, we explored an *in vivo* model of delayed hypersensitivity for 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.

Furthermore, within the liver is a population of resident phagocytic cells, the Kupffer cells, which may be involved in the regulation of fibroblast function and therefore liver fibrosis. Isolation and culture of these sinusoidal lining cells suggest that they too may release monokines and thus provide another pathway in the induction of pathophysiologic fibrosis.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

Cellular immune phenomena are the basis for the development of many chronic inflammatory lesions. There are certain pathologic conditions including scleroderma, pulmonary fibrosis, and schistosomiasis which have been characterized as cell-mediated which lead to excessive connective tissue formation or fibrosis. It is possible that the changes in the connective tissue could be mediated by the local production and release of lymphokines by antigen-activated lymphocytes. Lymphokines appear to regulate fibroblast function suggesting a pathway for enhanced connective tissue metabolism including collagen formation. Additionally, macrophages which are a predominant cell in these inflammatory loci can generate chemotactic signals responsible for the mobilization and recruitment of fibroblasts. Release of additional monokines stimulates fibroplasia and connective tissue deposition. Ultimately, collagen production and fibrosis terminates the inflammation. Whereas fibrosis is a normal repair process following tissue injury, in chronic inflammation, such as that found in granulomatous diseases, the irreversible fibrosis may lead to organ damage with a potentially fatal outcome. Activated lymphocytes may also 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 other cells. If continuously activated as in chronic inflammatory lesions like periodontal disease and rheumatoid arthritis, these cells may contribute to the pathologic tissue destruction by the release of lysosomal enzymes, collagenase, and prostaglandins. Thus, through an understanding of the way these cells are triggered, it may be possible to control or modulate their function.

PROPOSED COURSE:

Investigations are continuing into the mechanisms of induction of fibrosis associated with inflammation which in some cases may progress to chronic pathologic lesions. Areas of study will focus on the interaction between the lymphoid system and connective tissue components. Lymphocytes and macrophages appear to be able to influence fibroblast growth and thereby may provide a pathway for normal connective tissue repair 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 vitro* model and also in the *in vivo* model of schistosomiasis. Characterization of the soluble mediators

produced by the lymphocytes and macrophages will be correlated with the mediators released from active granulomas. The role of fibronectin produced by macrophages will also be explored both in the regulation of fibroblast function and in its mechanism of production by macrophages. The production of monoclonal antibodies will facilitate these investigations. That the same types of inflammatory cells are involved in lesions characterized by destruction as are in those characterized by excessive connective tissue formation is clear, but it is not known what mechanisms tip the balance in favor of one or the other. According to this dual role, inflammatory cells may contribute to tissue destruction and yet may also be critical in the initiation of fibroplasia and the remodelling of the fibrotic tissue. Regulatory disturbances of inflammatory cell function may create an exaggeration of one or the other role. Continuing research appears to be essential in this area to understand the immunologic pathogenesis of these and other connective tissue disorders.

Efforts will also be directed toward the identification of antagonists and / or promoters of monokines and lymphokines at the level of lymphocyte-macrophage interactions and at the level of their interaction with fibroblasts. Such information would have potential application in controlling the pathological manifestations of aberrant immunological regulation of connective tissue metabolism.

PUBLICATIONS:

1. McCarthy, J.B., S.M. Wahl, J. Rees, C.E. Olsen, A.L. Sandberg and L.M. Wahl. 1980. Regulation of macrophage collagenase production by 3' -5' cyclic adenosine monophosphate. J. Immunol. 124:2405.

2. Wahl, S.M. 1980. Inflammation and Wound Healing. In The Cell Biology of Immunity and Inflammation. (J.J. Oppenheim, D. Rosenstreich and M. Potter eds.). Elsevier North Holland Biomedical Press, New York. in press.

3. Wahl, S.M. and L.M. Wahl. 1979. Lymphokine modulation of connective tissue metabolism. Annals N.Y. Acad. Sci. 1979. 332:411.

4. Wahl, L.M., C.E. Olsen, S.M. Wahl, J.B. McCarthy, A.L. Sandberg, and S.E. Mergenhagen. 1979. Prostaglandin and cAMP regulation of macrophage involvement in connective tissue destruction, Annals N.Y. Acad. Sci. 332:271.

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6. Wahl, S.M., L.M. Wahl, and S.E. Mergenhagen. 1980. Lymphokine and monokine regulation of fibroblast function. In Biochemical Characterization of Lymphokines. Proc. of the Second Intl. Lymphokine Wordshop (A.L. deWeck, ed.) Academic Press, p.267

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10. Wahl, S.M. 1980. The role of mononuclear cells in the wound repair process. In The Biology and Management of Surgical Wounds (Shires, J.T. ed.) Lea & Febiger, Philadelphia. 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-00061-07 LMI

PERIOD COVERED
October 1, 1979 - September 30, 1980

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

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Wahl, Larry M.	Research Biologist	LMI NIDR
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COOPERATING UNITS (if any)

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LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	.75	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)

The mechanisms by which the complement system participates in the inflammatory response and the regulation of connective tissue metabolism continue to be examined. Several aspects of inflammation as well as resorption of bone are controlled by prostaglandins and current studies focus on the role of complement in the production of these mediators. In organ cultures of bone, complement activation initiated by antibodies reactive with cell surface antigens stimulates the synthesis of prostaglandins with resultant resorption of the bone. This effect is attributed to a macrophage-like cell present in the bone since prostaglandin production in cultures of peritoneal exudate macrophages is stimulated by antibody and rabbit complement. In the presence of the prostaglandin precursor, arachidonic acid, the synthesis of prostaglandins is markedly elevated, an effect which is inhibited by both steroid and non-steroid anti-inflammatory agents. Guinea pig and human sera are less effective complement sources. However, the stimulatory effects of complement from these species can be enhanced by the enzymatic removal of the sialic acid residues from the macrophage membrane, a process which favors activation of the alternative complement pathway.

PHS-6040

OBJECTIVES:

These studies are designed to further define the biological functions of the complement system in inflammation. Current emphasis is directed towards delineating the mechanisms by which complement activation at a cell surface results in the stimulation of prostaglandin synthesis in cultures of organs and cells and the modulation of prostaglandin production by pharmacological agents. The interactions of the individual components of the complement system with cell membranes which result in prostaglandin synthesis will be explored as will the effects of alteration of cell surface constituents on complement-mediated stimulation of prostaglandin production. The role of the complement system in inhibiting bone growth is also under investigation. Prostaglandins are apparently not involved in this process and attempts will be make to define the mechanism(s) by which complement activation is detrimental to the growth of bone, the complement components required and the possible alleviation of this effect. Other functions of the complement system which may influence macrophages and lymphocytes, such as mediator production, will be examined.

METHODS EMPLOYED:

Established techniques are used for tissue culture. Bone resorption is quantitatively assayed by determining the ^{45}Ca released into the media of fetal rat bone organ cultures. Prostaglandins are detected by specific radioimmunoassay. Bone growth is assayed by incorporation of ^{3}H proline into collagenase sensitive and collagenase resistant protein in rat calvaria in organ culture.

MAJOR FINDINGS:

Complement activation in organ cultures of fetal rat bones stimulates the synthesis of protaglandins by a cell present in the bone resulting in bone resorption. Rat and guinea pig peritoneal exudate macrophages, cells of similar origin to the osteoclasts which have been implicated in bone resorption, synthesize prostaglandins in the presence of normal rabbit serum and species specific antierythrocyte $F(ab')_2$ immunoglobulin fragments. Prostaglandin production is markedly decreased if the complement activity in the normal rabbit serum is destroyed by heating or the antibody fragments are omitted from the cultures. This effect is inhibited by the steroid anti-inflammatory agent hydrocortisone, and the non-steroid anti-inflammatory agents, indomethacin, RO-20-5720 and flufenemic acid. One mechanism by which complement exerts its effect on the production of prostaglandins by macrophages is by enhancing the incorporation of the prostaglandin precursor, arachidonic acid, into prostaglandins. The

stimulation of complement-dependent prostaglandin production by arachidonic acid is inhibited by the previously mentioned steroid and non-steroid anti-inflammatory agents, indicating that the effects of these drugs are due, at least in part, to the inhibition of prostaglandin precursor incorporation into prostaglandins. In view of the known effects of complement on cell membranes, it is likely that activation of this mediator system alters the cell membrane sufficiently to allow increased accessibility of prostaglandin precursors to the enzymes involved in prostaglandin biosynthesis.

The complement from rabbits genetically lacking the sixth component does not support complement-dependent synthesis of prostaglandins by macrophages. The complement components from this species are not available so the effects of other complement sources on prostaglandin production have been explored. Guinea pig and human complement, the components of which are available or can be readily purified are minimally effective. However, guinea pig complement does stimulate prostaglandin synthesis in the presence of arachidonic acid. These findings will permit future evaluation of the contributions of the individual components of the complement system to macrophage activation resulting in the production of prostaglandins as will as other inflammatory mediators.

Alterations of the composition of cell membranes or the cell surface contituents influence the susceptibility of the cell to the actions of complement. Therefore, macrophages were subjected to mild treatment with neuraminidase, a process which apparently favors activation of the alternative pathway. Enhanced levels of prostaglandins were synthesized by the enzymatically treated macrophages when cultured in the presence of antibody and complement. These findings suggest that membrane bound sialic acid may serve a regulatory role in the complement-mediated production of macrophage derived prostaglandins.

SIGNIFICANCE:

In certain areas of inflammation such as those which are found in periodontal disease and rheumatoid arthritis, fragments of components of the complement system and elevated levels of prostaglandins have been detected. These findings suggested that complement activation might be involved in initiating prostaglandin synthesis and thus mediated several aspects of inflammation, including the destruction of connective tissue, by this mechanism. The demonstration that complement activation does result in the destruction of bone in organ culture and, in addition, stimulates prostaglandin synthesis in bone and macrophage cultures implicates the complement system in those pathological events which are mediated by prostaglandins.

PROPOSED COURSE:

Studies will be continued to investigate the mechanisms by which immunological activation of the complement system influences several aspects of inflammation, including abnormal connective tissue metabolism. In investigations of bone resorption and stimulation of prostaglandin synthesis in macrophage cultures, isolated components of complement will be utilized to define their interaction with cell membranes. The specificity of the antibodies required for initiation of these complement-dependent events as well as the effects of alteration of cell surface components will be examined. Complement interaction with macrophages and other cells will also be examined to determine if this type of cellular stimulation can result in the production of mediators other than prostaglandins. A possible defect in prostaglandin production by complement stimulated macrophages from osteopetrotic mice and rats will also be explored as well as the effects of complement and antibody on resorption of bones from these animals and their normal littermates.

PUBLICATIONS:

1. McCarthy, J., S.M. Wahl, J.C. Rees, C.E. Olsen, A.L. Sandberg and L.M. Wahl. 1980. Regulation of macrophage collagenase production by 3'5' cyclic adenosine monophosphate. J. Immunol. 124: 2405.

2. Wahl, L.M., C.E. Olsen, S.M. Wahl, J.B. McCarthy, A.L. Sandberg and S.E. Mergenhagen. 1979. Prostaglandin and cAMP regulation of macrophage involvement in connective tissue destruction. Annals N.Y. Acad Sci. 332: 271.

3. Wahl, L.M., J.B. McCarthy, C.E. Olsen, S.M. Wahl, A.L. Sandberg and S.E. Mergenhagen. 1980. Regulation of macrophage collagenase by prostaglandins and cAMP. Proc. of the ASM Conference on Endogenous Mediators in Host Responses to Bacterial Endotoxin. Norfolk, Va. In press.

4. Sandberg, A.L., Complement. The Cell Biology of Immunity and Inflammation. Ed. by J. Oppenheim, M. Potter and D. Rosenstreich. Elsevier Press. Holland. 1980. 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 00216-04 LMI

PERIOD COVERED
October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Immunological Control of ConnectiveTissue Metabolism

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

Wahl, Larry M.	Research Biologist	LMI NIDR
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Wahl, Sharon M.	Research Microbiologist	LMI NIDR
Sandberg, Ann L.	Research Biologist	LMI NIDR
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COOPERATING UNITS (if any)

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LAB/BRANCH
Microbiology and Immunology

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Humoral Immunity Section

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.75	2.25	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)

The purpose of this project is to study the immune system in connective tissue metabolism. We have demonstrated that dexamethasone or colchicine inhibits macrophage collagenase production. 1) This effect of dexamethasone is attributed to the inhibition of phospholipase which liberates the precursors of prostaglandins, the required mediators of collagenase production. The inhibitory action of colchicine on collagenase production is most likely related to its effect on microtubules which may be essential in the transmission of the activation signal. Collagenase production can be restored in dexamethasone inhibited cultures by dibutyryl cyclic adenosine monophosphate and PGE_2. However, these compounds have no effect on colchicine treated cultures. Studies involving the role of the immune system in bone resorption defects have shown that the abnormally low proliferative response of T and B lymphocytes in these animals is due to macrophage suppression. This suppressive effect appears to be unrelated to prostaglandins since the macrophage from these animals produce normal amounts of PGE_2.

PHS-6040

OBJECTIVES:

Our goals in this project are 1) to determine the events in the sequence of macrophage activation which lead to the production of collagenase and how these events may be regulated, 2) to examine the role of macrophages and lymphocytes in connective tissue destruction utilizing model systems of adjuvant arthritis and defective bone resorption.

MAJOR FINDINGS:

We have previously demonstrated the sequence of events in guinea pig macrophage activation which lead to the production of collagenase. These essential steps included: 1) exposure of the macrophage to an activator such as lipopolysaccharide (LPS), 2) subsequent membrane movement resulting in cell spreading 3) production of prostaglandin E_2 (PGE_2) 4) PGE_2 induced elevation of intracellular levels of cyclic adenosine monophosphate (cAMP) and 5) synthesis and release of the enzyme collagenase. Some of our recent studies have focused on means by which this sequence of events can be blocked at various points resulting in the inhibition of collagenase. We have shown that dexamethasone and colchicine inhibit collagenase production by macrophages. Dexamethasone exerts its inhibiting effect on collagenase by blocking the synthesis of PGE_2. The inhibition of PGE_2 synthesis by dexamethasone appears to result from its inhibitory effect on phospholipase A_2 since the exogenous addition of this enzyme restores PGE_2 and collagenase production. The inhibitory effect of dexamethasone on collagenase can also be reversed by the addition of PGE_2 and dBcAMP. Colchicine inhibits collagenase production but does not effect the synthesis of PGE_2. Moreover, the exogenous addition of PGE_2 or dBcAMP to colchicine inhibited cultures does not restore collagenase production. Homogenates of macrophages treated with LPS and colchicine do not contain collagenase indicating that colchicine blocks the signal for enzyme synthesis and not the release of a stored enzyme. In light of the known effect of colchicine on microtubles it seems probable that this drug blocks collagenase synthesis by interrupting a signal which is normally transmitted by microtubules. Thus, while both of these drugs inhibit collagenase production they do so by blocking the activation sequence at different stages. Dexamethasone inhibits at the level of PGE_2 synthesis whereas colchicine, which has no effect on PGE_2 production, blocks at a later step, the transmission of the activation signal via the microtubles.

Our studies on the role of the immune system in bone resorption have been extended to an additional rat strain called the osteopetrotic (op) rat. We previously utilized a osteopetrotic rat strain, the toothless (tl) rat, and demonstrated that the macrophages from these animals, when compared to normal littermates, were defective in their regulation of lymphocyte proliferation and in their production of prostaglandins. Whereas the lymphocytes from tl rats were hyperproliferatie in response to T and B cell mitogens, the lymphocytes from op rats are hypo-proliferative. Moreover when the macrophages are removed from op spleen cells the lymphocyte

proliferation response to mitogens is normal. Thus, the macrophages from op rats have abnormally high suppressive effects on lymphocyte proliferation. This suppressive effect does not appear to be related to prostaglandins since macrophages from op rats produce similar amounts of PGE_2 to macrophages obtained from normal littermates. Thus, while the same etiology of excess bone formation occurs in these two rat strains the cellular defect in the two macrophage populations differ.

SIGNIFICANCE 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 demonstrated that collagenase production by guinea pig macrophages can be inhibited by dexamethasone which inhibits PGE_2 synthesis or by colchicine which interfers with microtubule integrity. Thus, the activation sequence that leads to the production of collagenase by macrophages can be blocked at different stages by these two drugs. These results may help in the understanding of the *in vivo* effects of these drugs on connective tissue metabolism.

The studies involving the osteopetrotic rat model indicate that there are defects in the immune system which may be related to the pathology of this disorder. Characterization of the exact manner in which these defects contribute to excess bone formation will increase our understanding of the role of lymphcytes and macrophages in bone resorption.

PROPOSED COURSE OF STUDY:

Over the last year we have developed the technique for separating subpopulations of monocytes or macrophages based on their density. Separation of these cells will permit the evaluation of functional heterogeneity among these subsets. Some of the functions to be compared will include prostaglandin, collagenase, fibroblast activating factor and chemotactic factor production. It is hoped to determine whether or not the different subsets are responsible for the production of these factors. In another series of experiments the ability of the macrophage to produce prostaglandins and collagenase when simultaneously exposed to an activator such as lipopolysaccharide and chemotactic factors will be examined. These findings will be useful in evaluating how the macrophage may respond the *in vivo* inflammatory response. The role of macrophages in adjuvant arthritis in rats will be studied in collaboration with Dr. Ronald Wilder (National Institute of Arthritis, Metabolism and Digestive Diseases). Some strains of rats are susceptible to the induction of arthritis by streptococcal cell wall products while others are very resistant. Macrophages from these various strains will be exposed *in vitro* to streptococcal cell walls and the media from these cultures will be assayed for prostaglandins, fibroblast activating factor and fibroblast chemotactic factor. Examination of the

effect of the immune system on bone resorption will continue with efforts focused on the osteopetrotic (op) rat. The extent of immune dysfunction in these animals will be evaluated by testing the ability of lymphocyte and macrophages to release a variety of mediators. These will include fibroblast activating factor, fibroblast chemotactic factor and monocyte chemotactic factor.

PUBLICATIONS:

1. Mergenhagen, S. E., S. Wahl, and L. Wahl. 1980. Regulation of fibroblast function by lymphokines and monokines. In Reticulo-endotehlial system and the Pathogenesis of Liver Disease. (H. Liehy and M. Grun, eds.) Elsevier/North Holland Biomedical Press, Amsterdam, International Biomedical Symposium, Wurzburg, West Germany, p. 69.

2. Wahl, L. M., J. B. McCarthy, C. E. Olsen, S. M. Wahl, A. L. Sandberg, and S. E. Mergenhagen. 1980. Regulation of macrophage collagenase by prostaglandins and cAMP. Proc. of the ASM Conference on Endogenous Mediators in Host Responses to Bacterial Endotoxin. Norfolk, Virginia. in press.

3. Wahl, S. M., and L. M. Wahl. 1980. Modulation of fibroblast growth and function by monokines and lymphokines. Lymphokine Reports: A Forum for nonantibody Lymphocyte Products, in press.

4. Schechter, G. P., Wahl, L. M. and Horton, J. E. 1980. In vitro bone resorption by human myeloma cells. In Progress in Myeloma. (Potter, eds.). Elsevier/North Holland, Inc., p. 67.

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-00254-03 LMI

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Microbial Antigens Associated with Specific Adherence

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

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Curl, Shelley	Microbiologist	LMI NIDR

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Floyd C. McIntire, Albert E. Vatter, and George Revis, University of Colorado Medical Center

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Humoral Immunity Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.00	1.00	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)
Studies are continuing to examine the mechanisms by which oral actinomycetes adhere to other plaque bacteria and to mammalian cells. The hybridoma technique has been used to produce several monoclonal antibodies against surface fibrils (i.e. fimbriae or pili) on Actinomyces viscosus T14V. These antibodies have detected the lectin activity associated with adherence by cross-linking isolated fibrils to form soluble immune complexes with lactose-inhibitable agglutination activity for streptococci and neuraminidase-treated human erythrocytes. The antibodies cross-react only with human strains of A. viscosus and A. naeslundii having a surface lectin activity similar to that of A. viscosus T14V and also cross-link the isolated fibrils from these heterologous strains to reveal their lectin activity. These findings indicate that the presence of antigenically related fibrils on a wide range of actinomycete strains accounts for their similar lectin activity.

PHS-6040
(Rev. 10-76)

OBJECTIVES: Z01 DE-00254-03 LMI

The continuing goals of this project are: (i) to identify, isolate and characterize microbial surface antigens which function in adherence; (ii) to characterize monoclonal and secretory antibodies against these antigens for their abilities to block adherence; and (iii) to examine the effects on mammalian cells of various microbial products. The present report concerns the use of monoclonal antibodies to identify and isolate surface structures from A. viscosus which mediate lactose-inhibitable adherence to certain oral streptococci and mammalian cells.

METHODS:

This project utilizes many standard biochemical, immunological, bacteriological, and electron microscopic techniques which have been defined. In addition, several monoclonal antibodies against surface antigens of A. viscosus T14V have been produced by the hybridoma technique and purified in high yield from ascites fluid. Highly specific affinity columns for the purification of bacterial surface structures have been prepared by immobilizing selected antibodies on beads of 1% agarose.

MAJOR FINDINGS:

Human strains of Actinomyces viscosus and A. naeslundii carry a lactose-inhibitable lectin which mediates bacterial coaggregation with certain plaque streptococci and hemagglutination of neuraminidase-treated human RBC. Monoclonal antibodies have been used to localize the lectin activity to a single type of structure on the surface of A. viscosus T14V. These antibodies react with fibrils (i.e. fimbriae or pili) on whole bacteria as shown by immunoelectron microscopy and reveal the lectin activity of these structures by cross-linking isolated fibrils into soluble immune complexes with lactose-inhibitable agglutination activity for streptococci and neuraminidase-treated RBC. Nine monoclonal antibodies against the lactose-sensitive fibril of A. viscosus T14V were produced, purified from BALB/c ascites fluid and screened for their reactivity with various actinomycetes. While each antibody differed in its ability to cross-react, only human strains of A. viscosus and A. naeslundii having a surface lectin activity like that of A. viscosus T14V were recognized. Moreover, the lectin activity of fibrils from heterologous strains was detected when these structures were cross-linked by monoclonal antibodies against A. viscosus T14V. These findings indicate that the presence of antigenically related surface fibrils accounts for the similar lectin activity on a wide range of bacterial strains. The different patterns of cross-reactions displayed by each monoclonal antibody suggests that these proteins may provide reagents for selectively covering up different antigenic determinants on the fibril of A. viscosus T14V. This would provide an approach for localizing the lectin binding site(s) on these macromolecular structures.

Crossed-immunoelectrophoresis of surface fibrils prepared from A. viscosus T14V and from other strains has revealed the presence of two distinct high molecular weight antigens. These were separated by the combined use of fractional ammonium sulfate precipitation and affinity chromatography using monoclonal antibody columns. One antigen was identified as the lactose-sensitive fibril and the other was found to be an additional type of surface fibril having no detectable lectin activity. These findings favor the idea that a single type of fibril mediates lactose-inhibitable coaggregation and hemagglutination and also raise the possibility that other surface fibrils mediate additional types of adherence.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH:

Specific adherence of microorganisms represents a critical initial event in many host-parasite relationships. The results of this project illustrate the use of monoclonal antibodies in the identification and isolation of antigens which mediate adherence. They also provide considerable support for the concept that lectin-carbohydrate interactions represent a significant and general mechanism of adherence. A better understanding of these phenomena at the molecular level may suggest new and useful approaches for the control of certain bacterial infections.

PROPOSED COURSE:

Studies will continue towards the precise identification of the lectin binding site on the lactose-sensitive fibril of A. viscosus T14V and related strains. These efforts will focus on the use of existing monoclonal antibodies and their Fab fragments to block adherence.

Monoclonal antibodies will be produced against the second type of fibril from A. viscosus T14V and will be employed to examine the involvement of these structures in various coaggregation reactions.

Purified fibrils of both types from A. viscosus T14V will be subjected to structural studies and analyzed for their amino acid compositions.

Certain additional ongoing studies will continue. These include attempts to isolate carbohydrates which form the streptococcal receptors in lactose-reversible coaggregation and experiments to evaluate certain effects of neuraminidase on immunologically competent cells.

PUBLICATIONS:

1. Costello, A. H., Cisar, J. O., Kolenbrander, P. E., and Gabriel, O. 1979. Neuraminidase-dependent hemagglutination of human erythrocytes by human strains of Actinomyces viscosus and Actinomyces naeslundii. Infect. Immun. 26: 563.

2. Cisar, J. O., E. L. Barsumian, S. H. Curl, A. E. Vatter, A. L. Sandberg and R. P. Siraganian. 1980. The use of monoclonal antibodies in the study of lactose-sensitive adherence of Actinomyces viscosus T14V. J. Reticuloendothel. Soc. (Symposium on Recent Advances in Oral Immunology, Sixteenth National Meeting, Reticuloendothelial Society, manuscript 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 00307-1 LMI

PERIOD COVERED: October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Tumor Specific Antibody with Chemotactic Activity

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

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Ann L. Sandberg	Chief, Humoral Immunity Section	LMI, NIDR
		LMI, NIDR

COOPERATING UNITS (if any)

LAB/BRANCH: Laboratory of Microbiology and Immunology

SECTION: Humoral Immunity Section

INSTITUTE AND LOCATION: NIDR, NIH, Bethesda, Md. 20205

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 administration of tumor specific antibodies which possess chemotactic activity might enhance tumoricidal host defense by directing the migration of inflammatory cells to a tumor site. The synthetic chemotactic peptide formyl-methionyl-leucyl-phenylalanine (f-MLP), has been covalently coupled to rabbit antibodies specific for a strain 13 guinea pig hepatoma. The tumor specific antibodies coupled to f-MLP (chemotactic antibody") retain their antigen binding properties and, in addition, are chemotactic for guinea pig peritoneal exudate cells. However, the "chemotactic antibodies" are relatively ineffective in inducing complement dependent cytotoxicity (CDC). The coupled antibodies are also chemotactic when bound to the tumor cells, implying that the antibodies, antibody-tumor antigen complexes or the f-MLP may be released and establish an effective chemotactic gradient for peritoneal exudate cells. These findings suggest that in vivo the chemotactic antibodies may localize in a tumor site where they would initiate the influx of inflammatory cells.

OBJECTIVES:

The participation of inflammatory cells in host defense against tumors may be augmented if the influx of the cells into an affected area could be enhanced. This might be accomplished by coupling a chemotactic factor to a tumor specific antibody. The goals of this project are to produce tumor specific antibodies; covalently couple them to the chemotactic peptide, f-MLP; characterize the complexes; evaluate the chemotactic and antibody binding activity of the complexes; and to determine the *in vivo* effects of the complexes on macrophage migration into the tumor site and tumor growth.

METHODS:

Standard methods are used for the production and purification of specific antibody, characterization of the antibody-peptide complexes, and the *in vitro* evaluation of the complexes (indirect membrane immunofluorescence, complement dependent cytotoxicity chemotaxis). ^{125}I-labeled antibody is used for *in vivo* pharmacokinetic studies.

MAJOR FINDINGS:

Membrane immunofluorescence and complement dependent cytotoxicity demonstrate that the IgG preparation used for the covalent coupling of f-MLP is specific for the target hepatoma and does not interact to a significant degree with normal guinea pig liver cells. f-MLP coupled to IgG or IgM contains 3-5 molecules of f-MLP per antibody molecule. The chemotactic activity of the modified antibody for guinea pig peritoneal exudate cells is dose-dependent in the concentration range of 25 - 200 µg protein/ml. Binding of IgG f-MLP to the tumor cell surface is comparable to that of native IgG as demonstrated by indirect membrane immunofluorescence. F $(ab')_2$ fragments also bind, excluding the possibility of attachment through Fc receptors. These results demonstrate intact antigen recognition and chemotactic function of the modified antibody. The complement activating properties of the chemotactic antibodies were tested by complement dependent cytotoxicity. Whereas native IgG and IgM show up to 70% specific killing of tumor cells, the f-MLP coupled IgG lost most of its complement activating properties, probably due to steric interference at the complement activating site. The f-MLP coupled antibody is also chemotactic when bound to the tumor cell surface, implying that the chemotactic antibody, antibody-tumor antigen complexes or the f-MLP are released and establish an effective chemotactic concentration gradient for peritoneal exudate cells.

In vivo the tumor-specific antibody is rapidly cleared from the blood after i.v. or i.p. injection during the first day ($T_{1/2}$ = ca. 18 h), then by a slower process for another 6 = 10 days ($T_{1/2}$=ca. 60 h). A guinea pig hepatoma cell line has also been established and characterized by karyotyping and growth kinetics. This cell line produces a variety of soluble factors, which induce fibroblasts to proliferate, produce collagen and migrate.

SIGNIFICANCE TO BIOMEDICAL AND DENTAL RESEARCH

Host-tumor interactions are of critical importance in the modulation of tumor growth. Inflammatory cells, especially macrophages, have been implicated as major effectors in in vivo tumor killing. Nevertheless, no direct in vivo model is available to modify the influx of macrophages into a tumor site. The in vitro results of this project suggest that direct in vivo modification (i.e. enhancement of local macrophage accumulation and subsequent tumor destruction might be possible.

PROPOSED COURSE

Studies are continuing to evaluate the in vivo effects of chemotactic antibody in tumor bearing animals. The accumulation of inflammatory cells in a tumor site will be quantitated and eventual therapeutic effects ascertained in a variety of clinically relevant animal models. Modification of tumor specific antibody with other biologically active agents (e.g. MDP) is also in a preliminary testing stage.

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 00034-12 LMI

PERIOD COVERED
October 1, 1979 to September 30, 1980

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

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Camargo, Sergio	Guest Worker	LMI NIDR
McGivney, Ann	Postdoctoral Fellow	LMI NIDR
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COOPERATING UNITS (if any)
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NCI, Laboratory of Cell Biology, NIH
NCI, Laboratory of Theoretical Biology, NIH
NIMH, Laboratory of Clinical Science, NIH

LAB/BRANCH
Laboratory of Microbiology and Immunology

SECTION
Clinical Immunology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.00	5.50	.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)

Histamine release from mast cells and blood basophils is being studied as one of the immunological mechanisms involved in inflammation. Among the histamine releasing agents employed are IgE antibody, environmental antigens, and the complement factor C5a derived from human serum. The relationships between IgE crosslinking, basophil desensitization and histamine release were analysed by kinetic studies. Cultured rat basophilic leukemia cells are used as a model for the studies of the IgE receptor, changes in phospholipid methylation in the cells and the role of lymphokines in modulating histamine release.

I. Project Description

A. Mathematical models for receptor cross-linking and histamine release in basophils.

Basophils sensitized to penicillin degranulate and release histamine when incubated with multivalent penicillin derivatives but not with monovalent hapten. The dose-response curve is biphasic with maximal release at a concentration of dimeric hapten of about 1 nM. The characteristics of the response generated using mixtures of monovalent and divalent derivatives, as well as a variety of other evidence, suggest that the rise and fall in the dose-response curve reflects the rise and fall in the concentration of receptors cross-linked by the multivalent hapten.

Extracellular Ca^{2+} is required for histamine release and cells may be desensitized to different degrees by incubation with various concentrations of ligand in the absence of Ca^{2+}. Washing and rechallenging cells with an optimal dose of ligand and Ca^{2+} indicate that the dose dependence of desensitization is also biphasic and is most pronounced at a ligand concentration which ordinarily stimulates maximal release. The implication is that cross-linking, will lead primarily to dimers and trimers, initiates signals for both degranulation and desensitization.

Kinetic studies reveal release curves which are sigmoidal, having delays in the onset of release that vary with ligand concentration with a maximum at approximately the same concentration as the peak in the dose-response curve. The results are interpreted in terms of a model in which cross-linked receptors are converted to an active, unstable intermediate which facilitates an increase in cytoplasmic Ca^{2+}, but which decays spontaneously into an inactive product. Dependence of histamine release on the concentration of the intermediate is nonlinear, suggesting either a positive feedback loop stabilizing the intermediate or the interaction of several aggregates. A fit of a simple mathematical formulation of the model indicates that it qualitatively explains the dose-response, desensitization, and release patterns.

B. Histamine release from human basophils by benzylpenicilloyl compounds.

Leukocytes from allergic patients were screened for their ability to release histamine with bivalent or multivalent benzylpenicilloyl (BPO) compounds. Ten subjects have been found whose cells release >10% of their available histamine with BPO in the presence of 44% deuterium oxide (to enhance the release). Eight of the 10 released histamine only at a BPO concentration of 10^{-5} or 10^{-6} M. Monovalent hapten (BPO_1 propyl) at 10^{-4} M inhibited release by either BPO_2 or BPO_{18} HSA. All subjects were tested with BPO-NH-$(CH_2)_n$-NH-BPO; where n=3 to 12 (Immunochem. 12:149, 1975). Optimal histamine release occurred when n=6 or 9, whereas when n=12 the compound was essentially inactive. One subject released optimally at 10^{-8} M with BPO_{18} HSA, but not with either BPO_3Lys_3 or $BPO_2(CH_2)_n$. Cells from this patient when incubated with BPO_{18} HSA in

the absence of Ca^{2+} were specifically desensitized to antigen but released normally to anti-IgE. Leukocytes from a second subject released optimally with 10^{-7} or 10^{-6} M $BPO_2(CH_2)_6$ and these cells were non-specificially desensitized with $BPO_2(CH_2)_6$ for both antigen and anti-IgE induced release. The results demonstrate that bivalent BPO haptens are incapable of activating the cells of some patients for histamine release. The inability of some cells to be activated by bivalent hapten does not appear to be due to low affinity of the anti-BPO IgE on the basophil surface.

C. Antigenic structural studies of rat and bovine serum albumin.

This study evaluated the capacity of rat serum albumin and of its proteolytic fragments to activate human basophils for IgE-mediated histamine release. The leukocytes from 8 out of 33 patients allergic to rats released histamine with rat serum albumin. Two proteolytic fragments of rat serum albumin, each constituting half of the molecule were used to study the IgE-reactive antigenic sites. These fragments released histamine with the cells of some of the donors, thus demonstrating the presence of at least 2 antigenic determinants on each fragment for a total minimum of 4 sites on the intact rat serum albumin molecule. Most of the allergenic activity, however, was not recovered in the two fragments (total recovery $\bar{x}$=6.4%, range between 0.1 and 31%). This loss could be due to cleavage of the rat albumin molecule in the middle of the third domain with loss of antigenic sites and/or due to minor conformational changes in the fragments as compared to the intact molecule. There was up to a 500-fold difference in the percent of activity recovered in the fragments when tested on cells from different patients. Therefore, there is no single immunodominant site on the molecule equally important for all patients. The cells of all 8 patients also reacted with mouse serum albumin but only 2 with bovine serum albumin. At least one determinant on mouse and rat serum albumin is cross reactive with IgE.

The antigenic sites on bovine serum albumin were studied utilizing peptic and tryptic fragments of the molecule. Rabbits were immunized with small doses of bovine serum albumin and their serum antibody response measured by a binding assay and the IgE response by antigen-induced histamine release from their basophils. The basophils from two bovine serum albumin allergic individuals were also used for histamine release studies. Serum antibodies bound large fragments of bovine serum albumin, these fragments also induced histamine release from basophils. Although about half of the antibody binding activity was recovered in the two halves of the albumin molecule only about 10% of the histamine releasing activity was present in the same fragments. The loss of activity of the bovine serum albumin molecule on proteolytic cleavage into two halves could be due to the breakup of the molecule in the middle of the third domain with loss of antigenic sites and/or due to minor conformational changes in the fragments as compared to the intact molecule. Large fragments of bovine serum albumin induced basophil histamine release, thus demonstrating the presence of at least 2 antigenic determinants on each of these peptides. This data therefore suggests the presence of at

least 4 antigenic IgE-binding sites on the bovine serum albumin molecule. By basophil desensitization experiments, unique IgE-reactive antigenic sites were demonstrated on each half of the molecule; however, some of the sites on the COOH-terminal half cross-reacted with antibodies directed towards the NH_2-terminal part of the molecule. The IgE-response of rabbits to bovine serum albumin was specific; there was no cross-reactivity with rat or mouse albumin. The present findings indicate a substantial loss in the IgE-reactive determinants of bovine serum albumin by cleavage into large fragments.

D. Efficacy and specificity of immunotherapy with laboratory animal allergen extracts.

The clinical and immunologic response to immunotherapy with laboratory animal allergens was evaluated. There were 22 patients; 11 had received immunotherapy with 12 different extracts (five mouse, six rat, one rabbit), and 12 were matched untreated patients. As a group, nine of 23 had seasonal hay fever. Among the treated patients nine of 11 subjectively improved with immunotherapy. Blocking antibody titers were determined by serum inhibition of allergen-induced histamine release. Treated patients had mean blocking antibody levels of $G_{30} = 59.3 \pm 38.7$. In the untreated patients the antibody level was low ($G_{30} = 4.6 \pm 3.6$). The difference between the two groups was highly significant (t test $p < 0.001$). The blocking antibody level correlates with both the final weekly allergen dose and also the cumulative allergen dose received during immunotherapy. When pretreatment sera were available a temporal rise in blocking antibody was demonstrated during immunotherapy. In patients allergic to several laboratory animals and treated with one allergen the blocking antibody response was predominantly specific to the allergen used in immunotherapy. This indicates a lack of cross-reactivity in the IgE response to the major animal allergens. When immunotherapy was discontinued in four patients there was a dramatic decrease in the blocking titer, and after 24 months the levels were the same as those of untreated patients.

E. In vitro studies of histamine release from leukocytes with food allergens.

Washed leukocytes from 174 subjects were tested for histamine release by challenge with 15 different food allergens. Histamine release >10% was considered positive. In 2593 tests there were 324 positive reactions (12%) by 77 different subjects (44%). However, only 24 of the positive reactions correlated with a positive clinical history to the specific food allergen. The allergen dilutions at which leukocytes released histamine were similar in the groups with and without clinical history of food allergy. Among donors who did not release histamine with food allergens there were 25 subjects with histories compatible with allergy to a total of 28 different foods.

Although only 59% of the subjects were atopic, 98% of the positive histamine release reactions with food allergens occured with cells from atopic individuals.

Serum from histamine release positive donors was used for passive sensitization studies. Leukocytes from non-atopic donors were incubated with normal serum, allergic serum or allergic serum heated at 56°C for 2 hrs. Only cells sensitized with non-heated allergic serum released histamine upon challenge with the food allergens.

Leukocytes from many subjects release histamine with food allergens, however, histamine release to these antigens does not correlate with clinical food allergy. The reaction is probably mediated by IgE and is more frequent in atopic subjects. The data suggest that a protective mechanism exists in individuals with positive leukocyte reactivity to prevent in vivo hypersensitivity reactions.

F. Enhancement of IgE-mediated histamine release from human basophils by immune-specific lymphokines.

Human leukocytes (basophils) release histamine when exposed to ragweed antigen E or anti-IgE. The present study shows that when leukocytes from BCG-positive donors are first incubated with PPD and then challenged with anti-IgE, histamine release is enhanced. In contrast, when leukocytes from BCG-negative donors are incubated with PPD and then challenged with anti-IgE there is no enhancement of histamine release. The enhancement of histamine release was detected within 24 hr after addition of PPD, but was maximal at 48 to 72 hr. Supernatant fluids collected from these leukocyte cultures revealed the presence of a soluble mediator(s) which, when incubated with leukocytes from BCG-negative donors, enhanced the release of histamine. Examination of the supernatant fluids from BCG-positive leukocyte cultures stimulated with PPD showed a correlation between histamine-release enhancing activity and interferon. Treatment of the culture fluids at pH 2.0 abolished the anti-viral activity, indicating that the interferon was of the type II or "immune" class. The same treatment only partly abolished the histamine-release enhancing activity. It is concluded that immune-specific stimulation of leukocytes results in the release of soluble mediators that are capable of enhancing IgE-mediated histamine release.

G. Effect of inhibition of transmethylation on histamine relase from human basophils.

Methylation reactions mediated by S-adenosyl-L-methionine (AdoMet) play an important role in a number of biological reactions including bacterial and human monocyte chemotaxis. This study evaluated the role of methylation reactions in histamine release from human basophils. Methylation was blocked by several methods. Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), an inhibitor of adenosine deaminase (EC 3.5.4.4.), blocked the IgE-mediated histamine release with an IC_{50} (concentration of drug required to produce 50% inhibition) of 0.33 mM. Preincubation of leukocytes with adenosine caused some inhibition of IgE-mediated histamine release. This inhibition by adenosine was potentiated by the addition of 1×10^{-5} M EHNA which alone did not affect histamine release.

Further addition of L-homocysteine thiolactone at 1 x 10^{-4} M potentiated the inhibitory effect of EHNA plus adenosine. The effect of L-homocysteine thiolactone was dose dependent.

3-deazaadenosine (DZA), an inhibitor of S-adenosyl-L-homocysteine hydrolase (EC 3.3.1.1.) inhibited IgE-initiated histamine release from human basophils with an IC_{50} of ∿ 1mM. This inhibition was potentiated by L-homocysteine thiolactone. The inhibition by DZA was not potentiated by two different phosphodiesterase inhibitors suggesting that the action of DZA is not through changes in intracellular levels of cyclic AMP. DZA inhibits during the Ca^{++}-independent activation step of IgE-mediated basophil histamine release. In contrast, when histamine release was induced by the calcium ionophore A23187, fMet-Leu-Phe or zymosan-activated serum there was either no inhibition or enhancement of histamine release by these inhibitors of transmethylation. These results suggest that AdoMet-mediated methylation plays an important role in IgE-initiated histamine secretion from human basophils, whereas release induced by A23187, fMet-Leu-Phe or C5a bypasses this reaction step.

H. Studies with the rat basophilic leukemia cell lines.

The rat basophilic leukemia cell lines have been used extensively for studies of the IgE receptor and of histamine release. However, these lines have not been previously compared for their chromosome number, IgE-mediated histamine release and for IgE-surface receptors. Furthermore, the heterogeneity of these sublines has not been determined. We found the cell lines to vary in both their chromosome number and their ability to release histamine by an IgE-mediated reaction. RBL-I and III have ∿ 44 chromosomes and did not respond to an IgE-mediated reaction, whereas RBL-II and RBL-IV have 68-73 chromosomes and showed moderate levels of histamine release (percent release $\bar{x}$= 5 ± 2 and 10 ± 4 respectively). The cloning of the RBL-IV line resulted in some sublines which were excellent histamine releasers (range 39% to 100%) and some which were relatively refractory (<10%) to IgE-mediated histamine release. These clones did not differ significantly in chromosome number. Recloning the releasing lines gave rise to poor releasers, whereas, the recloning of poor-releasers did not produce good releasers, indicating that the mutational drift in culture is toward loss of histamine releasing capacity. The number of IgE receptors, the rate of IgE association and dissociation were similar for the different cell lines. The study failed to disclose significant molecular weight differences in the IgE-receptor from the various clones and sublines, but disclosed a multi-component peak in SDS-PAGE analysis of receptors extracted from the sublines. The various cloned sublines are phenotypically stable and therefore useful for studies of the complex phenomenon of histamine release.

Receptors for IgE and IgG on rat basophilic leukemia cells (the 2H3 subline of RBL-IV) were detected by their ability to bind IgE and IgG in fluorescence or radioactive assays. The 2H3 cells have approximately two-thirds as many receptors for IgG as for IgE. The IgE receptors bind

monomeric IgE with high affinity, and IgE binding is uninhibited by high concentrations of monomeric or oligomeric IgG. In contrast, the IgG receptors bind both (rabbit and rat) IgG and (rat) IgE. The affinity of IgG receptors for monomeric IgG and IgE is much lower than that of the IgE receptor for its ligand; binding of radiolabeled IgE or IgG to IgG receptors can be detected only with cross-linked oligomers. While both receptors are sensitive to proteolysis by pronase, IgG receptors are more sensitive to tryptic digestion than IgE receptors. Dual laser flow microfluorometric studies revealed that the numbers of IgG and IgE receptors per cell varied considerably among 2H3 cells and that the distribution of the two types of receptors were independent of each other.

2H3 cells released histamine through an IgE-mediated system, but cross-linked IgG, even in saturating amounts, did not elicit histamine release, nor have any effect upon IgE-induced release. Moreover, 2H3 cells would not mediate ADCC of IgE-coated chicken red blood cells.

Two cloned sublines (2H3 and 1C1) of the rat basophilic leukemia cell line (RBL-IV-HR+) were mutagenized and selected for resistance to either thioguanine (TG), chloramphenicol (CAP), or both drugs and then recloned. All sublines were tested for: histamine content, IgE receptor number, non-cytotoxic histamine release in response to the Ca^{++} ionophore A23187 and to an IgE-mediated reaction. Both of the parental lines contained 100-500ng of histamine/10^6 cells, and $\sim$ 6 x 10^6 IgE receptors/cell and released histamine in response to ionophore or by an IgE-mediated reaction. From 1C1 subline 25 CAP resistant clones were selected: all 25 clones contained at least 200ng histamine/10^6 cells and 24/25 were capable of an IgE-mediated histamine release reaction; 1 clone with 10^6 IgE receptors/cell did not respond to either Ca^{++} ionophore or IgE-mediated reaction. From the 2H3 subline, 26 clones resistant to TG were selected: histamine content of 7/26 of these cloned lines was low, and 18/19 of the remaining clones did not demonstrate IgE-mediated histamine release. Several of these clones were capable of Ca^{++} ionophore induced histamine release. Most of these lines had IgE receptors, however 1/26 lines of 2H3 (TG^{res}) had no detecatable IgE receptors. Two clones were selected for resistance to both TG and CAP: both had a normal number of IgE receptors, however, neither line was capalbe of IgE-mediated or A23187 ionophore induced histamine release. These cells can provide a unique system for the study of the mechanism of histamine release.

A study evaluated the role of S-adenosylmethionine (AdoMet) mediated methylation reactions in histamine release from the 2H3 sublines of the rat basophilic leukemia cells (RBL-IV HR+). Several inhibitors of methylation reactions were used. 3-deazaadenosine (DZA), an inhibitor of methylation reactions, caused only 10% inhibition of histamine release at 1 mM. However, there was 50% inhibition of histamine release at 1.2 x 10^{-5} M of DZA in the presence of 1 x 10^{-4} M L-homocystein thiolactone. Another inhibitor or methylation reactions, 5'-deoxy-5' (isobutylthio)-3-deazaadenosine, also inhibited antigen-induced histamine release (IC_{50}=3.4 x 10^{-4} M). This inhibition was not enhanced by the addition

of 1×10^{-4} M L-homocysteine thiolactone. Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), an inhibitor of adenosine deaminase, inhibited IgE-mediated histamine release ($IC_{50} = 6.5 \times 10^{-4}$ M). The incubation of the cells with adenosine in the presence or absence of 1×10^{-5} M EHNA did not affect histamine release. However, the addition of 1×10^{-4} M L-homocysteine thiolactone to the adenosine plus EHNA caused marked inhibition. Incubation of the cells with a combination of DZA and L-homocysteine thiolactone resulted in the intracellular accumulation of S-adenosyl-homocysteine and 3-deazaadenosylhomocysteine. These results suggest that AdoMet-mediated methylation plays an important role in IgE-mediated histamine release from 3H3 cells.

Antigenic stimulation of rat basophilic leukemia cells sensitized with immunoglobulin E causes the release of histamine as well as arachidonic acid and its metabolites. The release of these substances is preceded by an increase in phospholipid methylation. Inhibiton of phospholipid methylation is correlated to the inhibition of histamine release. Inhibition of methylation also reduces arachidonate release. Phospholipid methylation appears to be associated with both histamine secretion and the release of arachidonate and its metabolites.

II. Significance to Biomedical Research and the program of NIDR

The purpose of the project is to study the immunologic release of mediators such as histamine which represent the effector mechanisms by which IgE, allergens, or microbial cells may interact with leukocytes or serum to cause inflammation. Desensitization mechanisms and pharmacologic agents are studied *in vitro* for their potential use in control of inflammatory responses.

III. Proposed Course

Experiments will pursue further pathways and regulatory mechanisms of immunologically-induced histamine release.

IV. Publications

1. Siraganian, R.P., Baer, H., Hochstein, H.D. and May, J.C.: Allergenic and biological activity of commercial preparations of house dust extract. J. Allergy and Clin. Immunol. 64:526-533, 1979.

2. Chabay, R., DeLisi, C., Hook. W.A. and Siraganian, R.P.: Receptor cross-linking and histamine release in basophils. J. Biol. Chem. 255:4628-4635, 1980.

3. Crews, F.T., Morita, Y., Hirata, F., Axelrod, J. and Siraganian, R.P.: Phospholipid methylation affects immuoglobulin E-mediated histamine and arachidonic acid release in rat leukemic basophils. Biochem. Biophysical Res. Comm. 93: 42-49, 1980.

4. Ida, S., Hooks, J., Siraganian, R.P. and Notkins, A.L.: Enhancement of IgE-mediated histamine release from human basophils by immune-specific lymphokines. Clin. exp. Immunol. 41: (In press).

5. Wahn, U. and Siraganian, R.P.: Efficacy and specificity of immunotherapy with laboratory animal allergen extracts. J. Allergy & Clin. Immunol. 65: 413-421, 1980.

6. Wahn, U., Peters, T. Jr. and Siraganian, R.P.: Studies on the allergenic significance and structure of rat serum albumin. J. Immunol. (In press).

7. Wahn, U., Peters, T. Jr., and Siraganian, R.P.: Allergenic and antigenic properties of bovine serum albumin. Molecular Immunol. (In press).

8. Segal, D.M., Sharrow, S.O., Jones, J.F. and Siraganian, R.P.: Fc (IgG) receptors on rat basophilic leukemia cells. J. Immunol. (In press).

9. Siraganian, R.P. and Hook, W.A.: Histamine Release and Assay Method for the Study of Human Allergy. In Manual of Clinical Immunology 2nd Edition (In press).

10. Siraganian, R.P.: Cellular, Immunological and Biochemical Basis of The Allergic Reaction. In The Cell Biology of Immunity and Inflammation Ed. Oppenheim, Potter and Rosenstreich (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 00205-04 LMI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Immunological Mechanisms in Periodontal Diseases

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

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Oppenheim, Joost	Medical Director	LMI NIDR
Siraganian, Reuben	Chief, Clinical Immunology	LMI NIDR
Metzger, Zvi	Visiting Fellow	LMI NIDR
Hoffeld, Jeffrey T.	Sr. Asst. Dental Surgeon	LMI NIDR
Meyer, Christine	Guest Worker	LMI NIDR

COOPERATING UNITS (if any)

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Laboratory of Microbiology and Immunology

SECTION
Clinical Immunology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryalnd 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.12	2.12	

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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)

Cellular immune reactions are being studied in patients with several types of periodontal disease, including juvenile periodontosis. Hybridomas secreting monoclonal antibodies against certain pathogenic oral bacteria are being developed.

I. Objectives

1. To define deviations of immune responses present in a group of patients with periodontal diseases.

2. To relate the degree of such immune deviations to the extent of the patients periodontal disease.

3. To differentiate the periodontal diseases based on differing immune responses.

4. To relate alterations in immune responses to the pathogenesis of periodontal diseases.

II. Project Description

Subjects have a complete dental evaluation including dental radiographs for screening purposes. Patients undergo venipuncture for certain baseline studies (CBC, Ig levels) and research purposes. Gingival fluid and dental plaque are also collected. Any subject with systemic disease diagnosed by history, tests or examination is excluded from the study.

Patients with periodontitis have been examined and classified according to the severity of their disease. The lymphocyte blastogenic response to dental plaque antigens and other antigens and mitogens has been determined. The role of the macrophage in this response has been the focus of this study. Patients with periodontitis show an increase in lymphocyte blastogenesis in response to dental plaque antigens as compared to individuals without periodontitis. The depletion of macrophages from lymphocyte preparations prior to culture and antigenic stimulation leads to an enhancement of the blastogenesis. This enhancement is most striking in patients with more severe disease as their lymphocyte responses to plaque antigens are initially lower. A similar enhancement of blastogenesis following macrophage depletion has been found in persons without periodontal disease but not of the same magnitude as our patient population.

The chemotactic response of monocytes to standard chemattractants has been studied in a group or young people with periodontosis. The monocytes of four of eight patients studied had a decreased chemotactic response when compared to controls from persons without periodontal disease. Sera from five of these patients were pre-incubated with autologous or heterologous dental plaque to generate chemotactic activity. These sera were then tested using monocytes from a donor without periodontal disease. Four of the patients sera showed a decreased chemattraction for normal monocytes when activated with autologous plaque as compared to activated sera from individuals without periodontal disease. Dental plaque alone was not chemotactically active. This decreased serum activation may contribute to the lack of a localized inflammatory response exhibited by these individuals.

Rats and mice have been immunized with the oral microorganisms, Capnocytophaga (a gram negative anaerobe), Streptococcus mutans and the

enzyme, glucosyl transferase (from S. mutans). These animals spleen cells are being fused to a cell line to produce hybridomas secreting monoclonal antibodies directed against the microorganisms or enzyme. Various subclasses of antibody are being isolated and purified.

III. Significance

Periodontal diseases are among the most widely prevalent inflammatory conditions of man. It has been estimated that over 90% of the adult population suffer from some degree of periodontitis. This is an excellent model for the study of local immunity. The immune responses of an individual to specific bacteria present around the teeth may be related to the susceptibility of that individual to periodontal diseases. The study of these responses may contribute to an understanding of the mechanism of the tissue destruction seen in periodontal diseases and other cellular immune interactions.

IV. Proposed Course

1. Additional patients are being examined and the periodontal status determined.

2. Studies of lymphocyte blastogenesis will continue with an emphasis on the role of macrophages and T-lymphocytes in mediation of the reaction.

3. Studies of chemotactic responsiveness of monocytes will continue with an emphasis on the interactions between serum factors, cells and dental plaque.

4. The in vitro production of immunoglobulins and other immunologic factors from gingival tissue will be investigated.

5. Hybridomas secreting monoclonal antibodies directed against; oral microorganisms will be developed. These will be used for specific identification of bacteria present in dental plaque and implicated in the pathogenesis of periodontal disease and caries.

6. Any immunological deviations found in our patient group will be further studied to determine a mechanism, if possible, and to develop therapy to locally or systemically overcome such a defect.

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 00290-1 LMI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Production of Hybridomas

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:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Hybridomas are being produced which secrete monoclonal antibodies of defined antigen specificity and antibody subclass. Studies are demonstrating means of improving the yield of hybridomas. An IgE producing hybridoma has been developed. Other hybridomas include those directed against oral micro-organisms, human IgE and the IgE receptor on the surface of mouse mast cells.

I. Objective

The aim of this project is the production of hybridomas secreting monoclonal antibodies of defined specificity. These hybridomas are being developed and purified for further studies utilizing sensitive radio-immunoassays, in vitro assays and tissue culture studies.

II. Project Description.

A) Development of IgE-producing hybridomas.

Mouse IgE myelomas have not been available until recently. An IgE hybridoma was produced by the fusion of spleen cells from parasitized mice and P3-NS-1 Ag4-1 plasmacytoma cells. Spleen cells from A/J mice infested 3 times with Nippostrongylus brasiliensis were obtained 3 days after the last dose of the parasite and the fusion carried out with 35% PEG 1000. IgE antibody activity was tested by the ability of culture supernatants to sensitize rat basophilic leukemia histamine releasing cells (RBL-HR+) for anti-mouse IgE-mediated release (anti-mouse IgE was prepared as in J. Immunol. 122:1719, 1979). At three weeks after hybridization, 40/72 wells from the first and 21/48 wells from the second hybridization contained IgE activity. One of the positive culture wells was expanded and cloned. High titers of IgE were present in both culture and ascites obtained after injection of the hybrid cells into pristane-primed CAF_1 mice. Culture fluid from this clone was capable of 1) sensitizing 10^5 cells at 1/10,000 for 40% histamine release; 2) blocking the ability of antigen-specific mouse IgE from sensitizing the RBL-HR+ cells for histamine release and 3) inhibiting the binding of radiolabeled rat IgE to the same cells. No specificity for this IgE has yet been determined. The monocolonal IgE was purified by $(NH_4)_2SO_4$ precipitation at 55% followed by DEAE chromatography and gel filtration on Bio-Gel A 0.5. Such mouse IgE preparations can be useful for studies of this immunoglobulin.

A rabbit antibody to this mouse IgE hybridoma has been prepared and is being purified. This will be useful in studies of mechanisms of immediate hypersensitivity reactions.

B) Methods for improving the yield of hybridomas.

One of the major drawbacks to hybridoma development has been the large number of cells which must be fused, maintained and screened for antibody production due to the low yield of antigen specific hybrids. This discouraging ratio has limited the use of hybridoma technology and made the development of further hybridomas cumbersome. Two techniques have been utilized in our laboratory which greatly enhance the yield of hybridomas following fusion. In the first method, spleen cells from immunized mice are cultured with antigen prior to hybridization. This increases the percentage of antigen specific antibody secreting hybrids formed while decreasing the number of cells available for hybridization. The second method, utilizes adoptive transfer of spleen cells from immunized animals into x-irradiated recipients followed by in vivo

antigen boosting. This results in a similar enhancement of hybrids and reduction in cell number.

The two methods depend upon antigen driven expansion of clones of pre-B or pre-plasma cells prior to hybridization. After three or four days only 15-27% of the original cultured or transferred cells are recovered as viable cells; however, on a per cell basis, when fused there is a 10-fold increase in positive wells. These methods appear to act as a "selective filter" to enhance the proportion of antigen specific, antibody producing, cells while decreasing the background non-speficic cells. These methods have been utilized in the production of hybridomas against the haptens dinitrophenyl (DNP) and benzylpenicilloyl (BPO) as well as other antigens.

C) Production of hybridoma antibodies against oral micro-organisms.

Hybridomas have been selected which secrete antibody against cell surface components (fimbriae) of the bacterium Actinomyces Viscosus T14V. Fusion of spleen cells directly from immunized mice yielded eight specific antibody secreting hybrids of a total of 216 wells. Adoptive transfer of spleen cells and fusion four days later yielded 11 secreting hybrids out of 34 wells plated. Various subclasses of antibodies are being selected for further studies on co-aggregation properties of these organisms.

D) Hybridomas directed against human IgE.

A hybridoma secreting antibodies against human IgE has been produced by the fusion of spleen cells from a mouse immunized with a human IgE myeloma and a mouse plasmacytoma cell line. The antibody is capable of triggering the release of histamine from human basophils. This hybridoma will be utilized in studies of human IgE allotypes.

E) Hybridomas directed against IgE receptors on mouse mast cells.

A hybridoma is being produced which is directed against the IgE receptor on the surface of a mouse mast cell line maintained in culture. The hybridoma antibody is capable of blocking the release of histamine from mast cells when they are incubated together prior to the addition of anti-IgE. An anti-receptor antibody will be valuable in studies of the mechanisms of IgE-mediated histamine release.

F) Production of anti-IgE idiotype secreting hybridomas.

Studies of how the immune response is regulated have focused on the phenomenon of idiotypes on antibodies and receptors. These structures act as specific markers of antibody producing cells which are recognized by the cellular components of the immune system (T-cells predominantly) leading to immune homeostasis.

The control of immunoglobulin E (IgE) synthesis is of particular interest since IgE is an obligate component of the allergic reaction. We are using a particular IgE myeloma product, SpE IV 7 IF, which is specific for DNP. This anti-DNP reactivity allows us to purify the antibody on affinity columns to assure a monospecific antigen. This system is particularly valuable because the immune response to DNP is well characterized for other immunoglobulin classes. Thus, we will be able to examine the IgE response to DNP and correlate it with parallel responses of other antibody subclasses.

III. Significance.

Hybridomas produce monoclonal antibodies of defined antigen specificity and antibody subclass at very high concentrations. The purity of this antibody and its highly defined nature make it a unique tool for immunologic studies. The hybridomas produced in these studies will be used to study interactions involved in the control and evolution of inflammatory responses.

IV. Proposed Course.

Experiments will continue to further purify and define existing hybridomas and produce new hybridomas of unique antigen specificities or antibody subclasses.

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 (salivary glands) and endocrine (pancreas) diseases with special emphasis on diabetes mellitus; and 4) premalignant and malignant lesions of the oral cavity.

Over the last year, in depth studies have continued on the projects discussed in previous annual reports. Strong emphasis has been placed on clinically-related research. In addition, two new projects have been initiated. The first is concerned with the use of recombinant DNA technology to make a vaccine against herpes simplex virus (HSV). This involves cloning the segment of the herpes genome coding for the viral glycoproteins that raise neutralizing antibody. The idea is to clone this gene segment into bacterial plasmids such that its expression into protein will be facilitated after introduction of the hybrid plasmid into E. coli. The herpes polypeptide (antigen) synthesized in E. coli will then be tested as a vaccine to prevent latency in the mouse model developed in our laboratory. The second project involves the anaylsis of immunological and nutritional factors that might be involved in the etiology and pathogenesis of aphthous ulcers.

As in the past, the program is disease oriented and we rely heavily on an interdisciplinary approach to study etiology and pathogenesis. The laboratory is made up of investigators with training in a variety of disciplines, including virology, immunology, pathology, cell biology, molecular biology and clinical medicine and dentistry. The staff is working well together and there is good cross-fertilization. This interdisciplinary approach continues to be extremely fruitful and we plan to add new disciplines or types of expertise as needed. This approach has given us tremendous flexibility to rapidly pursue promising leads.

The program greatly benefited over the last year by the presence in the laboratory of Dr. George Jordan from the University of California, who spent his sabbatical with us. Dr. Jordan is a physician and his specialty is infectious diseases. He interacted with many of the people in the laboratory and contributed both at the bench and in discussions with staff members. Two guest workers also added their special expertise to our program: Dr. Kathleen Melez, a pediatrician, who came to us from NICHHD, and Dr. Jan van Oirschot, a veterinarian, who comes from Holland. The in-depth strength of the laboratory, however, will be weakened over the next year by the loss of Dr. Bennett Jenson, a board-certified pathologist. Fortunately, there are now several people in our laboratory, both at the professional and technical levels who have training in pathology and electron microscopy and we anticipate no real interruption of on-going projects. Nonetheless, this position should ultimately be

refilled. In the interim, as backup and only if necessary, we will use consultants (e.g., several hours/weeks). Dr. Jenson, who will be at Georgetown, has already agreed to serve as a possible consultant.

One of the most gratifying aspects of our program, in recent years, has been the superb young scientists who have joined our laboratory through the NIH Visiting Fellow Program. These young scientists have contributed enormously both to the intellectual ferment and productivity of the laboratory.

The various service units of the laboratory continue to function well. A major effort is now being made to introduce new techniques and methods into the Tissue Culture Unit in order to increase flexibility (e.g., the types of cells being produced) and quality control. In the Office, the Lexitron - a word processing typewriter - which was obtained last year has proved invaluable and makes it easier and more timeefficient to accomplish our work.

A number of new techniques have been introduced and solidified into the on-going operations of the laboratory. This includes 1) recombinant DNA technology; 2) methods for preparing hybrid cells and making monoclonal antibodies; 3) extensive use of ELISA methodology; 4) assays for a number of hormones; 5) cultivation of a variety of specialized cells including neurons and myocardial cells; 6) extensive use of restriction enzymes; 7) preparation of cDNA for incorporation into plasmids; and 8) oligonucleotide fingerprinting of T1-digested RNA.

Fruitful collaboration continues with investigators from many of the Institutes at NIH, including NIAID, NIAMDD, NEI, NCI, NICHHD, NIDR, and also with colleagues from a number of universities.

Over the last year, good progress has been made in several areas. The significance and implications of some of our findings are briefly summarized below:

1. A new animal model for the reactivation of HSV has been developed. Mice are inoculated with HSV by the lip route and then passively immunized with antibody to HSV. In the passively immunized mice, neutralizing antibody to HSV is cleared from the circulation and cannot be detected in most mice after two months. Examination of the ganglia from these antibody-negative mice reveal the presence of latent virus in over 90% of the animals indicating that serum neutralizing antibody is not necessary to maintain the latent state. When the lips of these antibody-negative latently infected mice are traumatized by dry ice, phorbol, or other stimuli, viral reactivation occurs in up to 90% of the animals as demonstrated by the appearance of neutralizing antibody. This rise of neutralizing antibody in antibody-negatively latently infected mice, thus, provides a new model for identifying factors that trigger viral reactivation. Based on these and other studies, we have proposed a new hypothesis for explaining viral reactivation. According to this hypothesis, ganglion cells are primarily non-permissive for HSV replication. Various

stimuli act as signals to bring about a switch from the non-permissive to the permissive state. Replication of the virus at the epithelial surface and the ensuing inflammation may provide such a signal, making ganglion cells permissive for HSV. According to this model, during the course of the natural infection, the host's immune response shuts off this signal by decreasing replication of the virus at the epithelial surface. Ganglion cells then revert to the non-permissive state and latency ensues. However, a variety of other stimuli, such as epithelial trauma, can reactivate the latent virus, presumably, by switching the non-permissive latently-infected ganglion cells into permissive cells. Although the underlying molecular basis for latency and reactivation still remains to be defined, the idea that a variety of stimuli including the epithelial infection itself may make ordinarily non-permissive ganglion cells permissive for HSV replication has appeal and is being tested.

2. It has been amply documented that HSV persists in sensory (trigeminal) ganglia of the peripheral nervous system. In contrast, little is known about the latency of HSV in the central nervous system. This year, using molecular hybridization techniques, we have detected HSV DNA sequences in the brain stem and cerebral hemispheres of mice. This suggests that HSV travels from the peripheral nervous system to the central nervous system and in many cases is not eliminated, but establishes a latent infection. This raises the possibility that HSV may be involved in neurologic disorders of unknown etiology. If the viral genome or portions of it also persist in the brains of humans, the viral sequences might be expressed under the influence of various stimuli. In turn, the resulting viral proteins might interfere with nerve cell function. The possibility that HSV is latent in the brains of humans with various neurologic diorders will be explored in the future.

3. Last year we showed interferon was present in the circulation of a high percentage of patients with diseases such as systemic lupus erythematosus, rheumatoid arthritis, scleroderma and Sjogren's syndrome. Moreover, in the case of lupus erythematosus, the interferon was found primarily in patients with active disease. Over this last year, we have looked for interferon in the circulation of patients with a variety of other immunologic disorders. We now have found interferon in approximately 33% of patients with various types of vasculitis. Moreover, we have shown that the interferon is of the type II, or immune variety. Attempts are now being made to determine what role interferon plays in the pathogenesis of these diseases.

4. It has been known for many years that viral infections can trigger or enhance attacks of bronchial asthma. Viral infections also induce interferon. This year, studies from the laboratory showed that interferon can enhance the migration of basophils towards a variety of chemotactic factors. Since basophils are thought to play a role in bronchial asthma, interferon induced by viruses might enhance basophil migration and be one of the factors responsible for triggering attacks of asthma.

5. A new model has been developed for measuring receptors for viruses on the surface of cells. By use of this model, detailed information has been obtained on the rate of attachment and dissociation of viruses from cells and the number of receptors on the surface of these cells. Our studies suggest that both the number of receptors on the cell surface and the affinity of viruses for the receptors can influence virulence and pathogenicity. The relationship of receptors to virulence and the various factors that induce and modulate receptors on the surface of cells are now under investigation.

6. It is well known that viral infections can produce abnormalities in carbohydrate metabolism in normal subjects and profound changes in glucose homeostasis in insulin-dependent diabetics. It also is known that certain viruses can alter the plasma membrane of cells. In studies performed over the last year, it was found that monitoring changes in the number of insulin receptors on the surface of cells was a sensitive and quantitative method for evaluating the effect of viral infections on the plasma membrane. Several viruses, including HSV, now have been shown to markedly reduce the number of insulin receptors on the surface of cells. These findings suggest that abnormalities in glucose metabolism associated with some viral infections may, in part, be due to changes in the concentration of insulin receptors. These in vitro observations are now being extended to animal models.

7. Fresh insight into the pathogenesis of virus-induced diabetes has been obtained. For some time, we have been producing diabetes in mice with the M variant of encephalomyocarditis (EMC) virus. Over the last year, we found that the virus pool that we had been working with contains not one virus but at least two variants: one that produces diabetes and is referred to as the D variant and the other that does not produce diabetes and is referred to as the B variant. Even more important, co-infection of mice with the D and B variants showed that the induction of diabetes by the D variant was completely inhibited by the B variant. The mechanism by which the B variant inhibits the induction of diabetes by the D variant appears to be related, at least in part, to the different capacities of these two variants to produce interferon. Measurement of interferon levels demonstrated that the non-diabetogenic B variant produced considerably more interferon than the diabetogenic D variant. The interferon produced by the B variant is thought to protect beta cells from infection by the D variant.

These and related experiments have provided important new clues as to the approaches and potential difficulties that might be encountered in the isolation and identification of diabetogenic viruses in the human population. First, the D and B variants are antigenically indistinguishable by neutralization tests. This points to the possibility that similar problems might well be encountered in searching for diabetogenic variants of common human viruses by standard serologic techniques. Second, the D and B variants cannot be distinguished on a morphologic basis (plaque

size) in tissue culture. Thus, to isolate a diabetogenic variant in a virus pool that contains a mixture of diabetogenic and non-diabetogenic variants, it may be necessary to laborously test many individual virus plaques for diabetes in animals. Third, diabetogenic variants, even if present in a virus pool, may not always be detected when inoculated into animals because of the interference produced by non-diabetogenic variants. Lastly, our earlier studies with EMC virus and our present study with the D variant have shown that only certain inbred strains of mice will develop diabetes when infected and that susceptibility is genetically controlled. Although the strains of mice that are diabetes prone or diabetes resistant are known for EMC virus, other strains of mice or animal species may be required to demonstrate the potential diabetogenicity of human viruses.

8. Many different clinical types of hyperplasias and papillomas (e.g., focal epithelial hyperplasia, verruca vulgaris and planna, multiple and single oral papillomas, condylomata accuminatum, laryngeal papillomas) occur in the oral cavity. Some of these lesions are caused by human papilloma viruses (e.g., HPV). Over the last couple of years, an immunological technique (peroxidase anti-peroxidase) (PAP) has been adapted to identify HPV antigens in lesions of the oral cavity. HPV is the only virus that unequivocally causes hyperplasia and, perhaps, neoplasia in man. Until now, there has been no readily available test other than electron microscopy to effectively screen for HPV in hyperplasias and neoplasias suspected of having a papilloma virus etiology. By use of the PAP technique, it is now possible to readily screen and study the pathogenesis of papilloma viruses in the oral cavity. Future experiments will be directed towards evaluating the role of HPV in a variety of oral cavity lesions and, in particular, leukoplakia.

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 ZO1 DE 00080-07 LOM

PERIOD COVERED
October 1, 1979 - September 30, 1980

NO1 DE 92420

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

Notkins, Abner L.	Medical Director	LOM	NIDR
Yoon, Ji-Won	Research Microbiologist	LOM	NIDR
Dobersen, Michael J.	Staff Fellow	LOM	NIDR
Jenson, Alfred B.	Surgeon	LOM	NIDR
Aulakh, Gurmit S.	Expert	LOM	NIDR
Ray, Usharanian	Visiting Associate	LOM	NIDR
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Toniolo, Antonio	Visiting Fellow	LOM	NIDR
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COOPERATING UNITS (if any)
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SECTION

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TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
10.90.	5.90	5.00

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)

Biological characteristics of diabetogenic and non-diabetogenic variants of encephalomyocarditis (EMC) virus have been studied in tissue culture and in animal model systems. The underlying molecular mechanisms responsible for these properties are currently under investigation. The interaction of different environmental agents (e.g. viruses and chemicals) in the development of diabetes in experimental animals has also been studied. Cytopathology in human islet tissue as a result of acute viral infections has been the subject of a retrospective study.

Autoimmunological phenomena associated with juvenile-onset diabetes mellitus have been studied. This has included the identification and partial functional characterization of cytotoxic antibodies to islet cells in sera from diabetics and their first-degree relatives. An animal model system for virus-induced autoimmunity and diabetes has also been developed.

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 at an early age and is characterized by an acute onset, appearance of islet cell antibody, an infiltration of inflammatory cells in and around pancreatic islets (insulitis), and a reduction in the number of insulin-containing beta cells. Because of the abrupt onset and development of insulitis, an infectious etiology has long been suggested for JOD. Seroepidemiologic studies and numerous case reports have shown that a temporal relationship exists between the onset of certain virus infections and the subsequent development of diabetes. The recent isolation of a variant of coxsackievirus B4 from a boy who died shortly after the onset of diabetic symptoms is the strongest evidence to date that some cases of juvenile-onset diabetes mellitus might have a viral etiology.

Several lines of evidence also suggest an involvement of the immune system in the etiology of JOD. One of these has been the demonstration of humoral immunity against islet cells. Antibodies directed against intracellular components (islet cell antibody, ICA) and cell membrane antigens (islet cell surface antibody, ICSA) have been described. What role these autoantibodies play in the pathogenesis of JOD, however, is not clear.

Objectives

The objective of this research project is to investigate the role of viruses and the autoimmune phenomena in the pathogenesis of JOD.

Major Findings

Studies in animals: Inhibition of virus-induced diabetes mellitus. We have previously shown that plaque purification of the M-variant of encephalomyocarditis (EMC) virus resulted in the isolation of two stable variants; one diabetogenic and designated D variant and the other non-diabetogenic and designated B variant. When the D variant was inoculated into SJL/J male mice, hyperglycemia and hypoinsulinemia developed in over 90% of the animals. In contrast, none of the mice inoculated with the B variant developed diabetes. Histologic examination of pancreata from mice infected with the D variant revealed insulitis and necrosis of beta cells, while islets from mice infected with the B variant showed little

if any change. When islets were assayed for infectious virus, approximately 10 times more virus was recovered from animals inoculated with the D variant as compared to the B variant.

To see whether the non-diabetogenic B variant had any effect on the induction of diabetes by the D variant, the two viruses were mixed at various ratios and inoculated into SJL/J male mice. When the B variant was inoculated with the D variant at a 1:1 ratio, only 60% of the mice developed diabetes as compared to 95% with D variant alone. When the B and D variants were mixed at a ratio of 9:1, 11% or less of the mice developed diabetes and none of the mice developed diabetes when the B and D variants were mixed at a ratio of 99:1. When mice were first inoculated with the B variant and then given the same dose of the D variant at different times after infection, none of the animals developed diabetes. In contrast, all of the animals developed diabetes when inoculated with the D variant alone.

What is the possible mechanism of inhibition of the induction of diabetes? The B variant might inhibit the induction of diabetes by the D variant if the immune response to the former occurred more rapidly or resulted in higher antibody titer. To see whether there was a difference in the immune response between D and B variants, mice were infected with the B or D variant and antibody titers determined at different times after infection. No difference was found in the time of appearance or titer of antibody when the B and D variants were compared. Moreover, the B and D variants are antigenically quite similar. Antibody made against the D variant neutralized the D and B variants to about the same degree. Similarly, antibody made against the B variant neutralized the B and D variants equally well.

Further evidence that the immune response to the B variant is not responsible for the inhibition of diabetes by the D variant comes from cell culture experiments which bypass the immune response. Secondary mouse embryo cells were infected with the B variant, the D variant or a 1:1 mixture of the B and D variants. Close to 50 times more virus was produced in cultures infected with the D variant as compared to the B variant. Moreover, the mixture of the D and B variants produced considerably less infectious virus than the D variant alone.

When the supernatant fluids were examined for interferon, the D variant induced little if any interferon while substantial amounts of interferon were induced within 12 hours by the B variant. The mixed pool, containing both the B and D variants, induced intermediate amounts of interferon. Although there was a marked difference in the induction of interferon by the B and D variants, both of these variants were equally sensitive to the antiviral action of a standard mouse interferon when tested on secondary mouse embryo cells. To see whether the B and D variants induced different amounts of interferon in animals, mice were

infected and at various times thereafter, sera were drawn and assayed for interferon. Substantial amounts of interferon were induced by the B variant within 10 hours. In contrast, peak interferon titers were not reached until almost 30 hrs after infection with the D variant and the maximum titer was approximately one third that with the B variant.

These findings suggest that the rapid induction of interferon by the B variant protects beta cells from infection by the D variant, especially when both viruses are injected at the same time. The B variant also inhibited the induction of diabetes if inoculated from one to seven days before the D variant. Since interferon disappeared from the circulation within four days after inoculation of the B variant, the protection observed after four days is most likely due to the presence of cross-reacting neutralizing antibody. Thus, interferon and antibody, acting alone or in combination, appear to contribute to the B variant induced inhibition of diabetes.

Earlier studies in our laboratory showed that only certain inbred strains of mice developed diabetes when infected with the M variant of EMC virus. The demonstration that the M variant represents a mixture of the B and D variants raised the possibility that the D variant also might have a different host spectrum. To test this possibility, different inbred strains of mice were infected with the D variant and blood glucose indexes determined. Certain inbred strains of mice such as SJL/J, SWR/J, DBA/2J, NIH Swiss, NFS/N, and A/J developed diabetes, while others such as C57BL/6J, AKR/J, CBA/J, LP/J, and CE/J did not develop diabetes. The spectrum of host susceptibility, in general, was similar to that observed previously with the M variant, except the glucose indexes and the percentage of animals developing diabetes were higher with the D variant. In our previous experiments, the A/J strains of mice showed abnormal glucose tolerance tests but were not diabetic, while in the present experiment with the D variant, about 50% of the A/J mice developed diabetes.

The inhibition of diabetes by interferon raises the possibility that genetic factors might control the host's interferon response to infection with the D variant. Ongoing experiments, however, suggest that the time of appearance and the amount of interferon produced in response to the D variant is approximately the same in diabetes-resistant mice (C57BL/6J) and diabetes-prone mice (SJL/J). Interferon, nonetheless, may be important if beta cells from resistant mice as compared to susceptible mice are more sensitive to the antiviral action of interferon and thus rendered resistant to infection and the subsequent development of diabetes. Furthmore, the role of interferon in the prevention of virusinduced diabetes in susceptible animals is also very important. Both of these studies are now under way.

Molecular Studies. Nucleic acid hybridization studies were initiated to see if there were genetic differences between the D and B variants.

Using purified avian myeloblastosis virus reverse transcriptase, complementary single strand labeled DNA probes were generated to the RNA genomes of both variants. Preliminary results indicated that the genomic differences are too small to be detectable by nucleic acid hybridization, using total cDNA as a probe. Thermal melting studies of hybrids indicate that there are no detectable differences between the genomic RNA of these two variants. Experiments are in progress that will determine if there are any additions or deletions in the genetic makeup of these variants. In addition, using recombinant DNA technology, it may be possible to clone a DNA copy of the viral RNA genome. The cloned DNA could then be produced in sufficient quantities to conduct sequencing studies, restriction endonuclease analysis and could be used to determine gene order. This DNA also could be used as a probe in nucleic acid hybridization. Efforts are being made to synthesize a complete DNA copy of the viral RNA genomes of both variants. The complete DNA copies of these viruses will be cloned. The order of sequence would be determined. Restriction endonucleases analysis will be performed to detect any genomic differences between these two variants.

Another approach is to find structural differences between the D and B variants by analyzing the oligonucleotide fingerprints of T1digested RNA of these viruses. Preliminary findings indicate that the genome of the B variant may have a small deletion of about 20-25 nucleotides. The relationship between the expression of the deleted gene (if any) and the pathogenesis in susceptible animals will be studied if any differences in molecular levels are found.

Coxsackieviruses. Diabetogenic variants of Coxsackievirus B1, B2, B3, B5 and B6 have been studied in vivo in the animal model system. Coxsackieviruses originally obtained from the American Type Culture Collection have been passaged in cultures enriched for pancreatic beta cells to produce variants which induce a diabetes-like syndrome in SJL/J mice.

In preliminary experiments, the original unpassaged virus failed to raise the glucose index above control levels. Coxsackievirus that had been passaged several times in secondary mouse embryo cells also failed to raise the glucose index and failed to produce abnormal glucose tolerance tests. In those mice, viral antigens were found only in acinar cells by staining with fluorescein-labeled antibody to Coxsackievirus. In contrast, the passaged virus induced various degrees of abnormal glucose levels: 60% for Coxsackie B1, 50% for Coxsackie B2, 36% for Coxsackie B3, 50% for Coxsackie B4, 32% for Coxsackie B5, and 5% for Coxsackie B6. Infected SJL mice showed abnormal glucose tolerance tests at day 10, while none of the BALB/c mice showed abnormal glucose tolerance tests under the same conditions. Some of the Coxsackie B1, B2, B3, B4, or B5 virus-infected SJL mice also showed hyperglycemia, as evaluated by an increased glucose index.

Histopathology from animals infected with beta cell passaged Coxsackievirus revealed destruction of beta cells and infiltration of mononuclear cells in the islets. Frozen sections from the pancreas of infected mice also were stained with FITC-labeled antibodies to Coxsackieviruses. Coxsackie B1, B3 and B5 infected mice showed widespread viral antigens in the islet at 4 days after infection, but not in acinar cells. In comparison, Coxsackie B2 and B6 infected mice showed less viral antigens in the islet.

To be sure that the histologic and metabolic changes were not due to inadvertent contamination of beta cell-passaged Coxsackieviruses with EMC virus, immunofluorescence and neutralization tests were performed. Sera from beta cell passaged Coxsackie B1, B2, B3, B4, B5, and B6 infected mice neutralized strongly the original homologous unpassaged virus, but not EMC virus. Moreover, there was no cross reaction among sera from Coxsackie B1, B2, B3, B4, B5 and B6 infected mice.

It, therefore, appears that the various subtypes of Coxsackie differ in their ability to cause diabetes under similar conditions in an animal model system.

Cumulative Environmental Insults. So far, very little research has been done towards understanding how various factors (e.g., viruses, chemicals) interact in the development of diabetes. It is well known that normal animals have more than a sufficient number of beta cells to maintain glucose homeostasis and that hyperglycemia develops only after a severe depletion of insulin-producing cells has occurred.

We have previously shown that EMC virus induces diabetes only in certain inbred strains of mice, while in others, it only causes minimal alterations of glucose homeostasis or no effect at all. These differences have been shown to depend on the number of beta cells that become infected in different strains. This means that genetic factors govern the susceptibility of beta cells. To test this hypothesis, we minimally depleted the beta cell reserve of mice with streptozotocin, a well known beta cell toxin. In preliminary experiments, we determined a dose that would not alter glucose homeostasis. Twelve days after treatment, we infected several strains of mice with a small dose of the D-variant (diabetogenic) of EMC virus. In susceptible strains of mice, we observed a strong enhancement of the diabetic syndrome both in terms of serum glucose levels and in the percentage of animals developing diabetes. More striking results were obtained with diabetes-resistant mice: after pretreatment with sub-diabetogenic doses of streptozotocin, 18%, 30%, 53% and 100% of C3H/He, C57BL/6, CBA and AKR mice, respectively, developed diabetes after infection with EMC virus. It is noteworthy that none of these strains showed alteration in glucose homeostasis after infection with the virus alone. These results led us to test the effect of two widely distributed human viruses in mice treated with sub-diabetogenic

doses of streptozotocin. Sixty to 75% of SJL and NFS mice became diabetic after infection with Coxsackieviruses B3 and B5. The same strains of viruses only slightly affected glucose homeostasis in uncompromised animals. To check the validity of this system in showing the synergistic effect of chemicals and viruses in the induction of diabetes, we also infected mice with the B-variant (non-diabetogenic) of EMC virus. Diabetes did not develop in susceptible mice when streptozotocin was injected either before or after viral infection.

Therefore, in order to be diabetogenic, a virus must have a special capacity to infect and destroy beta cells even if the endocrine tissue of the host has already been damaged. To check whether this chemical treatment could influence the genetic susceptibility of beta cells to infection, we counted, by immunofluorescence, the number of beta cells infected in streptozotocin-treated and untreated animals. Very similar numbers were observed in both cases. Taken together, these results indicate that genetic factors are a constant with which environmental insults (chemicals, viruses) may interact to produce diabetes in experimental animals. Moreover, chemicals may have a synergistic effect with infectious agents eventually leading to overt diabetes.

Reoviruses and Autoimmunity. Reoviruses are widely distributed in the human population and it is well known that all the mammalian serotypes may replicate in a variety of tissues and organs (e.g., liver, pancreas, myocardium, respiratory and digestive tracts, etc.). Nevertheless, no clear cut clinical syndrome has ever been associated with these agents; actually, most of the infections are asymptomatic. Experimental work in animals has shown that reovirus type 1 may cause hydrocephalus and reovirus type 3 may cause active chronic hepatitis, biliary obstruction and runting; moreover, all three types seem to cause congenital infections and malformations. Recently, it has been found in our laboratory that reovirus type 1 may cause a diabetic syndrome in mice primarily by infecting and damaging insulin-producing beta cells. During these studies, we observed that neonatally infected animals showed high mortality, growth retardation, hypergammaglobulinemia and plasma cell infiltrates in various organs. These findings led us to investigate in detail the interaction of reovirus 1 with endocrine organs and the immune system.

Unlike EMC-induced diabetes, reovirus type 1 induces a slowly progressing glucose intolerance in mice which peaks at 14 to 20 days after infection and is associated with altered insulin and glucagon levels. Moreover, 30% to 60% of infected mice show a runting syndrome with ascites, oily hair, alopecia and increased levels of immunoglobulins. Using indirect immunofluorescence techniques, we observed the presence of autoantibody to pancreatic islet cells (ICA), cells of the anterior pituitary and parietal cells of the stomach. Fourteen days

after infection, 44% of neonatally infected NIH Swiss mice have ICA and 78% of them have antibody reacting with anterior pituitary cells. ICA and anti-anterior pituitary antibody were partially absorbed by purified insulin and growth hormone (GH), respectively; moreover, the same sera reacted with GH_3 cells, a cell line producing GH and prolactin.

To confirm the presence of autoantibody to hormones, we developed an enzyme-linked immunosorbent assay (ELISA): 100% of infected mice had antibody to GH 14 days after infection (range of titer 1:10 to 1:320) and 42% had antibody to insulin at day 30.

Histologically, we detected focal necrosis of pancreatic islets at day 5 followed by an extensive infiltration of plasma cells three weeks after infection. Sections of the anterior pituitary at day 14 also showed focal necrosis and inflammatory infiltrates with mononuclear cells and occasional polymorphonuclear leukocytes. These findings were confirmed by electron microscopy: crystalline assays of reovirus particles in different stages of morphogenesis were detected in pituitary GH cells as well as in alpha, beta and delta cells of pancreatic islets. These cells could be positively identified due to the presence of characteristic secretory granules. Developing virus particles were frequently associated with microtubles in infected cells. By radioimmunoassay, we measured GH levels in plasma of reovirus type 1-infected runted mice, non-runted mice and uninfected controls: 14 days after infection immunoreactive GH was strongly decreased in runted mice and approximately normal in non-runted animals.

At the present time, we are trying to evaluate the role of autoantibody to islet cells and insulin in the diabetic syndrome and also to relate the presence of anti-pituitary antibody to the runting syndrome. Many exciting questions remain to be answered; the most important being the mechanism of autoimmunity induction by reovirus and the possible role of viruses in human autoimmune pathology.

Immunological Studies. In the past five years, the role of humoral immune mechanisms in the pathogenesis of JOD has been intensively investigated. During this time, antibodies to intracellular components (islet cell antibody or ICA) and cell membrane antigens (islet cell surface antibodies or ICSA) have been identified. Our continuing study concerning the prevalence of islet cell antibody (ICA) in diabetics and their families has indicated that this antibody is more common in families having one or more diabetic children than in the general population. This has suggested that ICA may be more related to genetic factors (e.g., HLA types) or possibly to environmental factors (e.g., transmissible agents such as viruses) than to a direct role in the pathogenesis of JOD. The idea that ICA may be a useful predictive marker for JOD has been strengthened during the past year of follow-up studies by the finding that two siblings of diabetics, previously healthy but ICA positive, have developed JOD.

This study has been expanded during the past year to include gestational diabetics. These patients are characterized by glucose intolerance that has its onset or recognition during pregnancy and is distinct in classification from diabetics who become pregnant. It has been estimated that 35-50% of these gestational diabetics develop overt diabetes within ten years. Using sera from a longitudinal study performed between 1969-1972, we found that 73% of the ICA positive women are now being treated with insulin while only 8% of the ICA negative women are receiving an anti-diabetic agent. In addition, it was determined that 77% of ICA positive women also had HLA antigens HLA DR3 and DR4 (determinants which are strongly associated with JOD) compared with 17% of women who were ICA negative. The results of this study therefore seem to indicate that: (1) Gestational diabetes has features in common with JOD; and (2) ICA may be of some predictive value in determining the likelihood of developing JOD in gestational diabetics.

In the past year, we have embarked on a study of ICSA using two different approaches: (1) direct visualization of the antibody-antigen reaction by immunofluorescence techniques; and (2) a functional assay of the lytic properties of ICSA using conventional ^{51}Cr release. Viable rat islet cell monolayer microcultures were used in these experiments.

By the immunofluorescence technique, we found the prevalence of ICSA to be: JOD at onset, 88%; JOD less than 1 year after onset, 60%; JOD greater than 1 year after onset, 50%; maturity-onset diabetics 0%; and healthy controls 0%. We further demonstrated that only ICSA positive sera, in the presence of complement, were capable of mediating the release of ^{51}Cr from islet cell monolayers. Sera containing only ICA did not cause lysis of the cells. Since the islet cell cultures contain a number of different cell types, it was necessary to show specifically that beta cells were being damaged. Proof that the ICSA containing sera were lytic for beta cells was obtained by a new double-immunofluorescence technique in which lysed cells were identified simultaneously by their uptake of ethidium bromide into the nucleus (appearing red) and as beta cells by their staining with fluorescein-labeled insulin antibody (appearing green). Although these findings suggest that cytotoxic ICSA may contribute to the pathogenesis of JOD, family studies showed that approximately one-fourth of sera from non-diabetic first-degree relatives of diabetic probands were $ICSA^+$ and cytotoxic for beta cells. As mentioned above, we have found a similar relationship for ICA. In this way, JOD appears to be similar to other autoimmune diseases, such as thyroiditis in which approximately 40% of healthy first-degree relatives also have circulating thyroid autoantibodies. Evidence that non-diabetic $ICSA^+$ individuals are of a greater risk of eventually developing JOD awaits the results of long-term prospective studies.

Studies in Humans - Histopathology. We recently completed a survey to determine the effect of viruses on human islets of Langerhans by

examining pancreata from 250 children with fatal infections caused by at least 14 different viruses. Viral cytopathology was found in four of seven cases of Coxsackievirus B infections, 20 of 45 cases of cytomegalovirus infections, 2 of 14 cases of varicella-zoster infections, and 2 of 45 cases of congenital rubella. Degeneration of beta cells (lysis, necrobiosis) and acute insulitis with different cell types predominating, depending on the case, were found in neonates infected with the Coxsackievirus B group (1 B4, 1 B1, and 2 untyped B's).

Characteristic inclusion bodies without insulitis were observed in islets from cases with cytomegalovirus and varicella-zoster infections; obvious degeneration of islet cells was only seen with varicella-zoster. The rubella cases were remarkable for the relative absence of islet cell lesions; insulitis was only found in one islet of one case. These observations suggest that under certain conditions, islet cells can be infected in vivo.

Contract. A two year contract, "Screening for Diabetogenic Viruses" was awarded to Microbiological Associates and initiated in May, 1979. Forty candidate diabetogenic viruses are to be screened for their capacity to alter glucose homeostasis by infecting pancreatic islet cells of rats and genetically susceptible strains of mice. Each virus will be put through at least one part of a two-part screen procedure. The first screen measures the glucose indices and provides that frozen sections of murine pancreas be examined by immunofluorescence at different time intervals after infection. If the candidate virus infects pancreatic islet cells and induces hyperglycemia, then a second screen is done. The second screen measures concentrations of blood glucose and insulin and pancreatic insulin at different times after infection; it also provides pancreatic tissue for light and electron microscopic examination. The data supplied by these two screens provide the biochemical and pathological evidence necessary to determine if a virus is diabetogenic.

The well characterized diabetogenic virus, the D variant of EMC, was used as a positive control for the two screens. As expected, EMC caused diabetes in mice but not in rats. Mumps, a human virus, was passaged in rat beta cell cultures and then inoculated into neonatal and adult Fischer rats and neonatal SJL/J mice without development of diabetes. Reovirus type 1, a human virus diabetogenic for mice, was passaged in mouse beta cell cultures then in rat beta cell cultures before inoculation into neonatal rats that did not subsequently develop diabetes. Coxsackievirus B4, which is diabetogenic for both mice and man was passaged in rat beta cell cultures and then inoculated into young adult rats; none of the rats developed diabetes. Ten clones of the M variant of EMC were inoculated into susceptible strains of mice with results ranging from almost no diabetes for some clones to almost 100% diabetes for others. Finally, since none of the Fischer rats became diabetic after inoculation

with viruses (mumps, reovirus type 1 and Coxsackievirus) that were enriched for their beta tropic properties by passage through beta cell cultures, five other strains of rats are being tested for their susceptibility for developing diabetes after inoculation with these viruses.

Implications for Biomedical Research

The juvenile form of diabetes is the most difficult to control and the short and long term effects are enormous with respect to the patients health and economics. Recent evidence suggests that viruses and/or autoimmune mechanisms play a role in the initiation of this form of diabetes. Identification of diabetogenic viruses and preparation of a vaccine might ultimately prevent the disease. Further study of the immune phenomena present in juvenile-onset diabetics may result in a reliable predictive marker to identify those individuals at risk. Also, a detailed understanding of the immunology of diabetes may form the basis for immune intervention in the disease process.

Future Plans

Our future plans for research on virus-induced diabetes are aimed at (1) isolating viruses from patients with acute JOD and determining if these viruses are diabetogenic in animal model systems; (2) identifying diabetogenic variants of prototype viruses that are widely distributed in the human population by plaque-purification and by testing in animals; (3) identifying the molecular differences between diabetogenic and non-diabetogenic variants of EMC virus which can ultimately be used to isolate "diabetogenic genes" or gene products; (4) identifying inbred strains of animals that are susceptible and resistant to virus-induced diabetes with the goal of evaluating the genetic and non-genetic factors controlling susceptibility.

Attempts will be made to establish beta cell lines from humans so as to (1) study the effect of autoimmune antibody, and (2) select beta tropic viruses. Prospective studies will be carried out on ICA^+ and $ICSA^+$ individuals in order to better define the role of these antibodies in the pathogenesis of JOD. Also underway is the further characterization of ICSA by the use of the double-fluorescence technique, described above, with human islet cells. It should be possible to determine whether ICSA is cytotoxic to other hormone-producing islet cells (glucagon, somatostatin or pancreatic polypeptide). This adaption would be particularly useful since the supply of human islet cells is limited and preparation is difficult. Finally, using ICA and ICSA as affinity reagents, we will attempt to isolate and characterize the respective antigens.

Publications

1. Notkins, A.L., Yoon, J.W., Onodera, T., and Jenson, A.B.: Virus-induced diabetes mellitus: Infection of mice with variants of encephalomyocarditis virus, Coxsackievirus B4, and reovirus type 3. In Treatment of Early Diabetes, edited by R. A. Camerini-Davalos and B. Hanover, Plenum Publishing Corp., pp. 137-146, 1979.

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7. Yoon, J. W., McClintock, P. R., Onodera, T., and Notkins, A. L.: Virus-induced diabetes mellitus. XVIII. Inhibition by a non-diabetogenic variant of encephalomyocarditis virus. J. Exp. Med. (in press) 1980.

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 00094-07 LOM

PERIOD COVERED: October 1, 1979 - September 30, 1980 CT 0060114

TITLE OF PROJECT (80 characters or less)

Ulcerative Lesions and Tumors: Aphthous Ulcers - Clinical Trials

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)

Strober, Warren	Medical Director	MET NCI

LAB/BRANCH
Laboratory of Oral Medicine

SECTION

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.10	1.10	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)

The purpose of this project is to study the pathogenesis of recurrent aphthous stomatitis with the ultimate aim of preventing this condition by effective treatment and prevention. The recently initiated protocol aims at investigating the pathogenesis of aphthous stomatitis by both clinical and immunological studies. The clinical research involves investigating patients hematologically and from a general medical point of view to determine the incidence of precipitating factors involved in the pathogenesis of aphthous stomatitis. The incidence of food allergies is being explored and in vitro food allergy tests are being carried out in order to assess their specificity and sensitivity. The relationship between gluten-induced (celiac) disease and aphthous stomatitis is also being investigated. In addition, a double-blind trial into the effects of zinc sulfate tablets is being investigated. In vitro laboratory immunological tests are also being carried out in patients and in a control population. These tests involve lymphocyte transformation, assays for lymphokine and monokine and also assays of suppressor cell function. Finally, family studies are being carried out on these patients including HLA studies.

ULCERATIVE LESIONS AND TUMORS: APHTHOUS STOMATITIS

Background and Objectives

Recurrent aphthous stomatitis is a chronic recurring condition of the oral cavity characterized by single or multiple recurrent painful ulcerations which occur in approximately 20% of the population. In the past, these ulcerations have been thought to be autoimmune in nature. Recent studies have indicated that it is possible to modulate or eliminate some aphthous ulcers by replacing deficiencies of iron, folic acid or vitamin B12. In addition, some cases of recurrent aphthous stomatitis are associated with an increased incidence of celiac disease (gluten-induced) enteropathy.

The purpose of this project is to study the pathogenesis of recurrent aphthous stomatitis with the ultimate aim of treating and preventing this condition.

Streptococcus sanguis can be isolated from recurrent aphthous lesions. It is the purpose of this study specifically to investigate patient's immune response against this organism when compared with a normal control population without ulceration and to identify methods of modulating any deviations from normal in the immune response seen in these patients. We intend to investigate these hypotheses by using lymphocyte transformation as our index of immune function and to do detailed studies on subpopulations of immunocompetent cells using lymphocyte transformation as the basis assay. In addition, assays for lymphokines and monokines are being carried out and also tests for histamine release in response to various food antigens which are thought to be clinically relevant in the disease process. Furthermore, there is a distinct familial tendency in this condition and family studies are being carried out in conjunction with HLA and immunoglobulin allotyping. It is hoped that as a result of these investigations, a correlation will be found between HLA or immunoglobulin phenotypes and disease activity.

Major Findings and Implications

Initial results using lymphocyte transformation as an immune function test indicate that patients with recurrent aphthous stomatitis have an impaired blast transformation against streptococcus sanguis that is statistically significant. Monokine production, however (specifically lymphocyte activating factor), seems to be unaltered in these patients. Deviation in the production of chemotactic factor are being actively investigated. Further investigation is being carried out to identify the mechanism whereby patients have a suppressed blast transformation against strep. sanguis. It is hoped as a result of these investigations to establish whether this hypo-responsiveness is a result of suppression

either by macrophages or T suppressor cells or due to a lack of T cell help. Preliminary investigations suggest that macrophage suppression is not an important factor. More sophisticated suppressor cell assays are now being initiated.

Clinically important modulating factors are being investigated in a series of 100 patients attending the clinical center. This work involves the isolation of patients with nutritional deficiencies (iron folate and vitamin B12 deficiencies) and also the isolation of those patients who have a gluten-induced enteropathy (celiac disease). In addition, patients are being investigated for food allergy and it is hoped that food allergens, when isolated, will be relevant to the pathogenesis of the disease process. In addition, a double-blind trial is being initiated to assess the effects of zinc sulfate (660 mg daily) and its effectiveness in eliminating the disease. Family studies are also being carried out in conjunction with HLA and immunoglobulin allotyping in order to establish a genetic link between patients with the disease and their HLA and immunoglobulin allotypes.

Significance to Biomedical Research

Recurrent aphthous stomatitis is an extremely common condition affecting some 20 percent of the population and has many similarities with autoimmune diseases. It is hoped that as a result of the foregoing investigations, better insight will be gained into the mechanisms involved in tissue injury as a result of inappropriate immunological responses to exogenous agents. This may have further implications as far as understanding the pathogenesis of autoimmune diseases.

Proposed Course

It is proposed that when more evidence is available as to the nature of the suppression involved in the blast transformation of patient's lymphocytes against strep. sanguis, that the products of lymphocyte
activation (i.e., immunoglobulin synthesis and lymphokine production) can be more carefully assessed with the hope of ultimately identifying the pathogenic pathways involved which result ultimately in damage to the target tissue, the oral mucosa.

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 00123-07 LOM

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

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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7.75	3.55	4.20

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

The overall aim of this project is to study the immunology, molecular biology and neurobiology of herpes simplex virus (HSV) latency and reactivation in the peripheral nervous system and central nervous system. An immunological system for the detection of in vivo reactivation permits the evaluation of factors that induce reactivation. The molecular state of the latent HSV genome is being studied by DNA hybridization techniques.

HERPES VIRAL LATENCY

Introduction

Herpes simplex virus (HSV) is the most common cause of recurrent infection in this country. Estimates of the yearly occurrence include 98,000,000 cases of herpes labialis, 2,000,000 cases of genital recurrences, and a half million cases of corneal infection. In addition to this considerable morbidity, fatal cases occur both in newborns with a generalized infection and in adults with sporadic encephalitis.

Our principal interest is the pathogenesis of recurrent HSV disease. All of our work has been done in the mouse model. HSV is inoculated on epithelial surfaces (lip, cornea, footpad, vagina). The virus enters nerve terminals and spreads by axoplasmic flow to the nerve cell bodies in ganglia. Then an acute or productive infection occurs in ganglion cells. In about two weeks, the infection undergoes a transition from the productive acute infection to the latent state. Recurrent disease can occur only after HSV reactivates in ganglia and migrates down the axon to the epithelial surface.

The present report on herpes latency and reactivation will be divided into two parts: 1) Biological Studies: Immunology and Neurobiology and 2) Molecular Studies.

BIOLOGICAL STUDIES: IMMUNOLOGY AND NEUROBIOLOGY

Immunological Studies of Reactivation. In biological studies, we define latency in operational terms by the isolation of infectious virus in ganglionic explant cultures and cell-free homogenates. Cell-free ganglionic homogenates measure only infectious virus present *in vivo* in the ganglion. In contrast, explant cultures detect HSV either as a result of infectious virus or as a result of *in vitro* reactivation of latent virus in viable cells of the explant tissue. Therefore, the latent state is characterized by a virus-negative homogenate and a virus-positive ganglionic explant culture.

Over the past few years, we have studied three biological aspects of HSV ganglionic infection: 1) the immune conditions needed to establish latency, 2) factors required to maintain the infection in the latent state, and 3) stimuli that induce virus reactivation. Past annual reports have touched on all three aspects. The present section will be concerned with a new method that we have used in the last year to detect reactivation.

We designed *in vivo* experiments specifically to determine whether anti-HSV antibody is needed to maintain latency. Balb/CJ mice were inoculated with HSV by the lip or corneal route and injected (intraperitoneal) with rabbit anti-HSV serum 3, 48, 96, and 144 hours after virus inoculation. In control mice injected with normal rabbit serum,

serum neutralizing antibody increased and then maintained a plateau (64 units) for at least 12 months. In contrast, in passively immunized experimental mice, the antibody titer - initially high from passive immunization - decreased to low or undetectable levels two months after virus inoculation (less than eight units). Therefore, hyperimmune rabbit serum interfered with the normal humoral response of the mouse to HSV antigens.

To check the status of the infection, neutralizing antibody-negative mice were sacrificed four months after virus inoculation, and the trigeminal ganglia were assayed by homogenization and explantation. We found that the mice were infected (positive ganglionic explant cultures), and the infection was maintained in the latent stage (negative ganglionic homogenate cultures). Therefore, once latency was established, neutralizing antibody in serum was not required to maintain the infection in the latent state.

The negative neutralizing antibody titer in these mice could be explained either by immunological "tolerance" to HSV antigens or by insufficient antigenic stimulation to boost the humoral response. To exclude immunological tolerance, we reinoculated some antibody-negative, latently infected mice by another route. All of the reinoculated mice showed an increase in antibody titer. Therefore, the persistently low antibody titer suggests insufficient antigenic stimulation.

More recently, we have used a sensitive antibody binding assay (ELISA test using rabbit anti-mouse IgG antibody conjugated with alkaline phosphatase) to detect mouse HSV directed antibody. Sera from 15 latently infected mice previously demonstrated by microneutralization or plaque reduction to be seronegative were all found to possess HSV specific binding antibody. The magnitude of the immune response was, however, considerably lower than HSV infected mice that were treated with normal rather than with immune rabbit serum. These differences may merely be quantitative where the increased sensitivity of the ELISA test may detect antibody at levels below the sensitivity of the neutralization assays. Alternatively, qualitative differences in the immune response may produce antigen binding antibody directed only against non-neutralizing antigenic determinants. Future studies will attempt to elucidate the nature of the humoral immune response in passively immunized latently infected mice.

The ability of antibody-negative, latently infected mice to respond immunologically to HSV antigens was utilized as a method to detect reactivation. In these experiments, the reactivation was demonstrated by the development of neutralizing antibody (or a significant rise in HSV binding antibody as detected by the ELISA test) in latently infected mice inoculated with HSV by the lip route. In one series of experiments, 29 mice were treated with three ten-day courses of dry ice lip trauma. Reactivation occurred in 26 of these mice (90%) as detected by an increase in antibody titer. Similarly, electric shock to the lip

induced reactivation in 79% of the mice and phorbol myristic acid applied to the lip induced reactivation in 73% of the mice.

In these experiments, we used antibody-negative, latently infected control mice that were bled on the same schedule but given no other treatment. There was an increase in antibody titer in four of these 54 mice followed over a two to four month period (9%). It is unlikely that the repeated retro-orbital puncture resulted in viral activation. As described later, repeated eye stimulation of lip infected mice (or the converse) failed to reactivate virus. It is more likely that these results indicate a low frequency spontaneous activation.

In previous experiments, we detected reactivation by the recovery of infectious virus from ganglia at the latent stage of the infection. A major disadvantage of this method is that the mice must be sacrificed and only one point in time can be evaluated. In contrast, by monitoring serum antibody in neutralizing antibody-negative, latently infected mice, we can carry out long-term experiments. Since antibody titers remain elevated for long periods of time, reactivation can be detected weeks or months after the actual reactivation event occurred. Thus, this new sensitive technique should facilitate our attempts to study biological mechanisms of reactivation.

The results of our reactivation studies indicate that epithelial trauma induces reactivation in a high percentage of mice. Preliminary experiments indicate that the site of trauma is of major importance. The stimulatory treatments must be applied to an area innervated by the latently infected neurons. That is, lip but not corneal trauma will induce reactivation in mice inoculated with HSV by the lip route. Conversely, corneal scarification induces reactivation in mice inoculated with HSV by the corneal (7/15) but not the lip route (0/15). Therefore, it is probable that the mechanisms involve stimulation of nerve terminals that correspond to the latently infected ganglion cell. The fact that corneal scarification of a lip infected mouse or lip trauma to a corneal route inoculated mouse do not result in virus reactivation suggests further that generalized stress may not be an effective stimulus for reactivation.

How epithelial trauma induces reactivation is not known. Our working hypothesis is that ganglion cells are ordinarily non-permissive for HSV replication. However, certain stimuli may act as signals to switch the ganglion cell to a permissive state. Some stimuli may act directly on ganglia (e.g., neurectomy or the suggested cytotoxic effect of cyclophosphamide or X-irradiation). Other stimuli may act peripherally at nerve terminals.

This hypothesis can be used to explain the establishment of latency. With HSV inoculation on epithelial surfaces, the virus or associated inflammation may act on nerve terminals setting up a signal that switches the ganglion cells to a permissive state. This change enables the input virus to replicate in ganglion cells. In this particular model, the immune response acts predominantly at the epithelial surface. With control of the epithelial infection, the signal is removed and the ganglion cell - now containing HSV DNA - reverts to a non-permissive state. This hypothesis,

therefore, can be used to explain the rapid establishment of latency in passively immunized mice discussed in last year's annual report. During latency, there is a block in HSV transcription, and the ganglionic infection is quiescent. Epithelial trauma (e.g., with dry ice burn) can once again switch ganglion cells to a permissive state. Virus replication then ensues in ganglia, and recurrent disease may occur at the epithelial surface.

Neurobiological Studies. We have recently developed two techniques that will improve the detection and study of in vitro reactivation. During the course of reactivation in vitro, there is presumably a de-repression of the replicative block leading to virus expression in latently infected ganglionic cells. Standard explantation culture techniques result in extensive necrosis of the ganglion and hence a loss of many cells prior to viral reactivation. We have worked out a gentle procedure for the dissociation of adult trigeminal ganglion with excellent cell viability over the course of several weeks. The neurons are so well maintained that extensive neurite growth is observed in these cultures. Schwann cells, as well as fibroblastoid cells, have also been identified by immunochemical techniques in the cultures. Ganglia from both normal as well as from latently infected mice have been successfully cultured, permitting both the study and comparison of in vitro acute infection with in vitro reactivation of latently infected ganglia. The increase in sensitivity may allow detection of latent virus in other components of the peripheral nervous system as well as the central nervous system. In addition, this procedure will also allow the quantitation of the fraction of ganglionic cells that have reactivatable virus. Thus, the study of the effects of antiviral drug treatments, immunological manipulations etc., upon the percent of latently infected cells rather than the simple presence or absence of a latent infection will now be possible.

Standard fixation procedures (such as acetone) for the immunofluorescent detection of viral antigens, both in cell cultures and infected tissues, result in poor preservation of cellular morphology and architecture. We have developed a fixation procedure (4% para-formaldehyde + 0.1% glutaraldehyde) which provides excellent preservation of cellular ultrastructure without loss of viral antigenicity. The use of immunoperoxidase labeling techniques permit the detection and screening of labelled viral antigens by light microscopy prior to electron microscopic study. These techniques, used in conjunction with immunohistochemical markers, will allow the unequivocable identification of which subpopulation(s) of cells possess reactivating virus in our trigeminal ganglion culture system.

Future Studies

1. To continue evaluating the ability of different treatments to activate latent virus (as detected by a rise in HSV antibody) in vivo. These procedures will be selected for usefulness in appraising unifying hypotheses of activation such as local inflammation.

2. To determine the nature of the immune response (antibody detectable by ELISA but not neutralization procedures) to HSV in passively immunized HSV

infected mice. Sera from these mice will be compared with sera from control (HSV infected, treated with non-immune rabbit serum) mice in the PAGE profile of immunoprecipitated HSV polypeptides. In this manner, it will be determined whether passively immunized mice make antibody to some but not all antigenic determinants of HSV.

3. The culture procedure will be used to: (a) study reactivation of virus in latently infected ganglia by immuno-labeling methods, (b) detect latent virus in mouse CNS tissue such as cerebral hemispheres and trigeminal nuclei, (c) to quantitate the percentage of latently infected neurons in the trigeminal ganglion following different experimental procedures such as treatment with the anti-herpes drug acycloguanosine or induction of local inflammation during acute infection, (d) to search for latent HSV in human CNS tissues obtained during autopsy.

MOLECULAR BIOLOGY

State of the Viral Genome during Latency. As in past years, the major thrust of our work has been directed to the analysis of the HSV genome during latency, specifically to the determination of the physical state of the viral DNA in trigeminal ganglia of latently infected mice. The experimental strategy was described in last year's progress report and is only outlined here:

1. DNA from latently infected ganglia is digested with the restriction endonuclease EcoRI and enriched for viral DNA sequences by affinity chromatography and reverse phase chromatography.

2. The DNA species are further separated on the basis of their molecular weights and blotted from the agarose gel into nitrocellulose paper.

3. Paper-bound DNA is hybridized to a high-specific activity ^{32}P-HSV DNA probe. The probe is made from a terminal fragment of the viral DNA, corresponding to approximately 3% of the genome. Detection of the hybrids is done by autoradiography.

Using this experimental approach we found 18 molecular species of terminal fragments from the viral DNA in the trigeminal ganglia of latently infected mice. Nine of these DNA fragments had molecular weights ranging from 3.5 to 9.0 million daltons (mD); one has a molecular weight of 3.5 mD; the other eight had MW range of 0.7 to 3.5 mD. Since the true terminus of the EcoRI restricted viral DNA has a MW of 3.5 mD, these results indicated that some of the viral DNA molecules in the latently infected ganglia had cell DNA sequences covalently joined to them, and others did not. From these experiments it was tentatively concluded that a fraction of the HSV DNA in ganglia was integrated. The remainder was episomal. However, the presence of nine discrete integration sites, as well as of termini at MW's lower than the MW of free, non-integrated termini, was puzzling.

In order to rule out the possibility of genome rearrangements during latency, that would result in various combinations of viral DNA sequences not present in the infecting virus population, we decided to examine the restriction endonuclease pattern of virus reactivated from ganglia, and compare it with the pattern given by the inoculum. The reactivated virus gave restriction endonuclease patterns typical of the HSV-1 F strain used in the laboratory. To our surprise, the inoculum virus did not. Purification of the inoculum in isopycnic gradients, followed by separation of DNA species in cell gradients and analysis of these species by restriction endonucleases showed that a large portion of the viral inoculum (>80%) was formed by defective viral particles. These particles interfere with the replication of infectious viruses and are generated during high multiplicity passage of the virus. Most of the defective particles contain tandem repetitions of a small fraction of the viral genome (5-10%) that encompass the termini among other viral DNA sequences. The various arrangements in the defective particles may give rise to the profusion of viral DNA termini found in the ganglia as well as to the bonafide integrated sequences that were found. These findings made quite uninterpretable the results obtained for the integration experiments.

In order to repeat those experiments it was necessary to prepare viral inoculi free of defective viruses. This objective was achieved by low multiplicity passage of the virus. This pool has been used to inoculate 500 mice and obtain their trigeminal ganglia to repeat the experiments described above. A modification has been introduced in the experimental approach. Since the combination of affinity and reverse-phase chromatography often led to a large loss of DNA (usually 50%), we have designed with the collaboration of Mr. Clarence Sidney Johnson, NIDR Machine Shop, a simplified version of a preparative gel electrophoresis apparatus. We have tested it with reconstitution mixtures of viral and cellular DNA's. Its performance is excellent, equal to that of commercially available "gene machines" for a fraction of their cost, and the recovery rates are 90-100%. We plan to use it as an initial step in the enrichment of viral DNA sequences from the ganglia. Mr. Johnson has also designed a plastic "hybridization spot-tester" that allows the analysis of several hundred fractions by molecular hybridization at a time, and greatly simplifies time consuming techniques. Mr. Johnson's help and ingenuity have been invaluable to our progress this year.

HSV DNA in the Central Nervous System. Experiments initiated in 1978 to study latency in the central nervous system (CNS) continued during 1979 and 1980.

Cerebral hemispheres and brain stems of mice that had been corneally inoculated with HSV were examined for the presence of reactivable virus and/or viral DNA sequences at both 6 days and 8 weeks postinoculation. Six days after inoculation infectious virus was found in homogenates of 18 of 20 cerebral hemispheres and in 6 of 20 brain stems of the same mice. At this time 100% of the mice had viral DNA sequences in both organs, with a range of 1-5 copies/100 cells in the hemispheres and of 22-178 copies/100 cells in the stems. At 8 weeks postinoculation none of the mice had free virus in CNS homogenates and only 1 of 20 animals had cerebral hemispheres that

were positive by explantation. In contrast, 3 of 20 hemispheres and 4 of 20 brain stems had viral DNA sequences ranging from 0.5 to 9 copies/100 cells. These results show that viruses that progress from the peripheral nervous system into the CNS are not eliminated, but are capable of establishing a latent infection in the CNS that cannot be reactivated by standard explantation techniques.

Molecular Hybridization Detection of Latent HSV Genomes in Passively Immunized Mice. As described earlier, passive immunization results in a by-pass of the acute phase of the ganglionic infection without preventing the establishment of latent stage. Essentially all passively immunized mice are latently infected. Hybridization studies were conducted to determine whether passive immunization altered the quantity of HSV genetic information in the ganglion.

Viral DNA sequences were found at a level of 2 copies/cell in trigeminal ganglia of normal rabbit serum (NRS) treated mice at 5 days p.i., but only at a level of 0.14 copies/cell in immune rabbit serum (IRS) treated mice. Thus, passive immunization decreased the extent of viral DNA replication by a 14-fold factor to levels comparable to the latent stage in NRS treated mice. At the latent stage, viral DNA sequences were at a level of 0.17 ± 0.02 copies/cell in NRS treated mice and at a level of 0.08 ± 0.01 in IRS treated mice, corresponding to only a 2-fold difference.

To extend these findings and to determine whether the differences are due to fewer copies/cell or fewer cells that are infected, the quantitative explantation technique will be utilized. These results indicate that replication of the virus during the acute stage has little effect on the number of viral DNA molecules found in infected cells during the latent stage.

Future Studies.

Our immediate studies in molecular biology will be directed to continue the determination of the physical state of the viral genome during latency and cloning by recombinant DNA techniques the cellular joint sequences in the integrated viral DNA molecules. In the near future we will study the possibility that integration occurs for the input parental DNA molecules. To this end we will use passively immunized animals to eliminate virus multiplication and the presence of a large excess of progeny DNA molecules. Since the presence of latent viral DNA in the CNS of experimental animals has been confirmed, we will extend our studies to the naturally occurring HSV infection in humans. HSV is widespread in human populations and the presence of virus or of portions of the viral genome in the CNS of humans may be indicative of a possible role for HSV in neurological diseases of unknown etiology. To this end, DNA from brain stem and cerebral hemispheres of cadavers will be purified and examined for the presence of viral DNA sequences by the same preparative and analytical methods employed for experimental animals.

RECOMBINANT DNA

Molecular cloning techniques using recombinant DNA molecules have been introduced into the LOM and will be focused in two major areas of research.

Firstly, we have embarked on a collaborative inter-institutional project to produce a safe, effective HSV sub-unit vaccine using recombinant DNA technology. The conventional approach to this problem is to grow the virus in a suitable host and purify the desired vaccine components from extracts of infected cells. Such a sub-unit vaccine has to be free of HSV nucleic acids to avoid possible oncogenicity. This approach is tedious, time consuming, and costly, especially since relatively large amounts of infected cells have to be processed. Our approach to be described here will obviate most of the problems associated with producing a safe sub-unit vaccine to HSV.

The experimental approach, in brief, is to isolate the gene(s) coding for the major antigenic determinants (glycoproteins) of HSV-1 and clone these genes in a bacterial plasmid such that their expression into protein will be facilitated after introduction of the hybrid plasmid into E. coli. The HSV polypeptide (antigen) synthesized in E. coli cells will then be tested as a vaccine to prevent HSV latency in the mouse model system.

The gene coding for the HSV glycoprotein B2 has been selected as the ideal gene to be cloned initially for the following reasons: (1) it is a constituent of the virion envelope and also of the infected cell plasma membrane, (2) it appears to play a critical role in the process of penetration of the virus into cells, (3) antiserum raised against highly purified B2 also contains neutralizing antibodies, and (4) this antiserum also precipitates the non-glycosylated polypeptide precursors of B2.

The B2 gene has been localized in a fairly large HSV DNA segment (the Xba I-F restriction endonuclease fragment) by genetic and physical mapping techniques. In order to clone the B2 gene, it is necessary to identify the coding sequence of the gene within the Xba I-F fragment. A novel approach is to purify the B2 glycoprotein and sequence the NH_2-terminal portion of it. From this amino acid sequence data and knowledge of the genetic code, the corresponding DNA nucleotide sequence can be deduced and a short segment of DNA having this nucleotide sequence can be synthesized. This DNA segment will then be used as a probe to locate the NH_2-terminal (5') portion of the gene which is the start of the coding sequence. Precise identification of the carboxyl terminus (3') of the B2 gene is not as important, but can be achieved in much the same way. Once the coding sequence has been mapped, it will be isolated using restriction endonucleases and cloned in an expression plasmid. E. coli cells transformed by this hybrid plasmid will be selected and screened for the presence of the B2 gene (DNA) and evidence of its expression (protein).

Initial attempts to clone the Xba I-F fragment involved insertion of the fragment into the Pst I site of the plasmid vector PBR 322 using dG-dC homopolymer tailing. In this procedure the Xba 5'-sticky ends are first

removed by treatment with S1 nuclease and thereafter α ^{32}P-dGTP is added to the 3'-end of the duplex DNA to produce a homopolymer tail. The terminal transferase reaction had to be optimized so as to add between 12-18 dG residues per end. The Pst I cut vector DNA was similarly tailed with α ^{32}P-dCTP. Characterization of the reaction products, however, indicated firstly that >800 dG residues had been added per molecule of Xba I-F, due to the fact that the fragment contains naturally occurring nicks and/or gaps which serve as substrates for the terminal transferase. Secondly, a large proportion of the fragment had been digested randomly by the S1 nuclease to yield short sub-fragments of the Xba I-F fragment. These findings meant that the Xba I-F fragment could not be cloned in the Pst I site of PBR 322 using the homopolymer tailing. Attempts to repair the nicks and gaps in the Xba I-F fragments using Ecoli DNA Pol I prior to tailing were only partially successful and therefore this approach was abandoned.

The second approach involved digesting the Xba I-F fragment with Eco R1 and inserting these fragments into the Eco R1 site of the plasmid PBR 325. Since the Eco R1 site lies within the chloroamphenicol gene, successful cloning is indicated by isolating clones having the phenotype Amp^r Cam^s Tet^r. To date 12 such colonies have been isolated and are being screened for the presence of HSV 1 DNA sequences.

The third approach to clone the Xba I-F fragment was to ligate synthetic Hind III linkers to it and insert it into the Hind III site of the plasmid PBR 322. The Xba-F fragment was first treated with Ecoli DNA Pol I to produce a blunt end fragment. Next the Hind III linkers (5'-OH) were labelled with γ-^{32}P-ATP and T4 polynucleotide kinase to give 5'-^{32}P ends. The integrity of the linkers was checked by electrophoresing them in a 20% Acrylamide-7M Urea gel, which was autoradiographed to visualize the linkers. The kinased linker was then ligated to the blunt ended Xba-F fragment and then digested with Hind III to produce Hind III cohesive ends at each end of the Xba-F fragment. Free linkers were separated from the fragment by electrophoresis in a low melting point agarose gel followed by reisolation of the fragment, a step which produced considerable losses of the fragment. Finally, the fragment with the Hind III linkers was inserted into the Hind III site of the plasmid PBR 322. Several hundred colonies were isolated and these are being screened by in situ hybridization using a nick translated ^{32}P-HSV 1 DNA probe.

The Xba F fragment is about 5x larger (22 kb) than the plasmid vectors and is difficult to clone using these vectors since the cloning efficiency decreases as the size of the insert fragment increases. Also, the larger the cloned fragment is, the greater is the instability of the plasmid and the more likely are rearrangments and deletions to occur. In order to circumvent these potential problems the lambda (λ) vector Charon 28 was used to clone a large Bgl II-I fragment which is contained within the Xba F fragment and which is known to contain the B2 glycoprotein gene. The λ-Ch 28 vector contains two Bam Hl sites in which fragments ranging in size from 6 kb to 19 kb can be cloned; 6 kb being the minimum required to give a viable phage. Since the Bgl II and the Bam Hl cohesive ends are complementary,

the Bgl II-I fragment can be cloned in the Bam Hl site of Ch 28. The Bgl II-I fragment was ligated to the purified Bam Hl Ch 28 vector arms using T4-ligase. The ligated recombinant molecules were then packaged into viable λ Ch phage particles in vitro and 10 μl were plated onto λ-sensitive Ecoli. About 30 λ-plaques were seen indicating that a total of about 3000 viable phage had been produced in the packaging reaction. These recombinant phage are being screened for the presence of the HSV 1 Bgl II-I fragment using ^{32}P-HSV 1 DNA as a probe.

Once the desired cloned fragment has been obtained, the B2 gene contained therein will be isolated and cloned in an expression plasmid as discussed previously.

Secondly, the recombinant DNA techniques will be employed to clone the cellular joint sequences in the integrated viral DNA molecules isolated from latently infected mouse trigeminal ganglia.

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3. Openshaw, H. O., Puga, A., and Notkins, A. L.: Latency and reactivation of herpes simplex virus in sensory ganglia of mice. In Virus-Lymphocyte Interactions: Implications for Disease. Edited by Max R. Profitt, Elsevier/North-Holland, Inc., New York 7:301-306, 1979.

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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 00200-04 LOM

PERIOD COVERED

October 1, 1979 - September 30, 1980

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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Notkins, Abner L.	Medical Director	LOM	NIDR

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

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

Papillomavirus antigens were detected in verruca, multiple papillomas, condylomata and laryngeal papillomas of the oral cavity by the peroxidase-antiperoxidase technique.

PAPILLOMAS OF THE ORAL CAVITY

Background

The role of human papillomaviruses (HPV) in the induction of cutaneous and mucosal hyperplasias and papillomas was the subject of a recent review by us for "Current Advances in Oral Biology." The clinical appearance and natural history of individual papillomas appear to be determined, in part, by the serotype of HPV. At least five different HPV serotypes (HPV-1 through HPV-5) have been identified in cutaneous warts and an additional serotype (HPV-6) has been recovered from condylomata of the genital tract. Many different clinical types of hyperplasias and papillomas (focal epithelial hyperplasia, verruca vulgaris and plana, multiple and single oral papillomas, condylomata accuminatum, laryngeal papillomas and other papillomatous lesions) occur in the oral cavity. Although HPV particles (55 nm in diameter) have occasionally been seen in most of these lesions by electron microscopy, there have been no studies of oral cavity papillomas by immunologic tests using HPV antisera. We decided to conduct such a study and correlate it with electron microscopy with the idea it would provide us with data on the prevalence of HPV in the various papillomatous lesions of the oral cavity.

Development of Immunologic Tests. Rabbits were immunized with purified HPV obtained from plantar warts. This was the only papillomatous lesions that contained enough HPV to use for immunizations. All of the antisera that we collected from these rabbits were only reactive with plantar warts and a few common warts, but not with mucosal papillomas. We then disrupted the plantar wart virus with a detergent to produce antibodies against its internal structural proteins with the thought that one of these proteins might be common to all HPV. Antisera prepared against detergent-disrupted plantar wart HPV were cross-reactive with all virus-positive (by electron microscopy) papillomas from humans and other species (cattle, horses, rabbits, deer, dogs) by both indirect fluorescent antibody (FA) tests on frozen sections of papillomas and peroxidase-anti-peroxidase (PAP) tests on sections of formalin-fixed tissue. The ease and rapidity with which we were able to screen routinelyfixed pathology tissue for the presence of papillomavirus common antigens suggested that this or similar antisera would prove valuable in determining the role of papillomaviruses in naturally occurring hyperplasias and papillomas. Subsequently, we were able to prepare other antisera with the same specificity for the papillomavirus common antigen using detergent-disrupted bovine papillomavirus (BPV) instead of HPV as the immunogen.

Screening Oral Cavity Hyperplasias and Papillomas for HPV by Immunologic Tests. We first examined papillomas of the oral cavity that were likely to be caused by HPV. These included verruca, multiple papillomas and condylomata; keratoacanthomas of the oral cavity were also examined

because of their usual history of "wart-like" regression and experimental evidence that they might be caused by a transmissible agent in animals. Viral antigens were found by PAP in 19 of 29 verruca, two of five multiple papillomas, and three of five condylomata; they were not seen in the keratoacanthomas. HPV, when present, was localized to nuclei of the upper granular layer of well-differentiated squamous epithelial cells. Staining by PAP was much more prominent in the granular cells of verruca of the upper and lower lips than either verruca in other locations or multiple papillomas and condylomata. At this time, it is not known if the amount of structural antigens being produced in a particular papilloma is dependent on its anatomic site of location or the serotype of HPV that causes it, or both. These data show, however, that HPV can cause some oral cavity lesions.

We then examined recurrent laryngeal papillomas (102 from 41 patients) of childhood for the presence of HPV. Forty-nine of 102 (48%) laryngeal papillomas were positive for paipillomavirus common antigens. However, when two or more laryngeal papillomas from the same patient were stained by PAP, 27 of 30 (90%) patients were positive. These data present the strongest evidence to date that most laryngeal papillomas of childhood are caused by one or more of the different serotypes of human papillomaviruses. Inoculation of the neonatal larynx with HPV from maternal condylomata during childbirth probably causes most cases of laryngeal papillomas.

Relevance to Biomedical Research

HPV is the only virus that unequivocally causes hyperplasias and, perhaps, neoplasias in man. Its oncogenic potential, however, remains largely unknown. Until now, there have been no readily available tests other than electron microscopy to effectively screen for HPV in neoplasms suspected of having a papillomavirus etiology. Because of the wide variety of papillomatous lesions that occur in the oral cavity, their accessibility to surgical management and the availability of antiserum such as we have described here, it is now possible to study the pathogenesis and significance of papillomaviruses in oral cavity disease.

Future Plans

Attempts will be made to identify other oral cavity lesions containing HPV. Fresh tissue will be collected for the purpose of HPV isolation. After HPV has been isolated, HPV/DNA will be cloned in bacterial plasmids. Large quantities of HPV/DNA can then be obtained from oral cavity lesions for endonuclease restriction studies and molecular hybridization studies. Identification and characterization of the types of HPV in mucosal papillomas is necessary to help prepare a rational therapeutic approach, such as vaccination, for lesions like laryngeal papillomatosis, which is

often a life-threatening disease. It would also help us to understand the epidemiology of lesions that might occur with different HPV serotypes.

Publications

Jenson, A. B., Rosenthal, J. R., Olson, C., Pass, F., Lancaster, W. D., and Shah, K.: Immunologic relatedness of papillomaviruses from different species. JNCI 64(3):495-498, 1980.

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 00219-04 LOM

PERIOD COVERED

October 1, 1979 - September 30, 1980

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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☐ (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 observation that interferon is produced by lymphocytes as an integral part of the immune response and can regulate immunity suggested that interferon might be produced in certain autoimmune disorders. We found immune interferon in sera of patients with active immunologic diseases such as systemic lupus erythematosus, rheumatoid arthritis, scleroderma, Sjogren's Syndrome and vasculitis. The presence of interferon in immunologically-mediated diseases appears to be limited to only certain collagen-vascular diseases since we did not detect interferon in a variety of other diseases.

Earlier studies demonstrated that virus-induced interferon can enhance IgE mediated histamine release (immediate hypersensitivity). We have recently found that interferon can also augment basophil chemotaxis. These studies suggest that viruses and interferon may be involved in the pathogenesis of allergic reactions.

CELL-MEDIATED IMMUNE MECHANISMS AND INTERFERON

Background and Objectives

This research project is divided into two components: (1) the role of viruses and interferon in immunological processes, and (2) the production of monoclonal antibodies to viral antigens and interferon.

INTERFERON

Extensive studies during the past 20 years have clearly established the antiviral actions of interferon. Now, however, it is also evident that interferon can have a variety of other biological effects. At the cellular level, interferons induce alteration in macromolecular synthesis, inhibit cell multiplication and alter plasma membrane conformation. Both *in vivo* and *in vitro*, interferon can enhance or depress a variety of immune responses and may have an important role in regulation of the immune system. In fact, some believe that interferon inhibits tumor production through this mechanism of immune modulation.

Viruses are not the only inducers of interferon. Furthermore, in man, there are at least three distinct interferons. The type of interferon synthesized depends upon both the inducer and the kind of cell that manufactures the interferon. When fibroblasts and epithelial cells are stimulated by viruses, they produce Fe interferon. Lymphocytes, on the other hand, when stimulated with viruses, allogenic or transformed cells, produce Le interferon. Both Fe and Le interferons are antigenically distinct and are stable at low pH.

Lymphocytes produce a third type of interferon as an integral part of their immune response. Hence, it is called immune interferon. This interferon can be produced by sensitized lymphocytes in response to specific antigens. In contrast to Fe and Le interferons, this type of interferon is not stable at low pH. Recent evidence indicates that the three types of human interferon may be antigenically distinct from one another.

The objective of this research project is to determine the role of viruses and interferon in immunologic and immunopathologic processes. In last year's annual report, we described our findings on two aspects of the immunoregulatory actions of interferon: (1) the presence of interferon in the circulation of patients with autoimmune diseases; and (2) the enhancement of IgE-mediated histamine release (immediate hypersensitivity).

The production of interferon during the course of an immune response and its immunoregulatory activities led us to investigate its presence in autoimmune diseases. Systemic lupus erythematosus (SLE), and certain of

the other autoimmune connective tissue diseases are multisystemic chronic inflammatory diseases characterized by immunological abnormalities, particularly of an immunoregulatory nature. Recently, we described the presence of interferon in the circulation of patients with SLE, rheumatoid arthritis, scleroderma, and Sjogren's syndrome. The highest levels of interferon were detected in SLE patients and serial serum samples showed a good correlation between the presence of interferon and disease activity. Moreover, the presence of interferon correlated positively with two laboratory measures of disease activity, antibodies to DNA and low serum levels of the third component of complement.

Histamine and other mediators of anaphylaxis are released when IgE on the membrane of human peripheral blood basophils interact with specific allergens (e.g., ragweed antigen). This release of histamine from human basophils by allergens has proved to be a good in vitro model for allergic reaction. Using this model, we found that incubation of human leukocytes with certain viruses or antigens before challenge with ragweed antigen enhanced the release of histamine. Further studies showed that this enhancement of histamine release was in fact due to interferon induced by viruses (Le and Fe) and interferon induced by antigens (immune interferon).

Major Findings

Interferon in sera of patients with immunologically-mediated diseases.

During the past year, we have looked for interferon in the sera of patients with a variety of other immunologically-mediated diseases. Interferon (> 16 units) was detected in 33% of patients with vasculitis and in 57% of patients with SLE. This confirms and extends our earlier studies showing that interferon was present in 13 of 28 (47%) of SLE patients. In contrast, interferon was detected in only one of 28 patients with Wegener's granulomatosis. Sera from patients with sarcoidosis, infectious mononucleosis, minimal change nephritic syndrome, myasthenia gravis, uveitis, and juvenile diabetes did not contain detectable interferon. Moreover, 48 sera from 15 renal transplant patients were found to be negative for interferon. Two of 74 normal "healthy" individuals contained 16 units of interferon in their sera.

Patients with systemic or cutaneous vasculitis were divided into those with active and those with inactive disease. The patients with active disease were further divided into groups based on the severity of clinical symptoms ranging from 0 (remission) to 3 (acute disease). Only one of 17 sera from patients in remission and 1 of 5 sera from patients with equivocal clinical activity contained interferon. In contrast, 2 of 4 sera from patients with unequivocal disease activity (grade 2) and 5 of 6 sera from patients with acute disease activity (grade 3) contained interferon. These data show a clear cut correlation between the presence of interferon and the severity of disease activity ($p < 0.01$).

Our studies have demonstrated that the antiviral activity in SLE and vasculitis patients meets the criteria for identification of interferon. Since immune interferon is inactivated by treatment at pH 2, whereas Le and Fe interferons are resistant to such treatment, sera were tested at pH 2. Treatment at low pH completely or partially destroyed the antiviral activity of 7 of 11 SLE patient sera. On the basis of these studies, we conclude that there may be a mixture of interferons present and that immune interferon is frequently found.

Since antisera is available for two of the interferon species (Le and Fe) we performed antibody inhibition tests to determine if the interferon found in patient sera were serologically related to these interferons. Patient interferon is not serologically related to Fe interferon. However, all of the patient interferon was inhibited by anti-Le interferon antisera. Our studies serologically identify the patient's serum antiviral substance as interferon. These data also suggest that antiviral activity in the serum is either a mixture of Le and immune interferon and/or the pH 2 sensitive interferon is antigenically related to Le interferon. These data strongly suggest that the interferon found in SLE patients is produced by lymphocytes.

VIRUSES AND INTERFERON IN IMMEDIATE HYPERSENSITIVITY

Recent studies show that circulating basophils increase in number during the pre-attack stage of bronchial asthma and that basophils are present in nasal secretions from subjects with allergic rhinitis. It is also well established that viral infections may precipitate attacks of bronchial asthma and that viruses and interferon can enhance IgE-mediated release of the mediators of immediate hypersensitivity reactions. We, therefore, have investigated the effect of viruses and interferon on the in vitro migration of human basophils. We found that preincubation of human peripheral blood leukocytes with viruses enhanced the migration of basophils towards three unique chemotactic factors: a lymphokine (LDCF), a complement fragment (C5-peptide) and a synthetic tripeptide (f-met-met-met), which resembles chemotactic factors produced by bacteria. In addition, when supernatant fluids from virus treated PBL cultures were added to fresh PBL cultures, we again observed enhancement of basophil migration toward chemotaxins. Thus, we concluded that a soluble product causes the augmented chemotactic response.

Several lines of evidence indicate that the enhancing activity is mediated by interferon. First, there was a direct relationship between the time of appearance of IF in the culture fluids and the degree of enhanced migration. Second, there was a good correlation between the concentration of virus added to leukocyte cultures, the production of IF, and augmented chemotaxis. Third, all preparations of human IF, whether leukocyte or fibroblast derived, caused enhancement of chemotaxis. Fourth, the soluble factor causing enhancement could not be dissociated

from IF by standard physicochemical means. Fifth, there was a strong correlation between enhancing activity and IF titer when a partially purified preparation of IF was compared to two leukocyte culture supernatants.

We are also investigating the possible role of specific antiviral IgE antibodies in immediate hypersensitivity reactions. Mice have been immunized with herpes simplex virus and we have detected the presence of specific antiviral IgE antibodies by two assay methods; the antigeninduced response of histamine from immunized mouse mast cells and the use of an indirect enzyme-linked immunosorbant assay (ELISA).

Significance

Our studies show that interferon is produced in patients with certain immunologically-mediated diseases. The role of interferon in these immunologically-mediated diseases is unknown. However, it is known that interferon is an active modulator of the immune system. In certain autoimmune hosts, interferon may act to alleviate some of the over-active immune responses. On the other hand, it may contribute to the immunologic aberrations associated with these diseases, such as increased antibody levels, autoantibodies, immune complexes, depressed cell-mediated immune responses and kidney disease. A better understanding of the multiple aspects of the interferon system in these aberrant host responses should lead to a better understanding of immunologic control mechanisms.

Clinical disease activity in patients with SLE follows a course of exacerbations and remissions. Unfortunately, the laboratory measures of disease activity do not always correlate with clinical disease. Our findings suggest that interferon levels may be a better index of disease activity than the laboratory measures now in use.

Millions of people suffer from asthma and other forms of immediate hypersensitivity. Clinical studies suggest that upper respiratory tract infections can potentiate or augment asthmatic attacks. Our studies suggest that interferon produced by cells in response to viruses or bacterial antigens may be involved in the pathogenesis of asthma. The interaction of the viral antigens or allergens with IgE fixed on basophils in the presence of interferon may lead to the enhanced release of the pharmacologic mediators of anaphylaxis. Interferon also may augment the movement of basophils to the site of infection.

Course of Future Studies

The mechanisms by which lymphocytes from patients with autoimmune diseases are triggered to produce interferon will be investigated in greater depth. We also plan to further evaluate the use of interferon assays as a diagnostic tool for identification of clinical disease activity

in SLE and the capacity of lymphocytes from patients with certain immunoproliferative diseases to produce interferon will be investigated. Studies on the role of viruses and interferon in immediate hypersensitivity will be expanded. Specific antiviral IgE antibodies will be used to investigate the role of viruses in augmenting hypersensitivity reactions in a mouse model system.

HYBRIDOMAS: MONOCLONAL ANTIBODIES TO VIRAL ANTIGENS AND INTERFERON

Background

To obtain monoclonal antibody to specific antigenic determinants on complex antigens, recently developed hybridoma techniques have been used. For example, P3 x 63 Ag8 myeloma cells [which are resistant to 8azaguanine due to deficiency of hypoxanthine phosphoribosyle transferase and cannot survive in the media containing hypoxanthine, aminopterine and thymidine (HAT)] is hybridized to primed spleen cells from animals immunized with specific antigens. These hybrid cells can survive in HAT media, they proliferate continuously, and secrete homologous antibody to specific antigens.

This basic technique has been used during the past year to develop monoclonal antibodies against specific viral antigens and against interferon. A variation of this technique is being used in an attempt to produce a hybrid cell which could continuously produce hormones in vitro.

Major Findings and Future Course

Hybridomas have been established that produce antibodies to the diabetogenic variant (D clone) of encephalomyocarditis (EMC) virus. Characterization of these antibodies is now under way. These antibodies will enable us to identify antigenic variants and efforts will be made to relate antigenic differencesto virulence.

A hybridoma also has been established which produces antibody (detected by the ELISA assay) to human interferon. Studies are in progress to determine if this antibody will inhibit the antiviral actions of interferon. A monoclonal anti-interferon antibody can be used to purify interferon preparations and to develop rapid serological assays for human interferon. Efforts also will continue to develop a hybrid cell that can produce hormones.

Publications

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2. Hooks, J.J. and B. Detrick-Hooks: Spumavirinae: Foamy virus group infections. Comparative aspects and diagnosis. In Comparative Diagnosis of Viral Diseases. Vol. III. E Kunstak (ed.), Academic Press, Inc., New York (in press) 1980.

3. Ida, S., J.J. Hooks, R.P. Siraganian and A.L. Notkins: Enhancement of IgE-mediated histamine release from human basophils by immunespecific lymphokines. Clin. Exp. Immunol. 41:380-387) 1980.

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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 00255-02 LOM

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Receptors, Membranes and Diseases

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

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

The contributions of cell surface receptors for viruses and hormones are being investigated to determine their role in the pathogenesis of viral infections. Measurements of receptor activity and how they are related to susceptibility to disease are being complemented by studies of genetics of host susceptibility and analysis of the viral genome and capsid proteins. By means of these varying experimental approaches, a clearer understanding of the interaction of host and viral functions in the disease process is being gained.

RECEPTORS, MEMBRANES AND DISEASE

Background

The surfaces of cells are known to possess receptors which determine if and how those cells will respond to viruses, hormones, and other cells. In recent years, the relationship between cellular receptors and disease has become increasingly evident. Most viruses must attach to a cellular receptor in order to infect a target cell, and the absence of an appropriate receptor can render a cell resistant to those viruses. Moreover, antibody to hormone and neurotransmitter receptors can produce serious disease as in extreme insulin resistance, Graves disease and myasthenia gravis.

In recent years, it has become evident that many diseases are associated with specific histocompatibility antigens. The HLA (human) and H-2 (mouse) major histocompatibility loci govern the expression of the strongest and perhaps the most important of the cell surface antigens. Insulin deficiency, diabetes mellitus, multiple sclerosis, and a host of rheumatological disorders have been found associated with increased representation of certain HLA haplotypes. It is not unreasonable to consider that an apparently minor change in one or more cell surface components could lead to altered cellular function and eventually to multi-systemic disease.

We have been investigating the relationship between receptors and disease from several approaches. First, since the susceptibility of a given cell to a virus largely depends upon the presence and characteristics of a cellular receptor for that virus, we have been comparing cells of differing susceptibilities to encephalomyocarditis (EMC) virus with respect to the kinetics of receptor binding. Second, previous work from the Laboratory of Oral Medicine has demonstrated that a single virus can produce dramatically different diseases in different mouse strains. We have been attempting to determine the genetic basis of differential susceptibility by studying the segregation of susceptibility and resistance in crosses of susceptible and resistant strains of mice. Third, some viruses are known to modify the plasma membrane of their host cells. In some cases, viral antigens or new host antigens are displayed, while in others, normally present components are decreased. Therefore, we have been using a selected surface cell receptor, the one for insulin, as a probe of possible cell surface changes in viral infections. Fourth, a single serotype of virus such as EMC virus or coxsackievirus B4 can induce different syndromes in different individuals. In mice, EMC virus can cause either encephalitis, myocarditis, pancreatitis, or an insulitis. In humans, coxsackievirus B4 infection can be associated with meningitis, encephalitis, myocarditis, gastroenteritis, upper respiratory tract infections, and diabetes mellitus. In all probability, the type of

disease induced is at least partly dependent upon the genetic predisposition of the individual, however, different pools of serologically identical viruses may also contain variants with specific tissue tropisms in varying proportions. By plaque-purifying the M variant of EMC virus, we have isolated a large number of clones which differ in the types of diseases they induce when injected into mice. These have given us the raw materials with which to analyze susceptibility to viral infections in terms of the contributions of the virus and the contributions of the host.

Major Findings

Viral receptors on human and murine cells. The attachment kinetics of radio-labeled EMC virus were studied using established murine (Friend leukemia cells, L-929 cells) and human (HeLa-S_3, IM-9) cell lines and murine and human erythrocytes. A temperature of 0°C was found to be optimal for virus attachment. Unlabeled virus completely blocked the binding of labeled virus, and for HeLa cells, the number of receptors per cell was estimated to be in the range of 1 to 5 x 10^5. Virus inactivated at pH 6 was unable to attach to either murine or human cells, and labeled poliovirus was found to attach to human cells but not to murine cells. Thus, despite the low temperature optimum, the attachment was specific.

The initial rate constant at 0°C calculated for human erythrocytes was 3.2 x 10^{-10} cm^3 min^{-1} $cell^{-1}$. The rate constants for Friend leukemia cells and HeLa cells were, respectively, 200 and 2000 times faster. In contrast, no binding was observed to murine erythrocytes. The attachment of the virus to HeLa cells was found to be temperature independent over the range 0°C to 40°C. The attachment to murine cells (L-929 and Friend leukemia), however, was progressively reduced with increasing temperatures (0°C to 40°C) to about 1% of the rate at 0°C. The decrease in binding at temperatures greater than 0°C appeared to be due to an increased rate of dissociation of the virus. These results suggest that while presence or absence of receptors may determine cellular susceptibility, the ability of a virus to establish a severe systemic infection may be inversely related to the affinity of the receptor for the virus.

Induction of viral receptors. Once an infection is established in an individual, the cells of the immune system and the reticuloendothelial system are responsible for limiting the extent of infection. Presumably, this function could be compromised if the responding cells are themselves susceptible to infection. We have determined that normal splenic lymphocytes from SJL/J mice do not have detectable receptors for EMC virus and are not susceptible to infection with this virus. However, if the lymphocytes have been stimulated to undergo blastogenesis, by culturing them in the presence of mitogens, receptors for EMC virus can be induced. Phytohemagglutinin (PHA), concanavalin A (ConA) and E. coli lipopolysaccharide (LPS) are all effective at inducing EMC virus receptors. The maximum induction occurred after 48 to 66 hours in culture, and correlated

well with the ability of the cultures to incorporate [^{14}C]-thymidine, with an increase in the modal cell volume, and with the ability of the cells to be infected. The induction of receptors was prevented if blastogenesis was inhibited with mitomycin C and specificity for EMC virus was shown by the inability of the stimulated cultures to attach poliovirus. Both purified T cells (using PHA as mitogen) and purified B cells (using LPS as mitogen) were inducible for EMC virus receptors. Data obtained using the receptor assay and an immunofluorescence assay for EMC virus antigens suggested that the induction of EMC virus receptors was limited to a small fraction (less than 1%) of the lymphoblasts. The possibility existed that among cloned cell lines derived from T and B cell lymphomas, there might be receptor-positive and -negative lines. In collaboration with Dr. K-J. Kim, we have tested several lines derived from BALB/cJ mice for their ability to bind and replicate EMC virus. Thus far, one of two T cell lines has been found to possess EMC virus receptors and to be susceptible to infection, and one of four B cell lines has been found to be positive in both types of assays. Since these lines differ in their cell surface antigens and markers, we are continuing to screen the remaining available cell lines to determine if the presence of EMC virus receptor is associated with any specific marker or combination of markers, as a possible indication of which subsets of lymphocytes are inducible for EMC virus receptors.

In contrast to normal lymphocytes, normal SJL/J peritoneal macrophages appear to possess a small amount of EMC virus receptor, which is greatly increased after activation in vivo with thioglycolate broth. The attachment was not due to phagocytosis of the virus by the macrophages, since the cells did not bind labeled poliovirus and attachment of labeled EMC virus was completely blocked by a 10-fold excess of unlabeled EMC virus. Also, EMC virus attachment was maximal at 0°C and minimal at 37°C in contrast to phagocytic activity (as measured using latex microspheres) which was absent at 0°C. Cells pretreated with lidocaine HCl lost more than 90% of their phagocytic activity but retained the ability to attach EMC virus.

We are expanding these studies to determine if there are mouse strain specific differences in EMC virus receptor induction on lymphocytes and macrophages and whether these can be correlated with susceptibility or resistance to infection.

Effect of Virus Infection on Cell Surface Receptors. Using an in vitro radioreceptor assay with ^{125}I-labeled insulin and human amnion (WISH) cells, the effect of viral infections on the plasma membrane (insulin receptors) was examined. Both herpes simplex virus and vesicular stomatitis virus produced a 50% decrease in insulin binding. There was no evidence that this decrease was due to degradation of insulin. On quantitative analysis, this decrease in binding was found to be due to a decrease in receptor concentration with no change in receptor affinity. The decrease in receptors occurred early in the infection (4 to 12 h), at

the time when viral antigens were being inserted into the plasma membrane of infected cells. The decrease could not be explained solely by virus-induced shutoff of macromolecular synthesis; viruses such as encephalomyocarditis that do not insert new antigens into the plasma membrane did not cause changes in the number of insulin receptors. The most likely explanation is that virus-induced changes in the plasma membrane altered or displaced insulin receptors. These data suggest that in diabetic patients, abnormalities in glucose metabolism associated with some viral infections may be due, in part, to changes in the concentration of insulin receptors.

The effects of virus infection upon insulin receptors in vivo is being tested by assaying NIH/Swiss splenic lymphocytes for their ability to bind ^{125}I-labeled insulin at various intervals after infection with EMC virus. Based on our preliminary results, it appears that binding of radio-labeled insulin begins to increase three days after infection, reaching a maximum increase of 70 to 90% over control levels at four days post infection. After four days, the insulin binding decreases gradually to control levels.

The reasons for this increase are not known, but may be due to the virus-induced hypoinsulinemia and hyperglycemia. Some alternatives also being tested include the possible effects of interferon or other lymphokines on the regulation of insulin receptors, and the possibility of mitogenic effects of virus infection on sub-populations of lymphocytes leading to an increase in insulin receptors on the surfaces of these cells.

Genetics of resistance to virus infection. Earlier work in the LOM showed that C57BL/6J mice were resistant to EMC-induced diabetes mellitus, and that SJL/J and DBA/2J mice were highly susceptible. Furthermore, it was shown that resistance was most likely inherited as an autosomal dominant gene. Last year, we began a series of experiments designed to establish linkage of this gene with known enzymatic and antigenic polymorphisms. Using mice from the (SJL/J x C57BL/6J) F1 x SJL/J backcross, we have been systematically comparing the segregation of resistance to EMC-induced diabetes mellitus with a panel of known polymorphic traits also segregating in this cross. In a similar fashion, we have studied the genetics of Coxsackie B4-induced diabetes mellitus in animals of the (SWR/J x C57BL/6J) F1 x SWR/J backcross. The mice have been typed for several of the traits segregating in these crosses and, thus far, no linkage has been found. Notably, in both systems, the segregation of the major histocompatability locus (H-2) was independent of the susceptibility to virus-induced diabetes mellitus.

In collaboration with Dr. B. A. Taylor of the Jackson Laboratories, we have tested mice of B x D recombinant inbred strains for susceptibility to EMC-induced diabetes. These strains had randomly segregated DBA/2J

and C57BL/6J genes before they were inbred. By comparing the susceptibilities of these strains with the profiles of the known DBA/2J and C57BL/6J markers they possess, we had hoped to obtain additional information about the chromosomal location of the gene in question. The results of this study were somewhat disappointing. No association was found between induction of diabetes mellitus and any of the loci that have been identified in these strains.

Although the linkage data have, thus far, been negative, we have been able to strongly confirm the earlier hypothesis of a single autosomal locus determining resistance. We are continuing these studies with backcross progeny of different combinations of susceptible and resistant mouse strains.

Variants of EMC virus. Earlier studies in the LOM have shown that not only do mouse strains differ in their susceptibility to EMC-induced diabetes mellitus, but that different isolates of EMC virus with varied passage histories, but serologically indistinguishable, induce diseases in mice that involved different organ systems. One explanation for this could be that in nature a given virus may exist as a mixed population of viruses with different tissue tropisms. Last year, we reported the isolation of two clones of EMC virus, one of which (the D variant) was highly diabetogenic and the other (the B variant) was non-diabetogenic. When these variants were isolated, approximately 30 other clones were also produced and characterization of these has proceeded as time and available animals permitted.

We now have preliminary data on five additional clones and these have shown a surprisingly large degree of variation. Of the seven clones that have been tested (including the D and B variants), two are diabetogenic and non-lethal, two are non-diabetogenic and non-lethal, two are moderately diabetogenic and moderately lethal, and one is mildly diabetogenic and highly lethal. The clones that induce lethal disease appear to be highly cardiotropic. Upon completion of the in vivo screening of these clones, it is our intention to test their receptor-binding characteristics and how they relate to their tissue tropisms.

Significance to Biomedical Research

To a great extent, receptors are key intermediates in the response of cells to hormones, the infection of host cells by viruses, and the immune response. In man as well as in animal models, many debilitating diseases have been shown to be related to decreased number or function of receptors. Other diseases are associated with known cell surface antigens, and still other diseases are caused by agents that require an appropriate surface receptor. In some cases, the relationship between the receptor

and the disease has been characterized (e.g., adult obesity and insulin resistance). However, we are still left with a large degree of ignorance concerning the role of the cell surface in determining susceptibility to disease. By the appropriate study of selected membrane receptors, the relationship of receptor function and/or dysfunction to disease may be illuminated.

Course of Future Studies

We intend to continue our study of the physical-chemical nature of picornavirus-receptor interactions. This will focus on the relationship of receptor affinity to virulence and the chemical nature and regulation of the virus receptor. The significance of inducible receptors on immunocompetent cells will be evaluated using selected inbred strains of mice and identification of the nature of the responding lymphocytes will be attempted. During the next year, we will continue the study of the influence of viral infections on the cell surface receptors for polypeptide hormones, and will begin new protocols designed to test the ability of steroid hormones to modulate virus susceptibility and viral receptors. The study of the genetics of susceptibility to EMC virus and coxsackievirus B4-induced diabetes mellitus will be continued using new mouse strain combinations and larger numbers of mice in order to identify the chromosome which carries the gene determining resistance. We will continue to screen and characterize the variant clones of EMC virus with the aim of relating the *in vivo* activity of these viruses to their physical and chemical properties.

Publications

1. McClintock, P. R., Billups, L., and Notkins, A. L.: Receptors for encephalomyocarditis virus on murine and human cells. Virology (in press) 1980.

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ANNUAL REPORT OF THE
NEUROBIOLOGY AND ANESTHESIOLOGY BRANCH
NATIONAL INSTITUTE OF DENTAL RESEARCH

The Neurobiology and Anesthesiology Branch is concerned with the study of oral-facial sensation, with particular emphasis on mechanisms of pain and the development of new methods for controlling pain in humans. The Branch is composed of three sections that utilize anatomical, physiological, behavioral, pharmacological and psychophysical techniques to study neural function as it relates to the processing of sensory signals about the threat of tissue-damaging stimulation. The Neural Mechanisms Section includes the following research activities: 1) correlative morphological and physiological studies of the organization of the medullary and spinal dorsal horns and the response of specific neuronal cell types to innocuous and noxious stimuli; 2) correlative behavioral and physiological studies to determine the role of different peripheral and central neural populations in pain and temperature discrimination. The Neurocytology and Experimental Anatomy Section is concerned primarily with the study of the medullary and spinal dorsal horns utilizing light and electron microscopy and various tracer and marking methods for identifying putative neurotransmitters. The Clinical Pain Section develops new methods for measuring and assessing experimental and clinical pain and applies these methods to the study of various pharmacological and non-pharmacological techniques potentially useful in the control of anxiety, apprehension and pain associated with dental procedures and in the treatment of chronic pain.

There is extensive collaboration between investigators in the three research groups. Correlative studies on neuronal structure and function are being carried out to elucidate in detail the circuitry of the medullary and spinal dorsal horns 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. The effects of intravenous sedative drugs are evaluated utilizing the improved psychophysical assessment methods of pain and anxiety developed by the pain measurement group. Finally, our previous animal research has provided the conceptual framework for present studies on the role of endogenous pain-suppressing mechanisms in postsurgical pain following the extraction of third molars. This oral surgery model has proven to be extremely useful in providing a standard acute pain population in which the pain is localized to the same body region, within-subject controls are possible, and there is a large ambulatory patient population available.

This year we have continued to develop our chronic pain research program in collaboration with consultants in neurology, neurosurgery, anesthesiology, psychology and psychiatry. Patients receive extensive observation of their pain problem within a research environment. They are admitted to the Clinical Center, NIH, for 2-3 week periods and participate in research studies in which their responses to experimental

pain stimuli as well as their clinical pain are evaluated. This detailed experimental and clinical work-up forms the baseline for further evaluation of new pain control methods.

The clinical programs have been reorganized this year into one section (Clinical Pain Section) in contrast to the earlier separation of the anesthesiology and pain measurement programs. This was a natural union of two programs in which there already was extensive collaboration among investigators. The organizational change has facilitated the proper utilization of support personnel in the two groups.

Investigators in the Branch organized two symposia at the Society for Neuroscience meeting this year. Members of the Branch were invited speakers at the International Symposium on Oral Physiology held in Tokyo, at the International Colloquium on the Serotonergic Neuron in Marseille, and at the annual meetings of the Society for Neuroscience, the American Pain Society, and the American Psychological Association.

The research highlights of the Branch that follow will be presented by topic rather than by Section, because of the extensive interaction between the groups.

The Neural Circuitry of the Medullary and Spinal Dorsal Horns

The lower end of the spinal trigeminal nucleus, called trigeminal nucleus caudalis, is directly continuous with the spinal dorsal horn. However, it usually is considered a separate structure. Several findings in our laboratory have demonstrated a structural and physiological organization of nucleus caudalis similar to the spinal dorsal horn. First, the upper parts of both structures can be subdivided into six similar layers on the basis of neuropil texture, density and size distribution of neuronal cell bodies, and the distribution of small myelinated axons and axon bundles. Second, the morphologically-defined neuronal cell types in the two areas are similar as are the kinds of synaptic connections between primary axons, descending axons and the intrinsic neurons of the superficial layers (layers I and II). Third, projection neurons to the thalamus and propriospinal neurons are found in the same layers in both structures. Finally, the physiological properties of neurons in both structures are alike. Based on the above evidence, trigeminal nucleus caudalis more properly should be referred to as the medullary dorsal horn.

Our recent studies have examined the relationship between primary axons, descending axons from the brainstem, and the intrinsic neurons of the dorsal horn. In experiments in which horseradish peroxidase (HRP) was placed inside tooth pulps, the enzyme was transported in axons to two central termination sites in the brain stem. A long continuous column of the terminal axonal arbors extended rostrocaudally from the main sensory nucleus to layer V of the medullary dorsal horn. A second termination site was found in the dorsomedial parts of layers I and IIa

in the medullary dorsal horn. Thus, neurons innervating tooth pulps, and likely involved in transmitting pain signals, terminate in specific layers in the dorsal horn. These are the same layers that appear to receive other types of nociceptive afferent input.

We have performed correlative anatomical and physiological studies of the intrinsic neurons of the spinal dorsal horn. The response properties of these neurons are determined electrophysiologically. The neurons are then impaled with fine micropipettes and horseradish peroxidase iontophoresed into the cell. This powerful technique reveals the location and configuration of the dendritic and axonal processes of individual physiologically-characterized cells. We have concentrated our efforts on the two most superficial layers of the dorsal horn. Layer I contains neurons that respond exclusively to noxious stimuli (nociceptive-specific or NS neurons) and neurons that respond to both innocuous and noxious stimuli (wide-dynamic-range or WDR neurons). Some WDR layer I neurons receive input from mechanoreceptive afferents having small myelinated axons. Some have axon that generate extensive local arbors within layer I. Most layer I neurons have their dendritic arbors limited to that layer. Since layer I is known to contain many neurons that project to the thalamus, it is likely that layer II interneurons modulate the output from layer I.

Two morphological classes of neurons predominate in layer IIa, the superficial part of layer II. Both are physiologically-characterized as either WDR or NS neurons. Stalked cells have their cell bodies along the border between layers I and IIa. Their dendrites fan out rostrocaudally as they travel ventrally into the deeper layers. Their axons arborize mainly in layer I. Thus, stalked cells have a morphological configuration ideally suited for transferring primary inputs from layer II to layer I projection neurons. Layer IIa islet cells have dendrites that are compressed mediolaterally and extend rostrocaudally, mainly parallel to the layer borders. Their axons arborize in the immediate vicinity of their dendritic zones.

Layer IIb (deeper part of layer II) neurons only respond to innocuous or weak mechanical stimuli and the majority of them are islet cells. They differ from the IIa islet cells in their dendritic arbors which are 2-4X longer rostrocaudally than the dendritic arbors of IIa islet cells.

Thus, layer II can be divided functionally into a superficial part (IIa) that is concerned with the processing of nociceptive input and low-threshold mechanoreceptive input, and a deeper part (layer IIb) that appears to be concerned exclusively with low-threshold mechanoreceptive input. This physiological subdivision is consistent with the previously proposed anatomical division of layer II and suggests a morphological substrate for the often observed inhibitory effects of low-threshold input on nociceptive neurons, as well as the analgesia or reduction in pain reports in humans produced by such low intensity mechanical stimuli.

The intracellular HRP method also has provided an opportunity to examine the synaptic connections of these major neuronal cell types in the dorsal horn with the electron microscope. The layer IIa islet cell is a major source of synaptic vesicle-containing dendrites in the layer IIa glomeruli, the site of many primary afferent terminals. In contrast, the stalked cell does not contain synaptic vesicles in its dendrites and is a major source of non-vesicle-containing dendritic spines and shafts in the layer IIa and IIb glomeruli. These results indicate that the stalked cell receives primary afferent input in layer II and conveys it via its axon to the projection neurons of layer I. The layer IIa islet is thought to function as an interneuron inhibiting primary afferent input to stalked cells via its dendrodendritic and dendroaxonic synapses. Recent examination of layer IIb islet cells successfully filled with HRP indicates that they contain synaptic vesicles in their dendrites and form dendrodendritic synapses inside and outside of the layer IIb glomeruli. Presumably, IIb islet cells also function as inhibitory interneurons. These ultrastructural differences between islet cells and stalked cells as well as the differential distribution of their dendritic and axonal arbors, clearly indicates that stalked and islet cells are separate and distinct components of the neuronal circuitry of the superficial layers of the dorsal horn.

The descending afferents to the dorsal horn have been examined utilizing methods that identify neurotransmitters associated with these inputs. Serotonergic and noradrenergic axonal endings have been identified at the electron microscope level with autoradiographic techniques. Two categories of axonal endings were labeled with tritiated serotonin applied directly to the medullary dorsal horn. Dome-shaped endings form single synapses and scalloped endings form multiple synapses primarily on small caliber dendritic shafts and spines outside of primary afferent glomeruli in layers I and II. Dome-shaped and scalloped noradrenergic endings are found in layers I and II. The presence of these aminergic endings in layers I and II suggests that they have access to both the projection neurons in layer I and the interneurons in layer II. Our intracellular HRP studies revealed that stalked and islet cells and layer I cells receive synapses from axonal endings that resemble the serotonergic and noradrenergic endings observed in the autoradiographic studies. Other radioisotope studies, in which triated amino acids were injected into the medial brainstem and transported in an anterograde direction to their axons in the dorsal horn, revealed two dome-shaped endings in layers I and II, and one scalloped ending in layer IIa. Each type resembles serotonergic axonal endings identified in the tritiated serotonin uptake experiments, suggesting that these three endings arise from the serotonin-rich neurons in the medial brainstem.

This descending serotonergic system from the brainstem has been implicated in the mechanism of action of narcotic analgesics and endogenous opiate-like peptides present in the brain. We have extended our pharmacological analysis of the dorsal horn by examining the distribution of different neuropeptides using immunocytochemical techniques. Although both enkephalin

and substance P are found throughout layers I and II, the density of substance P containing axonal endings is greatest in layer I while enkephalinergic endings are greatest in layer II. In experiments in which axoplasmic transport is blocked using colchicine, a few substance P containing cell bodies are found in layer II whereas enkephalinergic neurons are found in layers I and II in addition to deeper layers. In future studies we will utilize immunocytochemical methods in combination with the intracellular HRP method to further delineate the organization of the dorsal horn and its role in nociception and sensory transmission.

Behavioral correlates of neural function in the medullary dorsal horn

As mentioned above, two general classes (WDR and NS) of dorsal horn neurons, studied in anesthetized animals convey information related to pain. A third class, referred to as low-threshold mechanoreceptive, only respond to weak or innocuous stimuli. Many of the WDR and NS neurons project directly to somatosensory thalamus. We have extended this analysis to examine neuronal properties in the medullary dorsal horn in awake, behaving monkeys. We asked the following questions: What are the functional roles of these neurons in animals trained to detect and discriminate noxious stimuli? Do they exhibit sensory-discriminative properties and code physical characteristics of the stimulus during behavioral tasks? Do behavioral variables such as stimulus relevance and selective attention influence the responses of these neurons? Finally, are there responses of dorsal horn neurons during behavioral tasks that are not observed in anesthetized animals?

Monkeys were trained to detect the termination of innocuous thermal stimuli (37^{o}-43^{o}C) and the onset of noxious heat stimuli (45^{o}-49^{o}C) applied to the face (thermal task). In a visual task, the same monkeys detected the onset of a visual stimulus while behaviorally nonrelevant thermal stimuli were presented. Neuronal activity in the medullary dorsal horn was correlated with a number of behavioral events such as panel press, temperature onset, temperature termination, panel release and reinforcement delivery. Our results show that medullary dorsal horn neurons differ along two response dimensions: (1) sensory-discriminative properties in response to thermal and mechanical stimuli; and (2) task-related responses independent of stimulus parameters and motor activity. Thermal nociceptive neurons had properties similar to those found previously in anesthetized animals. They were classified as WDR or NS based on responses to mechanical and thermal stimulation. Stimulusresponse functions were monotonic from threshold to 49^{o}C and neurons were most sensitive when stimuli were applied to the central portion of their receptive fields. WDR and NS neurons responded to noxious stimuli earlier than the shortest discrimination latencies of the monkey on 45^{o}-49^{o}C trials. For some neurons, the magnitude of the neuronal response to noxious heat stimuli was inversely related to discrimination latency. These findings suggest that activity in thermally-sensitive WDR and NS neurons provide signals related to the monkey's ability to discriminate noxious heat stimuli and support earlier conclusions that WDR and NS neurons code sensory-discriminative information.

The responses of these neurons, however, were modified by the behavioral situation. Behaviorally relevant thermal stimuli presented during performance of the thermal task usually produced greater neuronal responses than equivalent nonrelevant thermal stimuli presented during performance of the visual task. The effects of changes in selective attention were studied in the visual task by systematically reducing the intensity of the light cue. With a reduction in light intensity requiring monkeys to attend more vigorously to light onset, there was a reduction in neuronal responses to innocuous and noxious thermal stimuli. These findings indicate that the behavioral context in which stimuli are presented alters sensory-discriminative response properties of medullary dorsal horn neurons. Significant behavioral variables include the relevance of the stimulus to the behavioral task and the attentional demands of the task.

Some thermal nociceptive neurons, and mechanoreceptive neurons not responsive to thermal stimuli, exhibited responses that were not related to stimulus features or to parameters of movement and occurred only during the behavioral tasks. These task-related responses included an increase in activity when the panel is illuminated, sustained activity while the monkey presses the panel, and a further increase in activity when the relevant cue leading directly to reinforcement occurs. These task-related responses were independent of stimulus modality or intensity, since they were present in the thermal and visual tasks and occurred in the absence of thermal or mechanical stimulation of the face. These responses were absent within a task if a particular stimulus provided no information leading to reinforcement. Task-related responses also occurred in the absence of lip movements associated with reception of the liquid reinforcement and could be uncoupled from hand and arm movements associated with panel press and panel release. The above findings suggest that task-related responses result from the integration of environmental sensory input and the central neural evaluation of its behavioral relevance or significance. They are evoked by environmental sensory cues that reliably predict reinforcement.

Neurons with sensory-discrimination properties and neurons with task-related responses were found in the superficial and deep layers of the medullary dorsal horn. Both types of neurons project to nucleus ventralis posteromedialis in the thalamus. It appears that both types of responses are necessary for signalling the chain of neural events from stimulus detection to motor response responsible for the appropriate execution of goal-directed behaviors.

The Role of Endogenous Pain-suppressing Mechanisms in the Control of Postsurgical Pain

Psychophysical methods of pain assessment and measurement previously developed in this laboratory are now being used to evaluate pharmacological and non-pharmacological pain control agents. Pain following the extraction of third molar teeth was assessed before and after intravenous injections

of either 1) saline; 2) fentanyl, a short-acting narcotic analgesic, or 3) naloxone, a narcotic antagonist. The sensitivity of the visual analog and verbal descriptor scales used in this study was validated by the finding of a significant reduction in pain following administration of fentanyl and the reversal of the effect by naloxone administration. This study also showed that naloxone, in comparison to placebo, produced a biphasic effect on pain responses. Naloxone produced analgesia for 5-15 min after injection, followed by hyperalgesia at 60 min after injection. These results suggest that naloxone may have actions in addition to the antagonism of endogenous, pain-suppressing opiate-like peptides (endorphins). Our findings, however, support the hypothesis that there is a release of endogenous, pain-suppressing, opiate-like peptides following oral surgery either associated with the stress or trauma of the procedure, or in response to placebo medication.

An additional study examined the influence of naloxone on pain produced by electrical tooth pulp stimulation. Naloxone did not alter pain responsivity, but in comparison to placebo, did significantly increase the variability of verbal descriptor responses about the intensity of these stimuli. The finding that naloxone increases scaling variability indicates that investigators utilizing response measures based on discriminability factors need to carefully evaluate the effects of analgesic manipulations on the subject's psychophysical ability to perform the task as opposed to changes in pain perception. Our findings in awake monkeys provide additional evidence that the sensory-discriminative properties of "pain-transmission" neurons can be modified by factors such as attention, motivation and stimulus relevance (see above).

Assessment of Chronic Pain

We have evaluated the effects of narcotic analgesics and electrical brain stimulation on experimental and clinical pain in a group of chronic pain patients, some of whom received chronic brain electrode implants for the relief of their intractable pain. Two of three patients with brain implants appeared to receive relief of their pain lasting over 24 hours from electrical stimulation. However, our preliminary findings do not clearly differentiate between placebo effects and reduction in the perceived intensity and unpleasantness of clinical and experimental pain due to stimulation.

In experimental studies we assessed the effects of chronic pain on responses to experimentally-applied noxious heat stimuli. Pricking pain thresholds were significantly higher (50.5^{o}C) for 8 chronic pain patients than for 8 normal subjects (46.4^{o}C). Chronic pain patients and normal subjects also scaled the intensity and unpleasantness of noxious thermal stimuli between 45^{o} and 51^{o}C. Verbal descriptor responses of the unpleasantness of the stimuli were significantly lower in the chronic pain patients. These studies suggest that chronic pain patients find noxious heat stimuli of 45^{o}-51^{o}C less painful and less unpleasant than normal subjects. Thus, it is important to establish the range of heat stimuli reported as

painful by pain patients before evaluating the efficacy of pain control manipulations.

An additional study compared the effects of morphine on responses to noxious heat stimuli in chronic pain patients. Following morphine administration, verbal descriptor responses of sensory intensity and unpleasantness were reduced in the chronic pain patients in comparison to placebo. In a related study, the effects of fentanyl or placebo on sensations produced by electrical tooth pulp stimulation were examined in patients suffering from oral-facial pain (Myofascial Pain Dysfunction Syndrome). There was a significant reduction in unpleasantness following fentanyl administration as compared to placebo. These results in chronic pain patients support the hypothesis that the effect of a drug on perceived unpleasantness is dependent in part on the side effects experienced. We previously reported that pain free subjects usually experience dysphoria and nausea and report an increase in the perceived unpleasantness of experimental painful stimuli. In contrast, chronic pain patients report euphoria, warmth and relaxation along with their reduction in perceived unpleasantness of painful stimuli.

Control of Pain, Anxiety and Apprehension in Ambulatory Dental Patients

This project evaluates the efficacy and clinical toxicity of drugs given to dental outpatients to alleviate anxiety and apprehension associated with dental procedures. Measures of efficacy include relief of anxiety, analgesia, amnesia and patient cooperation. Measures of clinical toxicity include physiological responses such as arterial oxygen, blood pressure, cardiac output, etc., as well as psychological measures of sensory, psychomotor and cognitive function. In addition, new verbal descriptor scales are being developed to assess anxiety, relaxation, and alertness associated with different drug combinations.

Physiological studies have confirmed that the combination of diazepam, fentanyl and methohexital used for intravenous sedation, results in significant decreases in both respiratory rate and arterial oxygen saturation. Respiratory depression was not found with other drug combinations such as diazepam and methohexital, diazepam and fentanyl or diazepam alone. The diazepam-fentanyl-methohexital combination also resulted in a transient decrease in stroke volume and a reflex tachycardia. These results provide evidence that a widely used sedative combination is resulting in respiratory and cardiovascular depression. Other studies of the therapeutic effacacy of this drug combination indicate that it has little advantage over other sedative combinations in terms of anxiety relief, amnesia and patient cooperation. In contrast, a single agent, such as diazepam, results in near optimal anxiety relief without any detectable physiological impairment.

Preliminary data indicate that the descriptor scales for assessing sedative drugs appear to be sufficiently sensitive for differentiating between drug groups. Diazepam produced the greatest relief of anxiety

and increase in relaxation when compared to placebo. Diazepam also decreased the unpleasantness but not the perceived intensity of painful surgical stimuli.

Other studies have begun to evaluate the efficacy of preoperative administration of non-steroidal anti-inflammatory analgesics in the control of postsurgical pain. The analgesic activity of flurbiprofen is being compared to acetaminophen, and to the combination of acetaminophen and a narcotic, oxycodone. Preliminary data suggest that flurbiprofen produces greater pain relief than acetaminophen. Few side effects have been reported following either treatment. This study suggests that new pain control agents that are relatively risk-free, may be extremely useful for controlling pain associated with outpatient dental therapy.

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McGrath, P.A., Sharav, Y., Dubner, R. and Gracely, R.H.: Masseter inhibitory periods and sensations evoked by electrical tooth pulp stimulation. Pain, in press.

Price, D., Hayashi, H., Dubner, R. and Ruda, M.A.: Functional relationships between neurons of the marginal and substantia gelatinosa layers of the primate dorsal horn. J. Neurophysiol., 42:1590-1608, 1979.

Bennett, G.J., Abdelmoumene, M., Hayashi, H. and Dubner, R.: Physiology and morphology of substantia gelatinosa neurons intracellularly stained with horseradish peroxidase. J. Comp. Neurol., 1980, In press.

Bennett, G.J., Abdelmoumene, M., Hayashi, H., Hoffert, M.J. and Dubner, R.: Spinal cord layer I neurons with axon collaterals that generate local arbors. Brain Res., In press.

Ruda, M.A. and Gobel, S.: Ultrastructural characterization of axonal endings in the substantia gelatinosa which take up [^{3}H] Serotonin. Brain Res. 184:57-84, 1980.

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Ruda, M.A., Allen, B. and Gobel, S.: Ultrastructural analysis of medial brainstem afferents to the superficial dorsal horn. Brain Res., in press.

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Dubner, R., Hoffman, D.S. and Hayes, R.L.: Neural correlates of behavior in the monkey medullary dorsal horn. In: Oral-Facial Sensory and Motor Functions, edited by Y. Kawamura and R. Dubner, Quintessence, Tokyo, In press.

Abdelmoumene, M., Bennett, G.J., Hayashi, H., Gobel, S., Falls, W.M., Humphrey, E. and Dubner, R.: Substantia gelatinosa interneurons. Proc. of IUPS Satellite Symposium on Dorsal Horn, 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 00031-12 NA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 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

Brown, Frederick J.	Electronic Engineer (Instru)	NIDR NA
Medlin, Terry P.	Supv. Computer Specialist	NIDR OD

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Neural Mechanisms Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

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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)

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) An electronic logic system has been modified to allow the training of monkeys to a different behavioral paradigm. The training system uses monkey panel switch presses, fixed and random timing cycles, variable intensity light and thermal stimuli, sound and light cues, and liquid reinforcement. Built with IC logic and designed for use with moderate supervision, the system provides for some paradigm alternatives and instantaneous cue changes at each level of training. Training is provided for reaction time, paired stimulus, and sequential paired stimulus paradigms.

b) A DEC 11/34 computer which is now controlling neurophysiological-behavioral experiments on monkeys in one laboratory is being interfaced to the equipment in a second laboratory for similar experiments there. Scientists will interact with the 11/34 from each of the labs to control their experiments. The new lab interface and software will provide the scientists with on-line analysis of neural and behavioral data. An INTEL SBC 80-20 computer will assist in controlling the lab-equipment and buffer neural and behavioral data returned to the 11/34 from the lab.

c) A dual probe thermal stimulator has been built for the above laboratory. The two probe contact stimulator is similar to a previous design and uses circulating chilled water, nichrome heating elements, and electronic feedback control to regulate the probe temperatures between 20° and 60°C. The probes have individually settable rates of cooling and warming from 1° to 10°C/sec, and the probe temperatures are under computer control.

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 00165-05 NA

PERIOD COVERED
October 1, 1979 to September 30, 1980

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

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 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.15	.05	.10

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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 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 of 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.

Methods Employed: The 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. The animals had to concurrently perform two types of tasks: A detection task (reinforced response) and a discrimination task (nonreinforced response). This animal model of pain also is employed in NIRP Number Z01 DE 00291-01, "Neural Correlates of Behavior in the Monkey Medullary Dorsal Horn".

Major Findings: We have reported previously that morphine significantly increased the latencies at which monkeys discriminated 47°C and 49°C stimuli from innocuous temperatures of 37-43°C. Diazepam, in contrast to morphine, did not consistently reduce the discriminability of noxious heat (45-49°C) stimuli. In a second experiment, in addition, discrimination of 41° and 43°C stimuli was required. Morphine again suppressed the discrimination of noxious heat stimuli (45° and 49°C), and also increased discrimination latencies to 41° and 43°C stimuli. Diazepam had no systematic effect in this second experiment.

We concluded that the morphine effect was probably related to its attenuation of the perceived stimulus intensity apart from its effect on aversive aspects of the stimulus since the effects were not limited to noxious stimuli.

This study is now completed and is being prepared for publication.

Significance to Biomedical Research and the 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 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.

Proposed Course: This study will be terminated after publication of findings.

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 00247-03 NA

PERIOD COVERED

October 1, 1979 to September 30, 1980

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

Bennett, Gary J.	Staff Fellow	NIDR NA
Abdelmoumene, Mohammed	Visiting Scientist	NIDR NA
Hoffert, Marvin J.	Senior Staff Fellow	NIDR NA
Seltzer, Zeev	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 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.15	2.85	.30

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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)

Neurons in Rexed's layers I and II were physiologically characterized with respect to innocuous and noxious natural stimuli and percutaneous electrical stimuli applied to their cutaneous receptive fields. The neurons were then intracellularly stained with horseradish peroxidase which revealed their morphology for light and electron microscopic study. Layer I neurons either responded exclusively to noxious stimuli (NS neurons) or they responded to both noxious stimuli and innocuous mechanical stimuli (wide-dynamic-range; WDR neurons). Some layer I neurons were seen to have axon collaterals that generated extensive arborizations that overlapped their dendritic territories. Neurons with perikarya in the superficial one-third of layer II (layer IIa) were physiologically identified as either WDR or NS neurons. Layer IIa neurons were morphologically identified as stalked cells and islet cells. All of the islet cells in the deep two-thirds of layer II (layer IIb) responded exclusively to innocuous mechanical stimuli. These results show that stalked cells and islet cells are separate and distinct components of the neural circuitry in the substantia gelatinosa.

1. Project Description:

Objectives: The neuronal circuitry within the dorsal horn that subserves the sensations and reactions that are consequent to tissue damage is only vaguely understood. Although both electrophysiological and anatomical analyses of dorsal horn circuitry have a long history, these two bodies of data stand largely separate from one another.

This project uses a powerful technique (described below) that reveals the morphology of individual physiologically characterized neurons. Light microscopic examination of these neurons reveals the location and disposition of their dendritic and axonal processes. Electron microscopic examination reveals the synapses that the neurons receive and emit.

We have concentrated our attention on the two most superficial layers of the dorsal horn. Layer I is known to contain neurons that are innervated by nociceptors and that project to the somatosensory thalamus. Thus, layer I is an important output system sending information from the spinal cord to the brain. Layer II, the substantia gelatinosa, contains exceedingly few neurons that project to the brain. Instead, layer II neurons interact with other neurons in the spinal cord. A great deal of circumstantial evidence suggests that layer II neurons modulate the activity of projection neurons such as those in layer I.

Methods Employed: The lumbosacral spinal cords of anesthetized adult cats are prepared for electrophysiological recording in the usual manner. We use glass micropipettes with tip diameters of about 1.0 μm. Because these relatively large tips yield poor intracellular records, we physiologically characterize the neuron before penetration. The cell's responses to a battery of natural stimuli (touch, hair, movement, pressure, pinch, noxious heat, etc), and to percutaneous electrical stimulation of the cell's receptive field are determined. The percutaneous electrical stimulus allows us to estimate the conduction velocities of the cell's inputs. After physiologically characterizing the neuron, an attempt is made to introduce the micropipette into the neuron's interior. Successful penetrations are carefully checked in order to be certain that the impaled neuron is the same as the one characterized. HRP is iontophoresed into the cell for the duration of the impalement (1-15 min). At the conclusion of the experiment, the animal is perfused with fixative and the spinal cord removed. The cord is sectioned parasagittally into 150 μm thick sections. The tissue is then incubated in 0.5% $COCL_2$ and the HRP reaction product is developed by incubating with diaminobenzidine and hydrogen peroxide. The developed sections are cleared with glycerin and examined with the light microscope. Camera lucida drawings (1,650 X) are prepared from well-stained examples. The tissue is then removed from the glycerin and processed for electron microscopy in the usual fashion.

Major Findings: Our results have shown that layer I contains both NS and WDR neurons. While most WDR layer I neurons receive their low threshold mechanoreceptive input from primary afferents with large myelinated axons, we have documented that some WDR layer I neurons receive input from afferents with small myelinated axons (down hair afferents). This type of input has not been reported before. Morphologically, some layer I neurons are noteworthy because they have axon collaterals that generate extensive local arborizations. These arborizations appear to be largely within layer I and they overlap their dendritic territories. The collaterals in the local arborization issue many varicosities that are undoubtedly presynaptic boutons. This is the first demonstration that layer I neurons synapse in the dorsal horn. An intriguing possibility is that the layer I neurons that issue these local axonal arborizations are the enkephalinergic layer I neurons that have been seen in immunohistochemical studies.

Layer IIa neurons were physiologically characterized as either NS or WDR neurons. Their patterns of primary afferent innervation are very similar to those of layer I neurons. Two morphological cell classes were found in layer IIa and each class contained both WDR and NS neurons. Stalked cells had their perikarya along the superficial border of layer IIa. Their dendrites generally traveled ventrally while fanning out rostrocaudally. Their dendritic arbors were compressed mediolaterally. Their axons arborized largely in layer I. Layer IIa islet cells had dendrites that were deployed parallel to the layer borders and traveled rostrocaudally. Their arbors were also mediolaterally compressed. Previous work has shown that their axons arborize in the immediate vicinity of their dendritic territories.

Layer IIb neurons only responded to innocuous tactile stimuli. Nearly all of these neurons were islet cells. They differed from the islet cells in layer IIa in the size of their dendritic arbors which were 2-4X times larger rostrocaudally than the dendritic arbors of layer IIa islet cells.

The functional division of layer II into a superficial part (IIa) that is concerned with nociceptor and low threshold mechanoreceptor input, and an inner part (IIb) that is concerned exclusively with low threshold mechanoreceptor input is a new concept. It is consistent with the previously proposed anatomical division of the layer and suggests a morphological substrate for the well documented inhibition of nociception caused by activity in low threshold mechanoreceptors.

The electron microscopic analyses of intracellularly stained stalked cells, layer IIa islet cells, and layer IIb islet cells showed that all of these layer II neurons receive primary afferent synapses. In addition, they also receive synapses from axonal endings that resemble the tryptaminergic and noradrenergic endings of brain stem origin that have been studied by others in this laboratory. This observation suggests that both stalked cells and islet cells are involved in centrifugal control systems.

Importantly, the electron microscopic analyses have shown that islet cells, but not stalked cells, have vesicle-filled presynaptic dendrites. This ultrastructural difference, and the light microscopic observations about the different organizations of dendritic and axonal arbors, makes it clear that stalked cells and islet cells are separate and distinct components of gelatinosal circuitry.

Significance to Biomedical Research and the Program of the Institute: Many diseases, such as advanced carcinoma, are characterized by intractable pain. In addition several neuropathies (e.g., post-herpetic neuralgia, post-traumatic causalgia) present bizzare and excruciating pain sensations as their most salient symptom. Satisfactory therapy for these conditions is generally unavailable. We do not understand their etiology, largely because we do not understand the neural mechanisms that normally subserve pain sensations. The work described here is an effort to delineate the major neuronal circuitry that subserves nociception in normal skin. This work will contribute to our understanding of pain mechanisms in general and will be the foundation of our understanding of the etiology of pain pathologies.

Proposed Course: We plan to continue this work and extend our observations to the neurons in other parts of the dorsal horn. In particular, the physiology and morphology of neurons in Rexed's layer III are very little known and we have begun to analyse them. In the past our physiological characterization has been confined to the neurons' cutaneous input. We plan to extend our observations to orthodromic and antidromic electrical stimuli applied to higher levels of the neuraxis. In order to clearly identify tryptaminergic synapses onto intracellularly stained neurons, we have begun to apply a tryptaminergic neurotoxin after intracellular staining. Tryptaminergic synapses will degenerate and be easily seen in electron micrographs. In addition, we are continuing to develop immunohistochemical techniques that will allow us to neurochemically identify intracellularly stained cells and the synapses that they receive.

2. Publications:

Price, D., Hayashi, H., Dubner, R. and Ruda, M.A.: Functional relationships between neurons of the marginal and substantia gelatinosa layers of the primate dorsal horn. J. Neurophysiol., 42:1590-1608, 1979.

Bennett, G.J., Abdelmoumene, M., Hayashi, H. and Dubner, R.: Physiology and morphology of substantia gelatinosa neurons intracellularly stained with horseradish peroxidase. J. Comp. Neurol., 1980, In press.

Bennett, G.J., Abdelmoumene, M., Hayashi, H., Hoffert, M.J. and Dubner, R.: Spinal cord layer I neurons with axon collaterals that generate local arbors. Brain Res., In press.

Abdelmoumene, M., Bennett, G.J., Hayashi, H., Gobel, S., Falls, W.M., Humphrey, E. and Dubner, R.: Substantia gelatinosa interneurons. Proc. of IUPS Satellite Symposium on Dorsal Horn, 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 00289-01 NA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Morphological features of physiologically characterized primary afferent fibers

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

Seltzer, Zeev	Visiting Fellow	NIDR NA
Bennett, Gary J.	Staff 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 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.80	.70	.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)

Intracellular recordings from cat's trigeminal and dorsal root ganglion cells and from dorsal root afferent fibers were made under barbiturate anesthesia. Functional characterization of the response to natural stimuli and to electrical shocks, was followed by horseradish peroxidase (HRP) deposition in the cell body or fiber. The labeled cell and the central terminal projections of the axonal arbors, in the histochemically reacted tissue, were recovered at the light microscopical level. Analysis of the labeled structures, reconstructed in the parasagittal plane, enabled the categorization of different termination sites for various functionally-characterized afferent fibers.

1. Project Description:

Objectives: Previous studies have identified and classified the response properties of peripheral nerve fibers that innervate the face and body skin. Other studies have described the morphological features of the central terminal arbor of primary afferents in the dorsal horn. The aim of the present study was to correlate the physiological properties of these fibers with their terminal projections in different layers of the trigeminal medullary and spinal cord dorsal horn. (This project is a continuation of project # Z01 DE 00040-10).

Methods Employed: Under barbiturate anesthesia, intracellular recordings from trigeminal and dorsal root ganglion cells and from dorsal root afferent fibers were made in cats. Natural stimuli (thermal and mechanical both at noxious and nonnoxious intensities) and electrical shocks were delivered to the cutaneous receptive fields, enabled physiological characterization of the recorded units. This was followed by iontophoretic or pressure injected deposition of the HRP enzyme. After sacrifice and perfusion of the animal and sectioning of the spinal cord or brain stem, this enzyme catalysed in a histochemical reaction the polimerization of diaminobenzidine as a chromogen. The structures containing HRP thus labeled, were recovered and examined with light microscope and reconstructed in the parasagittal plane.

Major Findings: With intracellular injections of physiologically identified primary afferent ganglion cells, we were able to recover their stem axons at a considerable distance along the dorsal root. We are in a process of examining enhancing methods that will enable us to detect the HRP filled structures within the spinal cord. With intraaxonal injections of the afferent fibers near the dorsal root entry zone, we were able to label several physiological identified fibers. Preliminary results indicate that different functional types of primary afferents project to different layers in the spinal cord dorsal horn.

Significance to Biomedical Research and the Program of the Institute: These studies shed light on the way in which the peripheral nervous system channels the input which conveys coded messages that notify the presence of stimuli of various modalities. These studies mainly concentrate on studying the way by which the presence of tissue-threatening or tissue-damaging stimuli in the environment are channeled through specific primary afferent fibers to specific layers in the spinal cord dorsal horn. Thus, through better understanding of the neural circuitry which are involved in pain sensation we hope to develop new and improved methods of pain control.

Proposed Course: Utilizing the method described to extend the initiated study into a conclusive categorization of different modality classes of primary afferents correlated with their different layers of termination in the brain stem and spinal cord dorsal horn.

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 00291-01 NA

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Neural Correlates of Behavior in the Monkey Medullary Dorsal Horn

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

Bushnell, M. Catherine	Staff Fellow	NIDR NA
Dubner, Ronald	Chief, NAB	NIDR NA
Ziriax, John M.	Psychologist	NIDR NA
Taylor, Mark B.	Biological Aid	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH

Neurobiology and Anesthesiology Branch

SECTION

Neural Mechanisms Section

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NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.05	1.20	1.85

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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 effect of behavioral and environmental variables on responses of neurons in the medullary dorsal horn (trigeminal nucleus caudalis) to noxious and innocuous thermal stimuli. Rhesus monkeys were trained to detect the termination of innocuous heat stimuli and the onset of noxious heat stimuli. In a second task, the same monkeys detected the onset of a light stimulus. Neurons that responded to passive noxious mechanical and thermal stimulation also responded to other stimuli the monkey used for successful completion of a task. These task-related responses occurred to visual or thermal stimuli that predicted availability of reward. Neurons that reliably coded intensity of noxious thermal stimuli generally showed only small task-related responses, while neurons with large task-related responses often had imprecise sensory properties. Both types of neurons projected to the thalamus. These task-related responses may modulate sensory activity and thereby influence the perception of and response to oral-facial pain.

1. Project Description:

Objectives: This project was designed to study the modulatory influence of behavioral factors on the coding of nociceptive input in the medullary dorsal horn (trigeminal nucleus caudalis). Previous work in this project has demonstrated two classes of behavioral influence in nociceptive second-order neurons: (1) a modulation of the magnitude of response of nociceptive neurons to thermal stimuli by behavioral contingencies; (2) a separate response of some mechanically sensitive and heat sensitive nociceptive neurons to any stimulus that predicts the availability of reward, regardless of the sensory modality of that stimulus. The present studies provide a further exploration of these behavioral influences, in order to understand the functional significance to nociception of such activity. In addition to studying the sensory and behavioral response characteristics of these neurons, we also determined whether these neurons projected directly to the thalamus. The location of behaviorally influenced sensory neurons within the medullary dorsal horn also was determined.

This project is a continuation of NIRP DE 00151-05, with new emphasis on neural activity in behaving monkey.

Methods Employed: Monkeys are first trained to perform tasks involving thermal and visual stimuli. The tasks have been reported previously. In the thermal task, monkeys are trained to detect the termination of innocuous heat pulses delivered via a feedback-controlled contact thermode (1 cm diameter) applied to the upper hairy lip. The baseline temperature of the probe is 35°C. A panel is illuminated to signal the beginning of each trial, and the monkey depresses the lighted panel to initiate innocuous heat pulses of 37°, 39°, 41° and 43°C. After a random duration (2-8 sec) the temperature of the probe returns to 35°C, and the monkey receives a water reward for releasing the panel within 2 sec of the heat pulse termination. Water reward is sometimes delayed for 1 or 2 sec after panel release, in order to temporally separate the panel release from receipt of the reward. Additional heat pulses in the noxious range (45°, 47°, 49°C) are presented quasi-randomly. Release of the panel at the termination of noxious stimuli is not rewarded by water. However, panel release terminates the noxious heat pulse, thus allowing the monkey to escape the stimulus. Accurate performance on this task requires that the monkey discriminate noxious and innocuous temperatures, as well as detect innocuous temperature shifts in order to receive a reward.

A visual task is employed in which the monkey detects the onset of a light stimulus. As in the thermal task, a panel is illuminated to signal the beginning of each trial. The monkey depresses the panel, and after a random time (2-8 sec) a second panel light is illuminated. Panel releases of less than 2 sec after the light onset are rewarded by

water. Heat pulses are never presented in this task; either the thermode remains at 35° during the trial or is removed from the monkey's face. This visual task is used to assess task-related response characteristics of mechanically and thermally sensitive neurons that are separate from the responses to thermal stimuli.

After learning the behavioral tasks, the monkeys are prepared for single unit recording: a sealed chamber and head holding apparatus are chronically fixed to the monkey's skull, stimulating electrodes are stereotaxically introduced into the VPM thalamic nucleus and EMG electrodes are inserted in the musculature of the upper lip. After recovery from surgery, the monkey is placed daily in the recording apparatus, a microelectrode is introduced into the brainstem medullary dorsal horn and single unit activity is recorded while the monkey performs the behavioral tasks. Neuronal activity is correlated with the following behavioral events: (1) panel light onset indicating the start of the trial; (2) panel press; (3) thermal pulse onset; (4) thermal pulse offset; (5) light cue onset; (6) panel release; and (7) delivery of reward.

Major Findings: Medullary dorsal horn neurons differ along two response dimensions: (1) sensory discriminative properties in response to thermal and mechanical stimuli; and (2) task-related responses independent of stimulus parameters and motor activity. Thermal nociceptive neurons had properties similar to those previously found in anesthetized animals. They were classified as wide-dynamic-range or nociceptive-specific based on responses to mechanical and thermal stimulation. Stimulus-response functions were monotonic from threshold to 49°C and the neurons were most sensitive when stimuli were applied to the central portion of their receptive fields. Thermal nociceptive neurons responded to noxious stimuli earlier than the shortest discrimination latencies of the monkeys on 45°-49°C trials. For some neurons, the magnitude of the neuronal response was inversely related to discrimination latency. Thus, thermal nociceptive neurons provide signals related to the monkey's ability to discriminate noxious heat stimuli. These findings support earlier conclusions that wide-dynamic-range and nociceptive-specific neurons code sensory-discriminative information.

The responses of these neurons to thermal and mechanical stimuli, however, can be modified by the behavioral situation. Behaviorally-relevant thermal stimuli presented during performance of the thermal task produced greater neuronal responses than equivalent nonrelevant thermal stimuli presented during the intertrial interval in the thermal task or during performance of the visual task. Neuronal responses to mechanical stimulation produced by lip movements under the thermal probe were less during performance of the visual task than during the thermal task. The effects of changes in selective attention were studied in the visual task by systematically reducing the intensity of the light cue. A reduction in intensity of the cue required monkeys to attend more vigorously to light onset and was associated with a reduction in neuronal responses to noxious and innocuous thermal stimuli. Attentional demands

also were reduced in the thermal task by having the experimenter rather than the monkey initiate the trials and reward the monkey appropriately on innocuous thermal trials. Reduced neuronal responses to thermal stimuli were observed on experimenter-initiated trials. These findings indicate that the behavioral context in which stimuli are presented modulates the responses of thermal nociceptive neurons to thermal and mechanical stimulation. Significant behavioral variables include the relevance of the stimulus to the behavioral task and the attentional demands of the task.

Some thermal nociceptive neurons and mechanoreceptive neurons not responsive to thermal stimuli exhibited responses that were not related to stimulus features or to parameters of movement and occurred only during the behavioral tasks. These task-related responses include an increase in activity when the panel is illuminated, sustained activity while the monkey presses the panel, and a further increase in activity when the relevant cue leading directly to reinforcement occurs. These task-related responses were independent of stimulus modality or intensity, since they were present in the thermal and visual tasks and occurred in the absence of thermal or mechanical stimulation of the face. These responses were absent within a task if a particular stimulus provided no information leading to reinforcement. Task-related responses also occurred in the absence of lip movements associated with reception of the liquid reinforcement and could be uncoupled from hand and arm movements associated with panel press and panel release.

Some medullary dorsal horn neurons had robust task-related responses in the thermal task whereas others exhibited responses to a lesser degree, or no responses at all. Task-related responses usually were weaker in the visual task than in the thermal task. Most neurons that coded thermal intensity had weak or no task-related responses while these mechanoreceptive neurons that coded stimulus parameters less precisely exhibited substantial task-related activity. Neurons with precise sensory-discriminative properties and neurons with robust task-related responses were found in the superficial and deep layers of the medullary dorsal horn. Both types projected to nucleus ventralis posteromedialis in the thalamus.

The above lines of evidence suggest that medullary dorsal horn neurons exhibit responses in the behaving animal that result from the integration of environmental sensory input and the central neural evaluation of its behavioral significance. These responses are evoked by environmental sensory cues that reliably predict reinforcement. They appear to project to thalamic sites involved in sensory and motor activity leading to successful completion of the task and reception of the liquid reinforcement.

Significance to Biomedical Research and the Program of the Institute: These studies have characterized some behavioral modulatory influences on sensory neurons that relay information about noxious stimuli and thus could participate in the perception of and response to oral-facial pain.

Sensory neurons that show task-related activity project to the thalamus, suggesting a continued modulatory influence by environmental and behavioral factors at other levels of sensorimotor integration. These data show that the neural representation of oral-facial nociceptive input can be modified at the earliest stage of central nervous system integration. This modulation could influence the perception of oral-facial pain as well as facilitate rapid motor responses to noxious stimuli. The behavioral influences on sensory neurons that we show appear to be related to attentional, motivational and expectational manipulations. Thus, this work could suggest non-pharmacological approaches to the control of pain.

Proposed Course: Future experiments include the following: (1) introduce a new behavioral task designed to maximize the sensory-discriminative functioning of monkeys by requiring them to attend to stimulus intensity in order to chose the greater of two stimuli which are close in magnitude. This new task should maximize the responses of nociceptive neurons to noxious and innocuous thermal stimuli and will allow us with greater reliability to implicate certain classes of neurons in pain discrimination; (2) the study of possible mechanisms by which behavioral factors influence nociceptive neurons. This investigation includes assessing the effects of (a) pharmacological manipulations, such as systemic administration of various neuropeptides, and (b) electrical stimulation of reticular and cortical inputs to the medullary dorsal horn during behavioral responses in the thermal and visual tasks; (3) the study of nociceptive neurons in the ventroposterior medial thalamus in behaving monkeys.

2. Publications:

Dubner, R.: Peripheral and central mechanisms of pain. NIH Symposium on New Therapies for Pain and Discomfort, In press.

Dubner, R., Hoffman, D.S. and Hayes, R.L.: Neural correlates of behavior in the monkey medullary dorsal horn. In: Oral-Facial Sensory and Motor Functions, edited by Y. Kawamura and R. Dubner, Quintessence, Tokyo, 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 00132-06 NA

PERIOD COVERED
October 1, 1979 to September 30, 1980 CT 0060102

TITLE OF PROJECT (80 characters or less)

Psychophysiological Assessment of Intravenous Sedation

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

Dionne, Raymond A.	Staff Fellow	NIDR NA
Gracely, Richard H.	Research Psychologist	NIDR NA
Amato, Peggy R.	Clinical Nurse	NIDR NA
Clark, Barbara Ann	Clinical Nurse	NIDR NA
Streko, Thomas	Staff Dentist	NIDR NA
Sweet, James B.	Sr. Dental Surgeon	NIDR IR

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Clinical Pain Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.45	1.25	1.20

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 purpose of this project is to objectively evaluate the efficacy and clinical toxicity of drugs given to outpatients to alleviate apprehension associated with dental procedures. Measures of efficacy assessed include relief of anxiety, analgesia, amnesia and patient cooperation. Measures of clinical toxicity include respiratory and cadiovascular depression, psychomotor impairment and central nervous system depression. Previous work indicates that the combination of diazepam, fentanyl and methohexital causes a significant decrease in respiration rate and oxygen saturation while the combination of diazepam and methohexital does not depress respiration. A second study suggests that these two combinations are causing a transient decrease in stroke volume which may account, in part, for the subsequent reflex tachycardia seen. Recent work has been aimed at developing more sensitive and reliable scales for assessing the subjective effects of anti-anxiety agents. These newer scales are being employed, in conjunction with physiological and psychomotor assessment procedures, to evaluate traditional and novel anti-anxiety agents and combinations.

1. Project Description:

Objectives: The purpose of this project is to objectively evaluate the efficacy and clinical toxicity of drugs given to dental outpatients to alleviate apprehension and pain associated with dental procedures. Measures of efficacy assessed include relief of anxiety, analgesia, amnesia and patient cooperation. Measures of clinical toxicity assessed include arterial oxygen saturation, respiratory rate, blood pressure, cardiac output, stroke volume, heart rate, impairment of psychomotor and cognitive function and non-specific central nervous system depression. By combining the results of these individual assessments, it is possible to quantify the relationship between efficacy and clinical toxicity and determine the therapeutic ratio for various agents and combinations. This index provides an objective, rational basis for selecting the optimal drug or combination for the various therapeutic situations in which they are used. In addition, the therapeutic ratio provides an objective method for assessing new agents proposed for the relief of acute apprehension and pain.

Methods Employed: Patients undergoing the removal of impacted third molars serve as experimental subjects for these investigations. Patients are screened to confirm the need for extraction, a complete medical history and physical examination performed and the surgical procedure is conducted in the NIDR Dental Clinic in accordance with normal clinical care.

A recently completed study assessed the physiological effects of four commonly employed intravenous sedative regimens: diazepam, diazepam plus fentanyl, diazepam plus methohexital, and diazepam, fentanyl and methohexital. Physiological response was monitored non-invasively to avoid introducing anxiety and morbidity which can be associated with invasive techniques. Oxygen saturation was recorded via an earpiece oximeter, blood pressure via an automatic blood pressure, and heart rate, stroke volume and cardiac output with an impedance cardiograph. Continuous readings were taken during a baseline period, through surgery, and post-operatively.

A second study has been evaluating newly developed scales for assessing subjective effects of anti-anxiety agents. Differential descriptor scales which assess anxiety, relaxation, sleepiness (i.e., sedation) and alertness are administered in conjunction with a traditional ordinal ranking scale and Spielberger's State-Trait Anxiety Inventory. Previously validated differential descriptor scales for sensory intensity, unpleasantness and painfulness are administered along with an ordinal ranking scale to assess the amount of pain experienced during surgery. Testing is performed prior to surgery, after placebo administration and following administration of active drugs. Prototype drugs of the anti-anxiety class (diazepam), barbiturates (methohexital) and narcotic analgesics (fentanyl) are being employed to validate the new scales.

Major Findings: The physiological investigations have confirmed our earlier finding that the combination of diazepam, fentanyl and methohexital results in respiratory depression. Both respiratory rate and arterial oxygen saturation are significantly decreased. Respiratory depression was not seen following the other drug combinations or diazepam alone. No decrease was seen from baseline cardiac output following any of the four treatments. The diazepam-fentanyl-methohexital combination resulted in a transient decrease in stroke volume and a reflex tachycardia. This was seen to a lesser extent in the diazepam-methohexital group. No significant changes were seen following diazepam-fentanyl or diazepam alone.

These results provide further evidence that a widely used sedative combination, diazepam-fentanyl-methohexital, is resulting in respiratory and cardiovascular depression. Previous studies of this combination in comparison to diazepam-methohexital have indicated that patients' cannot differentiate between these two combinations in terms of efficacy. It has also been demonstrated that relatively large doses of intravenous diazepam result in near-maximal anxiety relief but without any significant cadiovascular or respiratory depression. These findings suggest that the use of the diazepam-fentanyl-methohexital combination may be resulting in little increase in efficacy while increasing the potential for clinical toxicty. In contrast, a single agent, such as diazepam, is resulting in near optimal anxiety relief without any detectable physiological impairment.

The differential descriptor scales for assessing the subjective effects of drugs employed for sedation appear to be sensitive for differentiating between drug groups. Diazepam, a relatively specific anti-anxiety agent, caused the greatest relief of anxiety and increase in relaxation when compared to placebo. Diazepam also decreased the painfulness of the procedure by decreasing the unpleasantness but not the perceived intensity of painful surgical stimuli. All three drugs resulted in an increase in non-specific CNS depression. These preliminary findings suggest that these scales may be useful for assessing the individual contribution different drugs make to the overall effects of sedative drugs.

Significance to Biomedical Research and the Program of the Institute: Research to date under this protocol has resulted in the development of techniques which are useful for assessing the therapeutic and toxic effects of sedative drugs given to dental outpatients. This research has also resulted in a body of knowledge on the various effects of the drug combinations tested.

The delivery of dental care and the maintenance of oral health is contingent upon regular professional care. It is generally accepted that apprehension about the pain associated with dental care causes a portion of the population to avoid or postpone dental care. Intravenous sedation is a technique for overcoming patient apprehension and minimizing any perception or memory of pain. Through these investigations, we have

been able to identify these drugs and combinations which are effective for achieving these goals with a minimum potential for clinical toxicity.

Proposed Course; Future studies are aimed at assessing the efficacy and potential toxicity of existing and novel anti-anxiety agents. The overall goal of these investigations will be to develop methods for controlling pain and apprehension which have minimal risk for the patient and require minimal additional training for the clinician. Various oral premedications will be evaluated for use in conjunction with nitrous oxide.

2. Publications:

Driscoll, E.J., Gelfman, S.S., Sweet, J.B., D.P. Butler, Wirdzek, P.R. and T. Medlin: Thrombophlebitis after intravenous use of anesthesia and sedation: its incidence and natural history. J. Oral Surg. 37:809-815, 1979.

Gelfman, S.S., Gracely, R.H., Driscoll, E.J., Butler, D.P., Sweet, J.B. and Wirdzek, P.R.: Recovery following intravenous sedation during dental surgery performed under local anesthesia. Anesth. Analg., October 1980.

Dionne, R.A., Driscoll, E.J., Gelfman, S.S., Sweet, J.B., Butler, D.P. and Wirdzek, P.R.: Cardiovascular and respiratory response to intravenous diazepam, fentanyl and methohexital in dental outpatients. J. Oral. Surg., 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 00133-06 NA

PERIOD COVERED
October 1, 1979 to September 30, 1980 CT 0060101

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

Gracely, Richard H.	Research Psychologist	NIDR NA
Dionne, Raymond A.	Staff Fellow	NIDR NA
Dubner, Ronald	Chief, NAB	NIDR NA
Heft, Marc W.	Postdoctoral Fellow	NIDR NA
Wolskee, Patricia J.	Psychologist	NIDR NA
Streko, Thomas C.	Staff Dentist	NIDR NA
Sweet, James B.	Sr Staff Dent (Oral Surg)	NIDR IR

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Clinical Pain Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.35	1.05	2.30

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.

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 the 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: A new study on the mechanisms of postoperative dental pain used verbal descriptor scales developed in this laboratory to assess pain following oral-surgical extraction of third molar teeth. Pain was assessed before and after intravenous injections of saline; fentanyl, a short acting narcotic analgesic; or naloxone, a narcotic antagonist. A new experimental design used both hidden and know intravenous injections to isolate the pharmacological action of the medications from the placebo effects produced by the knowledge that a drug had been administered. The sensitivity of the verbal measures was validated by a demonstrable pain reduction following administration of fentanyl and reversal of this effect following administration of naloxone. This study also showed that, in comparison to placebo, naloxone produces a biphasic effect on pain responses. Ten mg naloxone produced analgesia for 5-15 min after injection but produced hyperalgesia by 60 min after injection.

An additional study examined the influence of naloxone on pain produced by experimental electrical stimulation of intact incisors in oral-surgical patients. Naloxone did not alter responses made by either cross-modality matching to handgrip force or by choosing verbal descriptors to assess the intensity and unpleasantness of the tooth sensations. Naloxone did significantly increase the variability of sensory intensity handgrip responses in comparison to placebo.

Another study assessed the ability of volunteer subjects to measure and integrate an experience of pain and a linguistic symbol of that experience. Subjects were presented both a painful electrical tooth pulp stimulus and a verbal descriptor of sensory intensity or unpleasantness and asked to produce a response proportional to the average of the two stimuli. Responses to 25 pain-word pairs were analyzed by an analysis of variance according to Functional Measurement Theory. The results show that subjects can easily integrate an experimental pain experience with sensory intensity descriptors, but have difficulty integrating pain experience with unpleasantness descriptors.

Further studies examined the effects of fentanyl or placebo on the intensity or unpleasantness of pain sensations produced by electrical tooth pulp stimulation in patients suffering from chronic Myofascial Pain Dysfunction (MPD) syndrome. The results of these studies are presented in the NIRP Number Z01 DE 00246-03, "Masseteric Exteroceptive Reflex and Sensory Responses in MPD Patients".

Significance to Biomedical Research and the Program of the Institute: Previous studies suggest that naloxone reverses placebo-produced analgesia of postoperative dental pain. The study assessing the effects of naloxone and placebo on postoperative dental pain, when completed, will determine if this effect is dependent on placebo administration or if naloxone also increases postoperative pain when placebo is not administered. This result will provide evidence pertaining to an alternative hypothesis that naloxone increases pain by antagonizing the effects of endogenous opiate-like compounds (endorphins) released as a consequence of the stress or trauma of surgery and that placebo reduces pain by independent mechanisms. The demonstration of a biphasic response effect following naloxone administration suggests that lower doses of naloxone produce analgesia and higher doses hyperalgesia. This result, which is consistent with other evidence in human subjects, suggests that experiments designed to assess the action of endorphins must establish a dose/response relationship. These results suggest also that the action of naloxone is more complex than that implied by a simple model of endorphin antagonism.

The study investigating the effects of naloxone on experimental pain showed that mean pain was unaffected, a result consistent with previous studies using different experimental stimuli. The finding that naloxone increases scaling variability suggests that many effects observed with naloxone may be artifactual and irrelevant to pain or analgesia. Measures based on discrimination or influenced by psychological performance, such as sensory decision theory or cortical-evoked potentials, may show a naloxone-produced change in pain responses that represents altered psychophysical ability and not a change in pain perception. To control for this artifact, studies using such measures to assess pain responses should include non-noxious stimuli.

The study evaluating the ability of subjects to integrate pain experience and language shows that subjects use verbal descriptors of

sensory intensity reliably, and verbal descriptors of unpleasantness less reliably, to describe sensations produced by electrical tooth pulp stimulation. These results validate further the use of verbal descriptors to assess different dimensions of pain experience and show that facility with verbal descriptors varies with the type of dimension assessed.

The separate assessment of analgesic effects on pain perception and on the response labels attached to the perception is a major but elusive goal in the development of psychophysical pain measures. These results indicate that this cognitive integration model may provide a method to distinguish between the effects of a putative analgesic manipulation on either pain perception or on the verbal report of this perception. This model may be useful in separating the physiological effect of a pain treatment from the psychological effects resulting from the experimental situation, expectations of the subjects, and the side effects of the treatment.

Proposed Course: Future studies on the role of psychological and pharmacological factors that modify the perception of postoperative pain will further specify the influence of patients' expectations and the role of endorphin mechanisms in narcotic and placebo analgesia. The sensitivity of several techniques for assessment of postoperative pain will be evaluated in a separate study using narcotic analgesics and active placebo medications.

Studies on the role of endorphin modulation of experimental pain will continue with an examination of the interaction of naloxone and postoperative pain on responses to pain sensations produced experimentally.

The experiments with the pain-language integration model will continue with studies assessing the sensitivity of this model to pharmacological agents such as fentanyl and diazepam that have been used previously in this laboratory to evaluate psychophysical measures of pain evoked by experimental electrical stimulation of teeth.

2. Publications:

Gracely, R.H.: Psychophysical Assessment of Human Pain. In: Advances in Pain Research and Therapy, Vol. 3, edited by J.J. Bonica, J.C. Liebeskind and D. Albe-Fessard, New York: Raven Press, 1979, p. 805-824.

Gracely, R.H.: Pain Measurement in Man. In: Pain, Discomfort and Humanitarian Care, edited by L. Ng and J.J. Bonica, Proceedings of NIH Symposium, February, 1979, in press.

Heft, M.W., Gracely, R.H., Dubner, R. and McGrath, P.A.: A Validation Model for Verbal Descriptor Scaling of Human Clinical Pain. Pain, 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 00245-03 NA

PERIOD COVERED
October 1, 1979 to September 30, 1980 CT 0060117

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

Dubner, Ronald	Chief, NAB	NIDR NA
Gracely, Richard H.	Research Psychologist	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Clinical Pain Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.20	.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)

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 inhibitory period was recorded during tooth pulp stimulation at both non-pain and pain currents; 4) the effect of a narcotic on sensations produced by tooth pulp stimulation and on the masseter inhibitory period was evaluated; and 5) the effects of a conditioning electrical stimulus, applied to the tooth, on sensation and on masseteric inhibitory period were determined.

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 paresthesia and result from near threshold stimulation of an exclusive pain system. If there are two 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 two 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 inhibitory period, inhibition of masseter activity during substained contraction that occurs after the tooth pulp is stimulated. This inhibition may provide physiological correlates for the non-pain and pain sensations produced by tooth pulp stimulation. Inhibitory periods produced by currents that produce definite non-pain sensations may differ from those caused by currents that produce pain sensation. In order to assess the value of these inhibitory periods as physiological correlates of sensation: 1) the reliability and stability of EM 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 inhibitory period is determined; 3) the effects of narcotic (fentanyl) on sensations experienced and on the inhibitory period are evaluated; and 4) the effects of a peripheral electrical conditioning stimulus on sensation and masseteric inhibitory period are determined.

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: The results of this study were reported in detail last year and are summarized below:

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 occurred at higher stimulus currents. A strong 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. There was a multiplicative relationship between stimulus intensity and frequency, so that the strength of sensation increased both as stimulus current increased, and as frequency increased at higher currents.

The threshold for the masseter inhibitory period coincided approximately with an individual's detection threshold for the tooth pulp stimulation. Three configurations of masseter inhibitory periods (Single, Double, and Merged) were produced by different stimulus intensities. However, no particular configuration was associated unequivocally with pain sensation. Increases in stimulus intensity evoked changes both in the configuration of the masseter inhibitory period and in the quality of the sensation produced. Chi square analyses showed significant, but progressively weaker, associations between: (1) masseter inhibitory period configuration and stimulus intensity; (2) quality of sensation and stimulus intensity; and (3) quality of sensation and masseter inhibitory period configuration. The weakness of the association between the quality of sensation and masseter inhibitory period also was demonstrated in a double-blind study of the effects of a narcotic analgesic, fentanyl. Although the strengths of non-pain and pain sensations were reduced significantly after fentanyl, there were no changes in the masseter inhibitory periods.

We conclude that contrary to previous reports, the masseter inhibitory period cannot be considered a reliable correlate of pain. These findings also suggest that the reflex masseter inhibition acts via pathways in

the trigeminal brain stem sensory complex that are independent of opiate suppression of pain.

Significance to Biomedical Research and the 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 nociceptive 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 inhibitory activity (following chin tap) has been used as a diagnostic tool for assessing temporomandibular joint dysfunction (TMJ). This inhibitory period (following tooth pulp stimulation) has been used also as a nociceptive reflex, a physiological correlate for pain. However, the present results show that the inhibitory period often is not correlated with pain sensation. The inhibitory period may be used as an index of the current applied to an unanesthesized tooth, but not as a reliable index of pain.

Proposed Course: This study will be terminated after publication of the findings.

2. Publications:

McGrath, P.A., Sharav, Y., Dubner, R. and Gracely, R.H.: Masseter inhibitory periods and sensations evoked by electrical tooth pulp stimulation. Pain, 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 00246-03 NA

PERIOD COVERED

October 1, 1979 to September 30, 1980

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

Heft, Marc	Postdoctoral Fellow	NIDR NA
Gracely, Richard H.	Research Psychologist	NIDR NA
Dubner, Ronald	Chief, NAB	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH

Neurobiology and Anesthesiology Branch

SECTION

Clinical Pain Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.60	.50	.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 objectives of this project are: to establish sensory and pain thresholds to electrical tooth pulp stimulation in myofascial pain dysfunction (MPD) patients and to relate sub-sensory, sensory and pain producing stimuli of the tooth pulp to the masseteric inhibitory period in MPD patients, by means of electromyographic (EMG) recordings. In both 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 inhibitory period.

1. Project Description:

Objectives: The previous studies comparing the latencies and durations of the masseteric inhibitory period between patients suffering from the Myofascial Pain Dysfunction Syndrome (MPD) and normal subjects have been completed, and the results are being prepared for publication.

The purpose of the present study is to develop psychophysical measurement techniques and other more objective criteria for assessing chronic pain associated with MPD and the effect of various pharmacological and non-pharmacological pain control methods on the pain. Patients will independently assess the sensory and motivational dimensions of their chronic MPD pain using techniques described in NIRP Number Z01 DE 00133-06, "Assessment of Experimental and Clinical Pain".

Methods Employed: Experimental pain is produced by electrical tooth pulp stimuli applied to intact, vital incisor teeth. The pain intensity associated with these stimuli is assessed by verbal descriptor scaling techniques. Cross-modality matching procedures are employed to quantify the MPD pain. MPD pain is further assessed by verbal scaling and questionnaires. The results of previous studies have shown that MPD patients quantified the pain descriptors similarly to normal, healthy subjects, and they scaled their clinical pain as readily and reliably as pain produced by electrical tooth pulp stimulation. Furthermore, subjects reliably matched the pain intensity of the pain associated with their MPD and the electrical tooth pulp stimuli.

Major Findings: The reliable, objective, and valid verbal scales were used to assess MPD pain at two experimental sessions separated by three weeks. Pain was assessed before and after the intravenous administration of either a narcotic, fentanyl, or saline placebo, in a double-blind, cross-over design. Results of this study showed a significant reduction in pain unpleasantness with a non-significant reduction in pain intensity.

Significance to Biomedical Research and the Program of the Institute: The demonstration that fentanyl, a short-acting narcotic analgesic, reduced the pain unpleasantness associated with MPD is in contrast to a previous study which showed that fentanyl reduced the pain intensity but not unpleasantness of electrical tooth pulp stimuli in normal volunteers. This finding is consistent with previous observations that suggest that the amount of pleasantness or unpleasantness associated with narcotic analgesia depends on the situational context in which the drug is administered. For instance, the dysphoric side effects of narcotics in normal, healthy subjects probably offsets the placebo factors that reduce the unpleasantness of pain, and this probably accounts for the non-significant reduction in unpleasantness noted in the experimental pain study. On the other hand, patients suffering from pathological pain often report positive experiences

of warmth and euphoria contributing to a reduction in unpleasantness associated with their pain. Furthermore, in a previous study MPD patients showed a tendency to describe their pain experience more in terms of pain unpleasantness rather than pain intensity, in contrast to the descriptions of experimental pain by the normal, healthy subjects. Therefore, pain unpleasantness scales are probably more sensitive in assessing chronic MPD pain.

Proposed Course: Future studies will assess the relationship between the symptoms, i.e., subjective complaints, and the "objective" signs associated with MPD. Since the incidence of signs of MPD are more prevalent than the incidence of reported MPD, future studies will be concerned with assessing psychological and other factors which are important in the myofascial pain syndrome. These studies will be useful in diagnosis and treatment of patients suffering from MPD.

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 00276-02 NA

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Narcotic and Brain Stimulation Analgesia and Human Chronic and Experimental Pain

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

Gracely, Richard H.	Research Psychologist	NIDR NA
Dubner, Ronald	Chief, NAB	NIDR NA
Dionne, Raymond A.	Staff Fellow	NIDR NA
Hoffert, Marvin J.	Senior Staff Fellow	NIDR NA
Amato, Peggy R.	Clinical Nurse	NIDR NA
Wolskee, Patricia J.	Psychologist	NIDR NA
Lees, David E.	Deputy Chief	CC ANES

COOPERATING UNITS (if any)

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LAB/BRANCH

Neurobiology and Anesthesiology Branch

SECTION

Clinical Pain Section

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.70	.90	1.80

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)

The purposes of the study are (1) Assess the effectiveness of chronic electrical stimulation of midbrain sites for the relief of chronic pain in humans; (2) Evaluate the efficacy and mechanisms of traditional narcotic analgesia and compare these to chronic electrical stimulation of midbrain sites; (3) Validate experimental models of pain and their potential diagnostic use in chronic pain patients; and (4) Determine and compare the impact of both traditional narcotic and chronic electrical stimulation therapies on the functional, intellectual and emotional well being of these patients. Participants in this study will be (1) chronic pain patients receiving surgically implanted stimulating electrodes for pain control; (2) chronic pain patients maintained on traditional narcotic analgesics who will not receive implanted stimulating electrodes; and (3) healthy normal volunteers. The effects of chronic brain stimulation in surgical patients will be compared to the effects of narcotics previously administered to patients and to effects of narcotic regimes in nonsurgical chronic pain patients. In addition, the effects of narcotics on perceptual and neural mechanisms of experimentally induced pain and on normal psychological functioning will be compared between chronic patients and normal volunteers free of chronic pain.

1. Project Description

Objectives: These studies will assess both the mechanisms and relative efficacy of brain stimulation and narcotic analgesics for the control of chronic, intractable human pain. Stimulation of midbrain sites is a recent analgesic technique performed by a small number of neurosurgeons. The present studies address several issues pertinent to the use of this method. How does brain stimulation compare to conventional narcotic administration in terms of: (1) magnitude of analgesia, (2) mechanisms of analgesia, (3) adverse side effects, and (4) tolerance?

Methods Employed: Patients scheduled to receive implants are admitted to the NIH Clinical Center before and after surgery for extensive testing including neurological workups, psychiatric and psychological evaluation, appropriate laboratory tests, clinical pain questionnaires, responses to noxious tooth pulp and heat stimulation, and tests of cognitive and psychomotor functioning. Most patients are assessed before and after administration of analgesics, placebos, narcotic antagonists or brain stimulation. Patients not receiving implanted electrodes and normal volunteers are assessed in a similar manner.

Major Findings: Fifteen patients were assessed during this year. Four of these were evaluated in a two-week preoperative program. Of these, one did not complete the program, one was not a surgical candidate, and two received electrode implants and returned to NIH for postoperative evaluation. Results of radio-immunoassay of CSF removed from the third ventricle during surgery suggest an increase in concentration of endogenous opiate-like substances following surgery and stimulation. The effects of stimulation lasted for more than 24 hours.

One patient received an electrode last year and was reevaluated this year. Stimulation produced a relaxing effect but little relief of pain from cervical astrocytosis. Neither real nor sham stimulation produced significant reductions in clinical or experimental pain. Clinical pain and responses to noxious thermal stimuli were not elevated by administration of 0.4 mg naloxone, a narcotic antagonist.

Both patients (A&B) receiving electrodes this year also reported a relaxing effect during stimulation. However, the pain levels of both were reduced dramatically after surgery and both patients have resumed recreational and work activities that could not be performed before surgery. This analgesia was described as a longlasting effect produced by continued periodic stimulation. Patient "A" reported that several stimulation sessions were necessary to produce the effect and it would last for several days without stimulation. This patient could detect the difference between real and sham stimulation, but both produced no

short-term changes in clinical pain resulting from lumbar stenosis or in the perception of experimentally applied noxious thermal stimuli. A reaction time measure to repeated noxious thermal stimuli suggests that second (C fiber mediated) pain was suppressed after surgery in this patient.

The clinical and experimental pain parameters were not altered by either real or sham stimulation or by the administration of naloxone in patient "B".

The remaining 10 patients were evaluated in a new, 3 day, preliminary screening program. Five were rejected from further study for reasons of pending litigation, heavy drug dependence, poor psychophysical ability, organicity and psychiatric problems. Of the five accepted, one was weaned from narcotic medications and has received an alternative neurosurgical procedure. The remaining four are candidates for continuation in the study following surgical implantation.

Another study assessed the effects of chronic pain on responses made to experimentally applied noxious stimuli. Pricking pain thresholds determined by the method of double staircase were significantly higher (50.4°C) for 8 chronic pain patients than for 8 normal subjects (46.4°C). Five chronic pain patients and five normal subjects also scaled the intensity and unpleasantness of the sensations produced by noxious thermal stimuli ranging between 45-51°C and presented independently to 8 sites on the volar surface of the forearm. Handgrip responses and sensory intensity verbal descriptor responses were similar for the chronic and normal groups. The unpleasantness verbal descriptor responses were significantly lower in the chronic pain patients.

An additional study assessed the effects of morphine on responses to noxious thermal stimuli in chronic pain patients. Chronic pain patients scaled noxious thermal stimuli between 45°-51°C presented to the volar surface of the forearm both before and after the double-blind administration of morphine or a saline placebo. Following morphine administration, verbal descriptor responses of sensory intensity and unpleasantness were reduced in the chronic pain patients in comparison to placebo. In contrast, handgrip measures of sensory intensity and unpleasantness were not significantly reduced by morphine administration in comparison to placebo.

Significance to Biomedical Research and the Program of the Institute: The implant was a clinical success in 2 of 3 patients this year. It failed in a young female patient with cervical astrocytosis, progressive motor weakness, and impaired social and physiological functioning related to her illness. Pain was not the primary medical problem of this patient and evaluation was difficult. The present results stress the importance of patient selection. Conservative inclusion criteria will strengthen the significance of negative results and suggest important potential confounding variables if positive results are found.

The observation that stimulation produces analgesia for more than 24 hours indicates that experimental designs used for the assessment of morphine preoperatively are not appropriate for postoperative assessment of stimulation-produced analgesia.

The study investigating responses to noxious thermal stimuli in chronic patients and normal subjects provides additional evidence that the discrimination between sensory intensity and unpleasantness is facilitated by the use of verbal descriptors specific to different dimensions. The threshold results suggest that, in contrast to normal volunteers, chronic pain patients do not describe thermal stimuli less than 50.5°C as painful. The scaling results support the threshold findings and suggest that chronic pain patients find stimuli between 45-51°C less unpleasant than normals. Thus it is important to establish a range of painful stimuli with chronic pain patients because previously accepted values in normal volunteers are not applicable.

The results with morphine in chronic patients and normals supports the hypothesis that the effect of a drug on perceived unpleasantness is dependent in part of the side effects experienced. After narcotic administration, pain free subjects usually experience dysphoria and nausea, while chronic pain patients, in contrast, experience euphoria, warmth, and relaxation. These results further support the utility of the verbal descriptor method for the assessment of different dimensions of the pain experience. These findings also suggest that the verbal descriptor scaling of contact heat may provide an experimental model for the evaluation of pharmacological and non-pharmacological pain control methods.

Proposed Course: Assessment of patients scheduled to receive electrode implants will continue with an anticipated load of 20 patients yearly. The experimental design has been improved to include an initial 3 day screening admission and then a subsequent 2 week preoperative assessment for selected patients. During surgery, the sequence of CSF collections will be altered to assess separately the influence of surgical stress and electrical brain stimulation on the concentration of ventricular beta-endorphin. A new design for postoperative admissions will use one of three specific paradigms chosen on the basis of the duration of analgesia produced by 1 hour of brain stimulation.

The study comparing responses to noxious thermal stimuli in chronic pain patients and normals will continue with an evaluation of the influence of age, analgesic medication history and duration of pain experience. Results from these studies will be used to develop a method of chronic pain measurement based on responses to noxious thermal stimuli.

Studies on the effect of morphine on chronic pain patients and normal volunteers will continue. Additional subjects will be assessed in the heat scaling paradigm with special attention to confounding variables such as age and drug history. A new study using reaction time

measures to noxious heat pulses will assess the role of A-delta and C fiber primary afferents in pain perception and analgesia in both normal volunteers and patients with chronic pain syndromes.

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 00286-01 NA

PERIOD COVERED
October 1, 1979 to September 30, 1980 CT 0060133

TITLE OF PROJECT (80 characters or less)

Evaluation of Etidocaine and Flurbiprofen for Inhibition of Post-Operative Pain

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

Dionne, Raymond A.	Staff Fellow	NIDR NA
Gracely, Richard H.	Research Psychologist	NIDR NA
Amato, Peggy R.	Clinical Nurse	NIDR NA

COOPERATING UNITS (if any)

LAB/BRANCH
Neurobiology and Anesthesiology Branch

SECTION
Clinical Pain Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.80	.25	.55

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 consists of studies (1) to evaluate the efficacy of two novel therapeutic agents, alone and in combination, (2) to further document the clinical advantage of preoperative administration of non-steroidal anti-inflammatory analgesics for inhibiting postoperative pain and (3) to compare new analgesic assessment methods to existing analytical techniques. The analgesic activity of flurbiprofen given preoperatively and 4 hours later is being compared to the analgesic effect of acetaminophen. Flurbiprofen pre-treatment is also being compared to postoperative administration of a narcotic-mild analgesic combination, oxycodone plus acetaminophen. The anesthetic potency of a long-acting local anesthetic, etidocaine, is being compared to that of lidocaine. Flurbiprofen and etidocaine in combination will be compared to oxycodone plus acetaminophen and lidocaine. These investigations are also evaluating the sensitivity of newly developed scales for measuring analgesic activity in dental outpatients.

1. Project Description:

The project is a four part factorial comparison of two new therapeutic agents, flurbiprofen and etidocaine, alone and in combination, to standard agents. Flurbiprofen is first being compared to acetaminophen to establish analgesic activity in our model and to confirm the sensitivity of the methodology being employed. Flurbiprofen will then be compared to one of the more potent analgesic combinations used in dental outpatients, oxycodone plus acetaminophen. A parallel investigation will compare etidocaine, a new long acting local anesthetic, to lidocaine, the prototype of the amide type local anesthetics. If these investigations are successful in demonstrating a therapeutic advantage, or therapeutic equivalence with reduced toxicity, a fourth investigation will compare flurbiprofen and etidocaine to oxycodone plus acetaminophen and lidocaine.

These investigations will also be extending two previous investigations on the efficacy of preoperative administration of a non-steroidal anti-inflammatory analgesic such as flurbiprofen. These previous investigations have indicated that a significant inhibition of postoperative pain can be achieved by administering such a drug prior to surgery and that less side effects are encountered than if a standard narcotic analgesic combination is administered postoperatively.

Objectives: These investigations will evaluate the analgesic efficacy of flurbiprofen relative to two standard analgesics. A new local anesthetic, etidocaine HCl, will be evaluated for efficacy as a local anesthetic and for the ability to inhibit postoperative pain. These investigations will also evaluate the efficacy of pretreatment with a non-steroidal anti-inflammatory analgesic relative to standard treatment and the efficacy of long-acting local anesthetics for inhibiting postoperative pain. In addition, the sensitivity of novel scales for the measurement of pain, differential descriptor scales and verbal descriptor scales, will be compared to traditional analgesic scales.

Methods Employed: Patients in need of bilateral extraction of impacted third molars serve as subjects. Subjects are selected if the anticipated degree of difficulty of the extractions is approximately equal on both sides. Subjects receive one of the two treatments under study on a random basis at the first appointment and the alternative treatment at a second appointment, two to four weeks later. Neither the patient nor the clinical personnel involved in the study are aware of the agent being used at any time. Subjects complete the pain assessment questionnaires prior to surgery as a baseline observation and to ensure that they understand the scales. Two teeth are extracted at each appointment in the usual clinical fashion. Diazepam is administered intravenously prior to surgery in a variable dose depending on the patients' subjective response. A maximum dose of 20 mg is employed and each patient receives the same amount of diazepam at the second appointment.

In the analgesic studies, patients receive either flurbiprofen 50 mg or acetaminophen 1000 mg 30 minutes prior to surgery and 4 hours after the initial dose. Lidocaine 2% is used as a local anesthetic for both sets of extractions. For the comparison of local anesthetics, either 1.5% etidocaine with 1:200,000 epinephrine or 2% lidocaine with 1:100,000 epinephrine is administered in a standard fashion and the adequacy of the anesthetic block confirmed prior to initiating surgery. For the evaluation of the combination of preoperative analgesic plus long-acting local anesthetic, flurbiprofen or placebo will be administered 30 minutes prior to surgery. The flurbiprofen will then be followed by etidocaine as the local anesthetic while the standard treatment will consist of 2% lidocaine. Postoperatively, the experimental treatment will be a second dose of flurbiprofen and the standard treatment will be 10 mg of oxycodone plus 650 mg of acetaminophen.

Patients rate their pain intensity hourly starting two hours after the initial pretreatment dose and continuing to eight hours after the initial dose. Traditional measures of analgesic activity include ordinal ranking scales (none, slight, moderate or severe pain), a global evaluation scale, and a visual analog scale. Newer measures of analgesia being employed are the verbal descriptor scales and differential descriptor scales previously developed and validated at NIDR. Side effects are also recorded and patients indicate which of the two treatments they preferred at the end of the second appointment.

Major Findings: Preliminary data suggest that flurbiprofen is resulting in a greater inhibition of postoperative pain then is acetaminophen. Patients prefer the flurbiprofen over acetaminophen in most cases. Few side effects have been reported following either treatment. These preliminary data also suggest that the newer scales being employed are more sensitive measures of analgesic activity than the traditional ordinal ranking scales.

Significance to Biomedical Research and the Program of the Institute: The NIDR has played a prominent role in the development of pain control modalities for use in dental outpatients. General anesthesia and related sedative techniques have received most attention in past research. These techniques require extensive training to be used clinically and studies conducted at NIDR indicate that they can result in cardiovascular and respiratory depression. The studies being conducted in this project are aimed at assessing and developing novel methods of controlling the pain associated with outpatient dental therapy that do not require extensive training or cause an inordinate risk for the patients. Successful completion of these studies may result in novel methods for controling pain in dental outpatients. Utilization of such pain control modalities will facilitate the delivery of dental care and, hence, ultimately improve oral health.

Proposed Course: The results of each of the four studies outlined will be analyzed, evaluated and the methodology critiqued prior to initiating the next study in the sequence. Upon completion of these four studies, investigations involving other promising analgesics and local anesthetics will be proposed.

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 00020-15 NA

PERIOD COVERED
October 1, 1979 to September 30, 1980

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

Gobel, Stephen	Chief, NEA Section	NIDR NA
Arvidsson, Jan	Visiting Fellow	NIDR NA
Brown, Emma	Bio. Lab. Tech. (Ele. Mic)	NIDR NA
Allen, Barbara M.	Biologist	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 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.80	1.50	1.30

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, the Golgi method and the use of intraneuronal markers such as horseradish peroxidase. The objects of these studies are to delineate trigeminal pain-temperature pathways and to broaden our understanding of oral-facial sensation.

1. Project Description:

Objectives: Our anatomical studies during the past year had three major objectives. First, to draw together observations from several lines of experimentation which illustrate basic structural and functional similarities between the lower end of the spinal V nucleus, i.e., the medullary dorsal horn (MDH) and the dorsal horn of the spinal cord (SDH). Second, to determine the central termination sites of primary trigeminal neurons which innervate the pulp chambers of teeth. Third, to continue our analysis of the synaptic connections of the major neuronal cell types which comprise the neuropil of the substantia gelatinosa of Rolando (layers I and II).

Methods Employed: The protein, horseradish peroxidase (HRP) was used as an intracellular neuronal marker which could be visualized with both light and electron microscopes in three different ways. First, it was injected into the thalamus where it was picked up by axons in the thalamus and transported retrogradely back to their cell bodies. This approach provides an opportunity to compare the laminar locations of second order trigeminothalamic and spinothalamic projection neurons in the MDH and SDH. It was also injected into the MDH in order to compare descending brain stem projections and intrasegmental projections with those of the SDH. Second, it was applied to the cut peripheral ends of primary trigeminal neurons inside pulp chambers. In these experiments the HRP was taken up by primary trigeminal neurons, transported intracellularly to their cell bodies in the trigeminal ganglion and into their terminal axonal arbors in the central nervous system. Having these primary tooth pulp neurons filled with HRP provided an opportunity to study their central termination sites with far greater accuracy than ever before possible. Third, HRP was placed within single dorsal horn neurons using micropipettes whence it spreads into the axonal and dendritic arbors of the injected neurons. Having individual neurons filled with HRP allowed for detailed EM analyses of the synaptic inputs that these neurons receive from primary and descending aminergic axonal inputs.

Major Findings:

1. As a result of light microscopical studies conducted in the early 1950's, the lower end of the spinal V nucleus, i.e., the MDH, which is directly continuous with the SDH, was designated a separate and distinct nuclear entity and called subnucleus caudalis. Several different experimental approaches have been used to demonstrate similar structural patterns of organization in the MDH and SDH. These include similar patterns of lamination in which the head and neck of the MDH and SDH can be divided into six layers. Each of these layers in the two locations share anatomical similarities such as the texture of the neuropil, the density and size distribution of neuronal cell bodies and the distribution of small myelinated axons and small axon bundles. They also share

similar neuronal cell types and similar kinds of synaptic connections between primary axons and descending serotonergic inputs on one hand and neurons of layers I and II on the other hand.

Additional important similarities between the MDH and SDH are illustrated by results from experiments in which HRP was injected into the thalamus and the MDH. Long distance second order trigeminothalamic and spinothalamic projection neurons are found in the same layers, i.e., in layers I and V, in the MDH and SDH respectively. Shorter distance propriotrigeminal and propriospinal projection neurons are also found in the same layers in the MDH and SDH, i.e., in layers I, III, IV, V and VI.

2. In experiments in which HRP was placed inside of tooth pulps, two central termination sites of primary trigeminal tooth pulp neurons were identified. One consists of a long continuous column which extends from the main sensory nucleus at its rostral limit through subnuclei oralis and interpolaris into layer V in the MDH at its caudal limit. This column is situated dorsomedially within each of these structures. The second termination site is found in the dorsomedial parts of layers I and IIa in the MDH. Labeled axons were located ipsilaterally in the brain stem and did not cross the midline. Since labeled primary cell bodies in the Gasserian ganglion also were found only on the operated side, it appears that the tooth pulp projection is purely ipsilateral.

The central termination sites of the inferior alveolar nerve were more extensive than those of tooth pulp neurons and covered the dorsolateral and dorsomedial portions of the main sensory nucleus, subnuclei oralis and interpolaris and extended across layers I-V of the MDH. These studies indicate that layers I, IIa and V in the MDH and the dorsomedial portions of the main sensory nucleus and subnuclei oralis and interpolaris are termination sites of primary afferent tooth pulp neurons that most likely respond to painful inputs.

These experiments also reveal that a much higher percentage of small primary cell bodies (< 37 μm) were labeled in the inferior alveolar nerve experiments than in the tooth pulp experiments, i.e., 58.2% versus 20.8%. This suggests that nerve branches innervating skin and mucous membranes have a higher percentage of unmyelinated axons than nerve branches innervating the teeth.

3. In our laboratory, techniques have been developed for impaling neurons in layers I and II (which include some of the smallest neurons in the central nervous system) with microelectrodes and filling them with HRP (see NIRP Number DE 00247-03, "Cytomorphology of Functionally Characterized Spinal Cord Dorsal Horn Interneurons"). This important technical advance has provided an opportunity to examine the synaptic connections of the major neuronal cell types in the dorsal horn with the electron microscope and gain new insights into their functional roles. The first two cells examined were the stalked cell and the layer IIa

islet cell. We were able to show that the IIa islet cell is a major source of synaptic vesicle-containing dendrites in the layer IIa glomeruli. In contrast, the stalked cell does not contain synaptic vesicles in its dendrites and is a major source of dendrites without synaptic vesicles in the layer IIa and IIb glomeruli. These analyses indicate that the stalked cell receives primary afferent input in layer II and conveys it via its axon to the projection neurons in layer I. The IIa islet cells can modify primary afferent inputs before they reach the layer I projection neurons through their synaptic vesicle-containing dendrites at their dendrodendritic and dendroaxonic synapses as well as through their axons which arborize in layer IIa. The layer IIa islet cell is considered to function as an inhibitory interneuron which diminishes or inhibits the flow of primary inputs from layer IIa via the stalked cells to the layer I projection neurons.

Most recently, we have extended our EM analyses to several layer IIb islet cells successfully filled with HRP. The dendrites of IIb islet cells enter numerous glomeruli in layer IIb where they receive primary inputs. They contain synaptic vesicles in their dendrites and form dendrodendritic synapses inside and outside of the layer IIb glomeruli. The IIb islet cells are considered to function as inhibitory interneurons much like their counterparts in layer IIa.

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?

Recent technical advances have made it possible to selectively study individual neurons in trigeminal pain pathways at the light and electron microscopical levels. Data from such studies will provide more detailed information about the neural circuitry of trigeminal pain pathways and will permit us to design more critical experiments to approach the above as well as other questions concerning chronic pain states in the orofacial region.

Proposed Course: During the coming year, four lines of experimentation are planned. First, we will continue our analyses of how primary inputs are processed in the dorsal horn. This question will be pursued by filling the terminal axonal arbors with HRP by applying it to severed sensory roots. The laminar distribution and synaptic connections of different kinds of primary axons using light and electron microscopical techniques will be explored. Immunocytochemical techniques will be applied in an attempt to discover which neurotransmitters are present in primary neurons which respond to painful stimuli. Second, we will continue our electron microscopical analyses of the synaptic connections of the major cell types of the dorsal horn which have been intracellularly filled with HRP. We will attempt to introduce a second intracellular marker into these preparations which will mark either the primary axons or some of the descending aminergic inputs. Third, we plan to resume developmental studies which seek to define which components of the pain neural circuitry are already in place at birth and which components develop postnatally. Fourth, we will resume studies of the effects of peripheral nerve injury and the resultant primary axonal deafferentation on the neuronal cell types in layers I and II.

2. Publications:

Gobel, S.: Neural circuitry in the substantia gelatinosa of Rolando: Anatomical Insights. In: Advances in Pain Research and Therapy, Vol. 3, edited by J.J. Bonica, New York, Raven Press, 1979, pp. 175-195.

Gobel, S. and Falls, W.M.: Anatomical observations of horseradish peroxidase filled terminal primary axonal arborizations in layer II of the substantia gelatinosa of Rolando. Brain Res., 175:335-340, 1979.

Gobel, S., Falls, W.M., Bennett, G.J., Abdelmoumene, M., Hayashi, H. and Humphrey, E.: An EM analysis of the synaptic connections of horseradish peroxidase-filled stalked cells and islet cells in the substantia gelatinosa of adult cat spinal cord. J. Comp. Neurol., In press.

Gobel, S., Hockfield, S. and Ruda, M.A.: An anatomical analysis of the of the similarities between medullary and spinal dorsal horns. In: Oral-Facial Sensory and Motor Functions, edited by Y. Kawamura and R. Dubner, Quintessence, Tokyo, In Press.

Arvidsson, J. and Gobel, S.: An HRP study of the central projections of primary trigeminal neurons which innervate tooth pulps in the cat. Brain 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 00288-01 NA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Neuropharmacological Characterization of Synaptic Circuitry in the Dorsal Horn

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

Ruda, Maryann	Staff Fellow	NIDR NA
Gobel, Stephen	Chief, NEA Section	NIDR NA
Allen, Barbara M.	Biologist	NIDR NA
Blaes, Peter V.	Bio Lab Techn. (Biochem)	NIDR NA
Bennett, Gary J.	Staff Fellow	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 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.35	1.45	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)

Neurotransmitters were localized to specific populations of axonal endings and neuronal perikarya in layer I and II of the medullary dorsal horn. Monoaminergic endings were characterized using light and electron microscopic autoradiography following uptake of [^{3}H]serotonin or [^{3}H]norepinephrine. The aminergic endings synapsed primarily on dendrites and occasionally on neuronal perikarya. These observations suggest that monoamines modulate the response of second order projection neurons to nociceptive primary inputs either directly through synapses on layer I projection neurons or indirectly through synapses on the interneurons in layer II.

Experiments using anterograde transport of [^{3}H]amino acids demonstrated that three types of endings originate from the medial brainstem. Each of these resembled endings labeled in the [^{3}H]5HT uptake experiments.

Enkephalin and substance P were localized to axonal endings and neuronal perikarya in the dorsal horn using immunocytochemistry. Enkephalin neurons were found in layers I and II while substance P neurons were in layer II. A differential laminar distribution of endings was also observed.

1. Project Description:

Objectives: Since the localization and characterization of neurotransmitters in the dorsal horn forms the basis for understanding the modulation of nociceptive input in pain pathways, the objective of our experiments is twofold: (1) to describe the location and ultrastructural morphology of pharmacologically identified axonal endings and neurons in layers I and II of the medullary (MDH) and spinal (SDH) dorsal horn; (2) to develop a pharmacologically characterized circuit diagram of potential synaptic interactions in layers I and II of the dorsal horn. This project is a continuation of project number Z01 DE 00207-03.

Our initial studies focused on the monoamines serotonin (5HT) and norepinephrine (NE). Having ultrastructurally identified the 5HT and NE axonal endings in layers I and II, studies of dorsal horn afferents originating from brainstem nuclei rich in serotonin containing cell bodies were begun. These studies allowed us to correlate the morphology and synaptic interactions of the serotonergic endings identified in the [^{3}H]5HT uptake experiments with their nuclear origin.

During the past year, we developed the methodology for light (LM). and electron microscopic (EM) immunocytochemical analysis of neuotransmitters. Experiments now in progress will extend our analysis of the synaptic circuitry in the dorsal horn to an examination of the role of peptides. The development of this marker for neurotransmitters will allow us to label more than one system in the neuropil in the same experiment so that potential interactions can be determined.

Methods Employed: The pharmacological characterization of axonal endings and neuronal perikarya utilized four different approaches. The first experimental paradigm was designed to identify monoamine containing axonal endings by taking advantage of the active uptake process for monoamines at axonal endings which utilize monoamines as their neurotransmitter. In these experiments, radiolabeled monoamine, either [^{3}H] 5HT or [^{3}H]NE was topically applied to the surface of the MDH or injected into the cervical SDH of cats pretreated with a monoamine oxidase inhibitor. Following a survival time of 1-2 hrs, the animals were perfused with a mixture of aldehydes to bind the monoamine and the tissue was processed for LM and EM autoradiography according to the method of Kopriwa. This process results in the deposition of silver grains which can be identified at both the LM and EM level over processes containing the [^{3}H]neurotransmitter.

In a second series of experiments a serotonin neurotoxin, 5,6 dihydroxytryptamine (5,6DHT), was used to identify serotonergic axonal endings. The neurotoxin produced ultrastructural morphological changes in 5HT endings which were indicative of degeneration. The purpose of these experiments was: (1) to substantiate the localization and differential morphology of the serotonergic endings identified in the [^{3}H]5HT uptake

experiments; and (2) to identify potential interactions between 5HT and NE using degenerating 5HT endings and [^{3}H]NE labeled endings as two distinguisable EM markers in the same experiment.

The third experimental paradigm was designed to determine the morphology and distribution of afferent axonal endings to the MDH originating from medial brainstem nuclei rich in serotonin containing neurons. A comparison between these endings and those characterized in the [^{3}H]5HT uptake experiments will identify serotonergic medial brainstem afferents to layers I and II of the dorsal horn. In these experiments, a combination of [^{3}H]amino acids injected into the brainstem, was taken up by cell bodies in the area of the injection, transported in an anterograde direction to their axonal endings, and subsequently localized using EM autoradiography.

Our current approach to analysis of neurotransmitters involves immunocytochemical techniques developed in our laboratory over the past year. We employed antibodies made to a specific neurotransmitter to localize that neurotransmitter in tissue sections. Visualization of the neurotransmitter was carried out using the unlabeled antibody peroxidase-antiperoxidase immunocytochemical staining method of Sternberger. This technique resulted in the deposition of peroxidase in neuronal profiles which could be identified at both the LM and EM level. Normal cats were used to demonstrate immunocytochemically labeled axonal endings. To visualize neuronal perikarya, the animals are pretreated with colchicine to block axoplasmic transport and thus concentrate the amount of neurotransmitter present in the cell body. Initial experiments were aimed at identifying the location and morphology of enkephalinergic and substance P containing neuronal perikarya and axonal endings in the dorsal horn and the brainstem.

Major Findings:

Serotonergic Axonal Endings

LM analysis of the distribution of silver grains in the MDH and SDH demonstrated the presence of [^{3}H]5HT labeled endings in layers I and II with the greatest density in layer I. In EM autoradiographs, two categories of axonal endings were labeled: dome-shaped endings which form a single synapse and scalloped endings which form multiple synapses. Based on morphological criteria, the dome-shaped endings were divided into two different types while the scalloped endings were divided into four types. The dome-shaped endings were located throughout layers I and II and had either oval or flattened agranular vesicles. Each of the scalloped endings was confined to a single layer. The [^{3}H]5HT labeled endings synapsed primarily on small caliber dendritic shafts and spines and occasionally on a cell soma. The presence of 5HT axonal endings in layers I and II suggests that 5HT afferents have access to both the projection neurons in layer I and the interneurons in layer II.

Noradrenergic Axonal Endings

[^{3}H]NE labeled endings occurred with equal density throughout layers I and II of the MDH. Electron microscopic analysis of layers I and II yielded two categories of morphologically distinguishable axonal endings which took up [^{3}H]NE: dome-shaped endings and scalloped endings. The dome-shaped endings in layers I and II were of two types which could be distinguished by the presence of either flattened or oval agranular vesicles. Two types of [^{3}H]NE labeled endings were scalloped endings. One was found in layers I and II while the second was found only in layer IIb. [^{3}H]NE labeled endings synapsed primarily on dendritic shafts and spines and occassionally on a cell soma. The labeled endings were occasionally postsynaptic in axoaxonic synapses, suggesting that descending aminergic input may be presynaptically modulated.

In addition to the experiment in which [^{3}H]NE was used alone, in one experiment the animal was pretreated with the serotonin neurotoxin 5,6 DHT. This combination experiment offered the unique advantage of visualizing both autoradiographically labeled NE endings and blackened degenerating 5HT endings in the same preparation. Several examples of the same dendrite receiving synapses from both a labeled NE and 5HT ending were observed. The observation of both NE and 5HT axonal endings synapsing on the same dendrite indicates that both descending monoaminergic systems act, in part, on the same neuron.

Medial Brainstem Afferents

Axonal projections of medial brainstem nuclei to layers I and II of the MDH were identified. Following [^{3}H]amino acid injections into the brainstem, labeled myelinated and possibly unmyelinated axons were observed descending to the dorsal horn via the dorso-lateral funiculus. Labeled axonal endings were found throughout layers I and II but were most numerous in layer I. Three different morphological types of labeled endings could be distinguished: two dome-shaped endings in layers I and II which contained either flattened or oval agranular vesicles, and one scalloped ending in layer IIa. Each type resembled serotonergic axonal endings identified in previous experiments suggesting that these three endings originate only from 5HT neurons. The labeled descending afferents synapsed on dendritic shafts and spines and occasionally on a neuronal soma suggesting that the major site of action of descending medial brainstem afferents is the neurons in layers I and II.

Peptides in the Dorsal Horn

We have begun experiments aimed at extending our analysis of the pharmacological circuitry of the MDH and SDH by examining the distribution of peptides. The two peptides being investigated are enkephalin and substance P. In the dorsal horn, these peptides arise from intrinsic neurons in addition to descending brainstem afferents. Substance P also originates as a neurotransmitter in primary afferent axons. Preliminary

LM observations indicate that there is a differential laminar distribution of enkephalinergic and substance P containing axonal endings in layers I and II. Although both peptides are found throughout layers I and II, the density of substance P containing axonal endings is greatest in layer I while enkephalinergic endings are most dense in layer II. In animals pretreated with colchicine the intrinsic peptidergic neurons within the dorsal horn can be visualized. Substance P containing neuronal perikarya are found in layer II while enkephalinergic neurons are found in layers I and II in addition to deeper layers.

At the EM level, enkephalin immunoreactivity can be localized to dendrites and both unmyelinated and myelinated axons in layers I and II. Enkephalinergic axonal endings contain small round agranular synaptic vesicles and frequently form asymmetrical synapses on dendritic shafts. Substance P can be ultrastructurally localized to axonal endings which contain numerous dense core vesicles in addition to agranular vesicles.

Peptides in the Brainstem

In colchicine pretreated animals, we have demonstrated the presence of both enkephalinergic and substance P containing neuronal perikarya in n. raphe magus, n. raphe pallidus, n. raphe obscurus and adjacent reticular formation. The peptide containing neurons range in size from some of the smallest to some of the largest neurons in the area. Their morphology includes both fusiform and multipolar neurons. Enkephalinergic neurons occur with equal density throughout the raphe nuclei, while substance P containing neurons are most numerous in the caudal raphe nuclei.

Significance to Biomedical Research and the Program of the Institute: Several disease states such as cancer and stroke lead to intractable pain for which adequate therapy is unavailable. In other chronic pain states such as trigeminal neuralga, the ideology is unknown. Knowledge of the synaptic circuitry is of critical importance in understanding chronic pain states. Our recent technical advances developed to identify the mechanism of action of these pain states have allowed visualization of pharmacological characterized neurons and axonal endings. We are attempting to identify and characterize the pharmacology of the neural circuitry which subserves nociception. Our experiments have provided a circuit diagram of the monoaminergic and peptidergic inputs to layers I and II of the MDH and SDH. This analysis of 5HT and NE axonal endings is of particular significance since the activation of descending monoaminergic pathways are implicated in the production of analgesia. A more thorough knowledge of the synaptic circuitry involved in pain pathways will ultimately help answer questions concerning chronic pain states.

Proposed Course: The proposed course of experiments within the next year will have two major directions. The first will be to continue experiments on the characterization of peptides in the dorsal horn using immunocytochemistry. In addition to enkephalin and substance P, other

peptides such as vasoactive intestinal peptide, cholecystokinin and somatostatin which may be neurotransmitters in different classes of primary afferent axons will be examined. The analysis will continue at both the light and electron microscopic level.

The second avenue of investigation will focus on identifying interactions between different pathways in the dorsal horn. In these experiments, the intrinsic neurons of the dorsal horn will be labeled either by intracellular filling with horseradish peroxidase (HRP) or retrograde transport of HRP from the thalamus and brainstem. The tissue containing these characterized neurons will subsequently be processed for immunocytochemistry to determine the neurotransmitters of the afferent axonal endings which synapse upon these neurons. In addition to 5HT and NE, enkephalin and substance P will be investigated at the EM level. These experiments will provide the first unequivocal demonstration of the pharmacology of inputs onto characterized neurons in the dorsal horn.

2. Publications:

Ruda, M.A. and Gobel, S.: Ultrastructural characterization of axonal endings in the substantia gelatinosa which take up [^{3}H] Serotonin. Brain Res. 184:57-84, 1980.

Ruda, M.A., Allen, B. and Gobel, S.: Ultrastructure of Descending Serotonergic Axonal Endings in Layers I and II of the Dorsal Horn. J. Physiol. (Fr), in press.

Ruda, M.A., Allen, B. and Gobel, S.: Ultrastructural analysis of medial brainstem afferents to the superficial dorsal horn. Brain Res., in press.

Report of the Clinical Investigations Branch
National Institute of Dental Research
Summary Statement FY 1980

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 L. 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.

OPD continues its composite of studies of sensory, salivary and motor functions with recent emphasis on the methodology of taste testing. These latter studies demonstrate some of the elements of taste-testing procedures which have led to differences in results between other investigators in the field.

Clinical applications of taste testing have been extended also to the study of children and to comparison of subjects with and without caries experience. Another study, collaborative with the NIA, is concerned with changes in taste competence of aging persons, and those maintained on total parenteral nutrition.

Studies of salivary gland mechanisms continue in their dual pattern; those concerned with saliva and its influence upon mucosa and those concerned with internal secretions. Parotid salivary lysozyme and amylases are under study in normal adults, aging persons (in a project collaborative with NIA) and in persons who have oral mucosal disease.

Salivary gland secretions are being compared electrophoretically with those of pancreatic origin in continuing studies of salivary and/or pancreatic abnormalities, such as fibrocystic disease.

Recent studies of oral and pharyngeal disabilities in neurologically impaired persons have been concerned particularly with mechanisms of adaptation and compensation.

Two new anatomical reference books have been completed substantially and arrangements are being made to have them published with support from the National Library of Medicine. They are: Anatomy of the Infant Head by J.F. Bosma and Postnatal Development of the Rat Skull by M.J. Baer, J.M Ackerman and J.F. Bosma.

DMS activities are balanced between the investigation of new bases for quantitatively classifying information of diagnostic interest and the study of alternative technology via computer simulation and development of prototype systems.

The application of symmetric-axis geometry to the description of mandibular contours has been extended to a variety of animals in an effort to determine the extent to which the angular stability reported last year in humans is manifest in other species. To this end, a collaborative liason has been established between the Laboratory of Statistical and Mathematical Methodology, Division of Computer Research and Technology; and the Veterinary Resources Branch, Division of Research Sciences.

Work continues on problems associated with the demonstration of diagnostic utility obtainable from various x-ray systems including a second-generation CAT machine and various prototype systems of dental interest. Much of this work is being performed by visiting scientists in collaboration with permanent staff and clinicians in the Diagnostic Radiology Department, Clinical Center; and the Clinical Dental Services Section, NIDR.

Hardware development is likewise performed largely in collaboration with other groups both within NIH and outside. The application of rod-anode sources as a basis for dental fluoroscopic systems continues to be an area of research interest with emphasis being directed toward the establishment of clinically meaningful design specifications through the use of existing technology whenever possible. Efforts to facilitate quantitative analysis of sequentially obtained radiographs also is being pursued in association with the X-ray Physics Group, NBS. This effort involves the development of hardware designed to assure accurate reproduction of exposure geometry from one examination to the next and will make use of ideas recently patented by DMS investigators.

Considerable effort was expended this year by DMS programmers in association with personnel from the Scientific Systems Section and an outside contractor to bring about a significant up-dating of our primary computing facility. This involved a complete change of central processor and operating system including the rewriting of nearly all programs created within the Section since its inception.

Webber, R.L., and Blum, H.: Angular Invariants in Developing Human Mandibles, Science 206: 689 - 691, 1979.

Webber, R.L., and Nagel, R.N.: Proceedings from Workshop on Feedback Control and Exposure Geometry in Dental Radiology. U.S. Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, Education and Welfare, Public Health Service, National Institutes of Health Publication No. 80 - 1954, Washington, D.C., U.S. Government Printing Office, 1979.

Webber, R.L.: On Modeling Dental Radiographic Systems. Proceedings of the Bureau of Radiological Health Symposium on Biological Effects and Dosimetry of Ionizing Radiation (In press)

Yin, L.L., Tromka, J, Seltzer, S., Webber, R.L., Farr, M., and Renny, J.: Proceedings of the Lixiscope Conference, July 1978.

Webber, R.L.: Simulating the Effects of System Components on the Appearance of Dental X-Ray Images. Oral Surg., Oral Med., Oral Path. (In Press)

Webber, R.L., and Nagel, R.N.: Image preprocessing as an aid to visual interpretation. IADMFR Proceedings (1978) In press)

Webber, R.L., and Nagel, R.N.: Three-Dimensional Enhancement of Two-Dimensional Images. Journal of Clinical Engineering. 5: No. 1, 41 - 50, 1980.

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 00065-09 CI

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Development 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

Webber, Richard L.	Dental Director	NIDR	CI
Grondahl, Hans-Goran	Visiting Scientist	NIDR	CI
Grondahl, Kerstin	Visiting Fellow	NIDR	CI
Okano, Tomohiro	Visiting Fellow	NIDR	CI
Wiebe, John D.	Clinical Dent Assoc	NIDR	IR
de Rijk, Waldemar G.	Sr Staff Fellow	NIDR	CI

COOPERATING UNITS (if any)
Division of Electronic Products; Bureau of Radiological Health; X-ray Physics Group, National Bureau of Standards

LAB/BRANCH
Clinical Investigations Branch

SECTION
Diagnostic Methodology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.05	2.8	1.25

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) Factors influencing the clinical performance of x-ray systems both dental and medical are being modeled and evaluated in vitro using computer simulations and quantitative measurements derived from radiographic phantoms.

Specifically included in this effort are measures of the variance associated with the radiographic assessment of file position in endodontically instrumented root canals using a variety of calibrated film-screen systems.

The significance of the spectral composition of the x-ray beam, exposure geometry, lesion size, and detector linearity also are being explored through measurement of associated image modulation, signal-to-noise ratio, transinformation and perceived lesion detectability.

Preliminary findings suggest that endodontic determinations can be made reliably with significantly fewer noise equivalent quanta than conventionally used. Simulations also indicate that control of exposure geometry and detector sensitivity permit improved detection of small radiographic changes in dental lesions at greatly reduced x-ray doses (100x).

Project Description

I. Objectives

The purpose of this project is to study existing and new radiographic systems through in vitro modelling, and the development of prototypes suitable for clinical evaluation. Much of the effort is concerned with determining the limits imposed by ideal systems in order to assess rationally relative efficiency measured in terms of dose, turn-around time, and diagnostic accuracy.

II. Methods and Major Findings

Promising x-ray sources and image-detector configurations are being studied in collaboration with other government agencies and private interests. Much of the work involves computer simulation coupled with clinically meaningful in vitro measurements in order to rationally relate system elements to the diagnostic task to be accomplished.

Previous results suggest that isotopic x-ray sources generally are not well suited to fluoroscopic applications so recent efforts have concentrated on the potential of miniaturized rod-anode devices and scanning sources as promising alternatives.

One such effort involves collaboration with the X-ray Physics Group at the National Bureau of Standards through a pending interagency agreement sponsored by the extramural Restorative Materials Program, NIDR. This work involves the development of a state of the art, source-detector scanning system designed to improve detector efficiency and to permit diagnostically meaningful subtraction of serially obtained dental radiographic images via computer control of exposure geometry.

Modelling investigations have been concerned with the effects of detector nonlinearity on radiographic image quality produced from a variety of x-ray spectra. The output is in the form of clinically meaningful images suitable for controlled study of lesion detectability. Another model simulates the effects of quantum noise on radiographic images through the computation of Poisson random deviates from mean photon intensities derived from a linear attenuation model. The detectability of small carious and periodontal lesions induced in a realistic radiographic phantom has been determined psychophysically as a function of mean photon fluences using a display system having a noise-equivalent band pass comparable to that of available film-screen systems.

Results from these simultions and confirmatory psychophysical experiments indicate that factors other than information capacity of the system limit detectability of radiologic changes of dental interest. This appears to be true for diagnosis of active caries and periodontal disease in spite of the fact that horizontal changes in angulation of the primary beam of up to ten degrees do not appear to influence significantly the amount of observed overlap in images of adjacent crowns in dental radiographs.

Exact registration of a simulated, quantum-limited system permits lesion changes to be isolated by simple subtraction. For changes larger than the resolution limit, ideal performance is limited only by the signal-

to-noise ratio of the primary beam reaching the detector. Imperfect registration significantly reduces performance but not so much as to preclude substantial improvement relative to the status quo.

Other work considers measures of statistical variance associated with the radiographic assessment of file position in endodontically instrumented root canals. A variety of film-screen systems which are calibrated in terms of noise equivalent quanta are used to provide an indication of the minimum photon fluence required to reliably perform a radiographic evaluation of file position. Preliminary results suggest that the variance in the assessment of endodontic file position is not determined generally by image quality measured in this way. Most of the observed variance is associated with context-dependent interpretative differences. Nonlinear effects of exposure variation also were shown to influence the associated variance of these measures but here significance was limited to clinically unrealistic extremes so that this effect can be controlled easily. Noisy film-screen systems increased the observed variance somewhat but the amount appears to be small relative to that produced from factors unrelated to the detector.

Finally, a computer program has been written which calculates transinformation as a function of lesion size (expressed as a difference in attenuation) and average photon fluence reaching an ideal detector in order to determine absolute limits of diagnostic performance in quantum limited systems. The influence of substantial changes in a priori probability of lesion occurence is small compared to changes in photon fluence and/or lesion size in the effect on the amount of information passed by a quantum-limited radiographic system. This amount is nearly proportional to the mean number of photons reaching the detector, the function being asymptotic for small values.

III. Significance

These studies show that factors other than information capacity limit diagnostic performance obtainable from both ideal and existing system components for a variety of specific diagnostic tasks. Of particular importance are factors such as exposure geometry and mode of display which determine how diagnostic information is sampled and interpreted respectively. Unlike signal-to-noise ratio and noise-equivalent band pass these factors are not limited by photon fluence so that there is room for substantial improvement in diagnostic performance without a concomitant increase in exposure to ionizing radiation.

Methods for measuring the degree of spatial registration possible between successive radiographic images appear to be particularly important for early diagnosis of largely irreversible, degenerative processes such as caries and periodontal diaseses.

Publications:

Webber, R.L., and Nagel, R.N.: Proceedings from Workshop on Feedback Control of Exposure Geometry in Dental Radiology. U.S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health Publication No.80 - 1954, Washington, D.C., U.S. Government Printing Office, 1979, 33 pp.

Webber, R.L.: On Modeling Dental Radiographic Systems. Proceedings of the Bureau of Radiological Health Symposium on Biological Effects and Dosimetry of Ionizing Radiation (In press)

Yin, L.L., Trombka, J., Seltzer, S., Webber, R.L., Farr, M., and Renny, J.: Proceedings of the Lixiscope Conference, July 1978.

Webber, R.L.: Simulating the Effects of System Components on the Appearance of Dental X-Ray Images. Oral Surg., Oral Med., Oral Path. (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 00158-06 CI

PERIOD COVERED
October 1, 1979 to September 30, 1980

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

Webber, Richard L.	Dental Director	NIDR	CI
Blum, Harry	Gen Physical Scientist	LSM	CR
Grondahl, Kerstin	Visiting Fellow	NIDR	CI
Goldberg, Susan	Bio Lab Aid	NIDR	CI

COOPERATING UNITS (if any)
DCRT, NIH

LAB/BRANCH
Clinical Investigations Branch

SECTION
Diagnostic Methodology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.57	.8	.77

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 geometric method for isomorphically transforming the shape of biological structures expressed as two dimensional projections is being applied to cephalograms and anatomical specimens to determine constraints underlying growth and development. The rationale underlying this effort is based on the assumption that the relatively small amount of systematic progress in quantitative morphology may be attributed to a lack of coordinate independent bases for mathematical description.

Symmetric-axis geometry permits angular relationships between symmetrically determined branches to be quantitatively assessed by superposition of homologous elements. When this method is applied to lateral projections of the human mandible, branch angles determined by the condyle, coronoid, and upper ramus have been shown to be relatively independent of age within and between individuals. Recent measurements on certain primates and various strains of mice confirm that this homologous angular invariance is not limited to humans, but that the angles can vary with species.

I. Objectives

Characterization of the shape of biological structures which grow in predictable ways is difficult using conventional techniques since most common mathematical tools are based on Euclidian geometry which does not take into account developmental redundancy. Attempts at systematic description are often complicated by coordinate-dependent transformations which yield different results depending on the arbitrary selection of fiducial references. A coordinate-independent transformation originally described by Blum permits the shape of closed forms which grow incrementally in time to be efficiently described by means of a central, as opposed to peripheral pattern called the symmetric axis. The result is a new geometry which capitalizes on the redundancy of linear growth processes and therefore provides an efficient way to describe shapes which are of interest in studies of biological growth and development. The purpose of this investigation is to continue to explore the utility of this new geometry in the description of biological shape, and to use it as a tool to study factors constraining growth and development in controlled biological experiments.

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 to 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 two-dimensional projections of biological structures recorded at various stages of development, in man and homologous species it is possible to identify and analyze processes underlying biological growth and development.

Recent work involves high-contrast photography of reproducibly positioned, surgically extracted, mouse mandibuli. The resulting pictures are digitized directly using a calibrated video system coupled to a digital frame buffer. They are then preprocessed with automatic edge-detecting algorithms to preclude any human error in the process of data transfer.

III. Major Findings

An analysis of human mandibuli from lateral cephalometric data serially obtained from a variety of individuals confirm the observations made last year. The angles between homologous branches of the symmetric axis determined by the junction of three axis segments near the mandibular foramen are remarkably invariant both within and to a lesser extent between all individuals studied when compared with other components defining mandibular shape.

An effort has been made to determine factors underlying this observed invariance by transcription of grossly different mandibular shapes from sources readily available in the literature. These include acromegalics, micrognathics, edentulous mandibuli, even mandibuli from various primates and lower mammals including gorilla, orangutan and rodents. In many cases the homologous angles appear to superimpose to an extent limited only by transcription accuracy. In those instances when the angles can not be precisely superimposed such as when comparing humans with mice the discrepancy is usually associated with a lack of homology produced by the interposition of an additional axis segment in one of the shapes being compared. Smaller variations require more sophisticated measures of angular congruity to adequately define statistical parameters.

Recent data produced directly from photographs of mouse mandibuli having known genetic characteristics show similar angular invarance within and between strains. Mandibuli from mice having different genetic profiles differ in certain aspects of shape as measured by segment length and radius function in ways not correlated with their degree of genetic similarity.

IV. Significance

The discovery of a precisely defined, relatively stable property which follows directly from symmetric-axis description of homologous projections of the human mandible provides a solid justification for future application of this new analytical tool. The fact that this invariant pattern was discovered on the first application of the method to developmental projection suggests that other invariant properties are likely to emerge from comparable analysis of other biological structures characterized by similar growth dynamics. The existence of a relatively invariant angle in the jaw implies that certain aspects of mandibular growth and development can be reliably predicted. This necessarily reduces the number of possible alternatives that an Orthodontist must consider when taking into account the role of mandibular growth in his treatment plan.

V. Proposed course

An effort will be made to define angular invariance more rigorously to facilitate statistical analysis of small variations. Symmetric-axis derived measures of mandibular shape will then be used in the taxonomic description of mice with particular emphasis on the relationship of these measures to genetically determined traits. This approach has the advantage of accuracy made possible by sacrificial exposure of the bones of interest, plus virtual identity of siblings associated with common laboratory strains. These data will be compared with existing human cephalometric data transferred from the Center of Human Growth and Development, The Univeristy of Michigan.

Publication:

Webber, R.L., and Blum, H.: Angular Invariants in Developing Human Mandibles. Science 206: 689 - 691, 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 00211-04 CI

PERIOD COVERED
October 1, 1979 to September 30, 1980

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

Webber, Richard L.	Dental Director	NIDR CI
Grondahl, Hans-Goran	Visiting Scientist	NIDR CI
Dwyer, Andrew J.	Staff Radiologist	DRD CC
Cramer, Harry J., Jr.	Staff Radiologist	DRD CC

COOPERATING UNITS (if any)
Diagnostic Radiology Department, Clinical Center

LAB/BRANCH
Clinical Investigations Branch

SECTION
Diagnostic Methodology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	.8	.95

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 an extension of previous work involving the creation, development and testing of image processing techniques designed to improve diagnostic performance.

Recent work has centered around the use of context-dependent contrast enhancement coupled with low-pass spatial frequency filtering to enhance the images of induced nodular radiolucencies in CAT scans of the liver. Statistically significant results have been obtained from double-blind psychophysical comparisons of processed video displays and unadulterated CAT scans used as controls.

Other studies deal with the effects of computerized manipulation of second-order, grey-level statistics via a coocurrance analysis of adjacent pixels.

When an algorithm based on the relative frequency of adjacent pixels is applied to serially obtained radiographic data, diagnostic accuracy in detecting tiny interseptal and proximal dental lesions is improved significantly over the status quo.

1. Objectives

This project is concerned with further study of factors underlying diagnostic performance obtainable from images with emphasis on the effect of mode of display on performance by human interpreters. Previous work has shown that image processing can improve diagnostic performance obtainable from dental radiographs for specific tasks not limited by the information capacity of the system. This work also demonstrated that monocular cues to depth can be used meaningfully to augment perceptibility of complex images of diagnostic interest.

Current objectives are based on logical extensions of these findings with emphasis on performance testing of promising enhancement schemes.

11. Methods and Major Findings

The emphasis on technology development within DMS as reported in previous years has largely given way to studies designed to formally evaluate selected enhancement techniques within the context of specific diagnostic tasks. The one exception is a continuing effort to develop a generalized diffuse reflectance model to be used in association with the existing pseudo-solid transformation to facilitate contour perception in a way which is less sensitive to illuminant projection geometry.

An automated scheme for randomly presenting test and control images in a way that facilitates formal evaluation of the effect of mode of display on the performance of a variety of diagnostic tasks has been developed and applied to selected data, both dental and medical.

Theoretical analysis of factors known to influence the amount of information available from CAT scans on second-generation machines suggests that visual interpretability may be enhanced by a suitable position-invariant filter designed to eliminate noise expressed at high spatial frequences.

This hypothesis is formally tested in a study involving an evaluation of context-dependent, contrast enhancement (histogram equalization) and low-pass, spatial frequency filtering (uniform aperture convolution) on the detectability of computer-induced nodular, radiolucent lesions in CAT scans of the liver obtained from patients examined by the NIH Clinical Center department of Diagnostic Radiology. This source of images was considered appropriate for preliminary study because there is no loss of information capacity associated with the digitization. A battery of radiologists and untrained observers were exposed to a randomized sequence of paired displays using an unbiased, double-blind paradigm under reproducible conditions. The data indicate a statistically significant improvement in diagnostic performance obtainable from low-pass filtered CAT scans of the liver irrespective of unrestricted manipulation of image brightness and contrast.

Another study deals with the use of an enhancement algorithm designed to rank pixel frequences of adjacent grey-level pairs as a means for creating a two-dimensional description of second-order image redundancy. Images so produced are characterized by the fact that those grey levels in the original image having the least second-order spatial redundancy are coded brightest in the transformed image.

Such an algorithm is particularly well suited to task-dependent applications involving the enhancement of small differences in otherwise identical images. It is being applied to serially obtained dental radiographs containing known lesions. The lesions induced in interseptal bone and in proximal dental enamel are too small to be detected reliably by conventional radiographic techniques.

When applied to subtracted radiographic images of such lesions obtained from in vitro specimens, coocurrence processing likewise has been shown to produce a significant improvement in diagnostic accuracy over unprocessed controls. In this case dentists rather than radiologists participated in the evaluation. These data confirm that factors other than quantum statistics limit performance under these conditions. They also indicate that with suitable geometric registration and image enhancement, the detectability of incipient dental disease can be significantly improved using less radiation than currently employed.

Another image enhancement scheme is also being evaluated for clinical applicability. Continuing study of the pseudo-solid technique includes preliminary comparisons with more conventional methods of enhancement for a variety of filtered images having various information capacities and contrast ranges. Detectability of obscure patterns is enhanced substantially by a suitable pseudo-solid transformation but angular dependence appears to limit the practical application of this enhancement modality at this time.

III. Significance

Recent concern for exposure to ionizing radiation coupled with the technological progress made in diagnostic medicine renders this research particularly timely. The fact that the two major dental diseases, caries and periodental disease, are characterized by insidious, asymptomatic onset with largely irreversible consequences, likewise emphasizes the need for developing improved methods for early diagnosis of these disorders.

The results of the controlled studies reported above, demonstrate that improved diagnostic performance can be obtained in a variety of applications which do not require any increases in exposure to x rays. By matching mode of display to factors known to be limiting the transfer of diagnostic information in the performance of explicitly defined tasks, it is theoretically possible to make substantial improvements in diagnostic performance measured in terms of risks and benefits.

V. Propcsed course

Further activity will continue to emphasize formal evaluation of selected enhancement techniques within the context of specific diagnostic tasks. It will include also modification of the pseudo-solid algorithm to permit diffuse reflectance to be used as a perceptual cue to aid in the detection of changes in surface contour induced by the pseudo-solid transformation. In this way it is anticipated that the strong, angular dependence demonstrated by recent application studies can be circumvented in the future.

Publications:

Webber, R.L., and Nagel, R.: Image preprocessing as an aid to visual interpretation. IADMFR Prcceedings (1978) (In press)

Webber, R.L., and Nagel, R.N.: Three-Dimensional Enhancement of Two-Dimensional Images. Journal of Clinical Engineering. 5: No. 1, 41 - 50, 1980.

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 00287-01

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Laser Doppler Velocimetry in Oral Structures

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

de Rijk, Waldemar G.	Senior Staff Fellow	NIDR IR
Bowman, Robert L.	Director	H IR
Bowen, Patrick D.	Technician	H IR
Bonner, Robert F.	Physicist	BEI R

COOPERATING UNITS (if any)
Laboratory of Technical Development, National Heart, Lung and Blood Institute, NIH; BEIB, DRS.

LAB/BRANCH
Clinical Investigations Branch

SECTION
Diagnostic Methodology Section

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.88	.7	.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)

The purpose of this project is to develop and evaluate a method for quantitative measurement of bloodflow in gingival tissues.

A new non-invasive optical method for measuring bloodflow; Laser Doppler Velocimetry (LDV) has been developed by NHLBI. This method has been modified and adapted for intra-oral use by fabricating special probes which reproducibly position the fiber optic source-detector system in the mouth. Preliminary results indicate that the system can reliably detect changes in gingival blood flow induced by pressure occlusion and vascoconstricting drugs.

I. Objectives

The purpose of this project is to explore the applicability of laser doppler velocimetry as developed by the NHLBI, to intraoral tissues. The initial goal is to adapt the existing prototype device to intraoral use and then determine whether the bloodflow is correlated with existing clinical measures of gingivitis and periodental disease.

II. Method

The instrument consists of a coherent light source (laser) fiberoptically coupled to a probe and signal analyzer. Light from the laser is directed onto the tissues via an optical fiber. The directly-reflected light, and light scattered by the RBCs are conducted to a photocell by a second optical fiber. Beat frequencies are observed in the electrical signal, and an on-line frequency analysis is performed. The integral over frequency and amplitude provides the measure of flow. Flow is defined as the product of volume and velocity. Intraoral probes have been devised for dental applications and special probe holders have been fabricated to insure reproducibility of probe placement over time.

III. Major Findings

Preliminary results have been obtained, indicating that the instrument is sufficiently sensitive to display the pulsatile component of the blood-flow (heartbeat) in the gingivae of a normal volunteer. Venous and arterial blockage could be differentiated reliably.

The effect of vasoconstrictors in local anasthesia was shown to produce a measurable reduction in peripheral bloodflow in the injected area.

IV. Significance

The instrumentation is developed to provide a new diagnostic tool in the evaluation of periodontal disease. The role of bloodflow in periodontal disease has received scant attention in the literature, mainly because of the absence of adequate instrumentation. This project explores the potential of a new non-invasive diagnostic technique which hopefully will contribute to a better understanding of gingival physiology in vivo.

V. Proposed course

During the coming year a clinical protocol will be developed, and, following approval, a formal clinical evaluation will be initiated.

Measurements are anticipated in two areas:

1) Measurement of bloodflow in attached and free gingivae including any correlation with an independently assessed clinical status of the periodontium.

2) Measurements before and after oral prophylaxis, scaling and rootplaning, to assess the influence of therapeutic manipulations on the peripheral bloodflow in gingiva.

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 00048-09 CI

PERIOD COVERED
October 1, 1979 to September 30, 1980

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

Bosma, James F.	Chief, Oral Pharyn.	OPD CI NIDR
Murayama, Diane	Illustrator	OPD CI NIDR

COOPERATING UNITS (if any)

LAB/BRANCH
Clinical Investigations Branch

SECTION
Oral and Pharyngeal Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

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 twelfth year of anatomical studies of the face, pharynx and cranium of the human fetus at term.

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. Negotiations for its publication are in progress.

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.

III. Major Findings

Preparation of book: The Head of the Newborn Infant. This general descriptive anatomy is in the process of expansion of its original manuscript, with additional illustrations, to total of approximately 240, and corresponding enlargement of text. Its illustrations are essentially completed. Its critique is simultaneously in progress.

IV. Significance

These studies provide information for description of normal development of anatomical form and structure. 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.

V. Proposed Course

The book, The Head of the Human Infant, has been submitted to a publisher, the Johns Hopkins University Press. The Press will submit a grant application to NIH-NLM for partial subvention sponsorship.

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 00124-06 CI E

PERIOD COVERED
October 1, 1979 to September 30, 1980

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

Weiffenbach, James M.	Research Psychologist	CI	NIDR
Cowart, Beverly	Psychologist	CI	NIDR

COOPERATING UNITS (if any)

Department of Pediatrics, National Naval Medical Center, Bethesda, MD

LAB/BRANCH
Clinical Investigations Branch

SECTION
Oral and Pharyngeal Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: .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)

The discrete elicited lateral tongue movement first described under this project is the basis for psychophysical studies of oral sensation in the human newborn. This transverse reflex is elicited by touching the tongue or placing a drop of fluid on its dorsal surface. The reliable adaptation of this reflex with repeated elicitation has been incorporated into a demonstration of taste sensitivity. Adaptation to water stimuli leaves responding to sugar solutions unimpaired, but adaptation to sugar solutions suppresses responding to water drops. The newborn's sensitivity to both sweet and salty tastes are viewed in the context of the subsequent modification of these sensitivities with maturation and experience.

Project Description

I. Objectives

This project explores the oral sensory competence of the newborn human infant. A novel reflex technique has been developed as part of this project. It is equally well suited to measuring the local sensitivity of the tongue to touch and to taste stimulation. In the beginning, project investigations were primarily concerned with developmental changes in touch sensitivity. Progressively greater emphasis is now being placed on studies of the sensory mechanisms underlying neonatal taste experience. Since infants are sensitive to taste at birth it is possible, as 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. Postadaptation increments in responding above that found with continued water stimulation have been demonstrated for both glucose and sodium chloride test solutions.

III. Major Findings

The major share of research effort assigned to this project during the current year has been invested in completing an extensive critical review of the literature relating to the development of human taste perception throughout the life span. The review defines the context for the newborn project and clarifies the relation of it to other ongoing investigations (Z01 DE 00212-04 CI).

There were no new major findings from experimental investigations during the report period.

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 later food preferences should lead to the design of effective intervention strategies.

V. Proposed Course

Alternative means of achieving test and control data from the same subjects will be developed and tested with the end of providing a clinically useful individual difference measure of taste sensitivity.

Publications:

Weiffenbach, J.M, Daniel, P.A., and Cowart, B.: The Development of Saltiness Perception. In Kare, M.R., Fregley, M.J. and Bernard, R.A. (Eds.) Biological and Behavioral Aspects of Salt Intake, New York, N.Y. Academic Press, 1980.

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 00181-04 CI

PERIOD COVERED

October 1, 1979 to September 30, 1980

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

Bosma, James F.	Chief, Oral Pharyn.	OPD CI NIDR
Murayama, Diane	Illustrator	OPD CI NIDR
Sapperstein, Selma	Secretary	OPD CI NIDR
Koback, Anna	Secretary	OPD CI NIDR

COOPERATING UNITS (if any)

National Library of Medicine
Philadelphia Children's Hospital
University of Michigan

LAB/BRANCH

Clinical Investigations Branch

SECTION

Oral and Pharyngeal Development

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.95	.20	.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)

A PHS-NLM Grant Application for subvention of publication of the book, Postnatal Development of the Rat Skull has been approved. The book is now at the University of Michigan Press.

In Part 1 of the book, the cranium and mandible of the rat are demonstrated by drawings and photographs at successive postnatal ages. In Part 2, development of the cranium and mandible are demonstrated by alizarin 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.

Project Description

I. Objectives

Normative demonstration of postnatal development of the individual bones and teeth and of the cranial composite and mandible in the rat.

II. Methods

Of Part 1. Rat skulls of days 1, 15 and 60 were dissected after papainization. The individual bones were then photographed and drawn.

Of Part 2. 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 in 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 of Part 2 as prepared for publication, combines color photographs (in 120 projection slides) with tracings of the corresponding anatomical transsections Each of these atlas units 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

Publication of book, Postnatal Development of the Rat Skull by the University of Michigan Press, under subvention provided by NIH-NLM Grant Application, I-R01 LM03464-01.

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 00182-04 CI

PERIOD COVERED
October 1, 1979 to September 30, 1980

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

Bosma, James F.	Chief, Oral Pharyn	CI	NIDR
Weiffenbach, James M.	Research Psychologist	CI	NIDR
Wolf, Robert O.	Dental Investigator	CI	NIDR
Geoffrey, Virginia	Speech Specialist	CI	NIDR
Murayama, Diane	Illustrator	CI	NIDR
Sapperstein, Selma	Secretary	CI	NIDR
Koback, Ann	Secretary	CI	NIDR

COOPERATING UNITS (if any)

NICHD, NIH
NINCDS, NIH
Clinical Center, NIH
Johns Hopkins Medical Center

LAB/BRANCH
Clinical Investigations Branch

SECTION
Oral and Pharyngeal Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.35	.70	.65

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 motor and/or sensory impairments of feeding or of speech or other upper respiratory performances, either of malformation, of inflammatory or neurological disease or following cancer surgery, are studied by sensory testing, cinephotography, cineradiography and speech analysis.

Project Description

I. Objectives

The study of clinical variations which are associated with malformations of inflammatory disease, or of surgical 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 designed to define anatomical abnormalities and their physiological correlates. And, for the purpose of devising and applying therapies for disabilities of feeding and of speech and other respiratory functions.

II. Methods

Standard procedures of cinephotography and cineradiography, as well as acoustical and other physiological recordings are employed. The regional performances of feeding, speech and other respiratory actions are described in infants, children and adults. Selected patients having sensory disorders of the oral and pharyngeal regions are also evaluated by sensory testing. Patients who have surgical excision for cancer are studied as examples of the modification of sensory input by ablation.

III. Major Findings

During this year, we have continued in study of oral and/or pharyngeal sensorimotor disorders of various etiologies, but we have directed particular attention to study of two clinical categories:

a. Impairments in association with rheumatoid arthritis or related collagen disorders. Standardized history and physical examination of the mouth and pharynx demonstrates articular, fascial or motor abnormalities which are undetected or neglected in usual clinical work. These include specific involvements of mandibular, hyoid and/or laryngeal articulations.

   Remarkably, extensive involvement and disability of the cervical spine articulations are not generally associated with pharyngeal impairment.

   An apparently new clinical category of abnormality is that of laxity of the pharyngeal aponeurosis in certain of the collagen disorders. By observation routines which include both pharyngeal inflation and swallowing, during radiographic observation, laxity of the aponeurosis is found at characteristic sites in the meso- and hypopharynx. This abnormal mobility is distinguishable from weakness of the pharyngeal constrictor muscles.

b. Initial manifestation of progressive motor disease and disability in the oral and pharyngeal area. In previous years, we had noted examples of initial evidences of amyotrophic lateral sclerosis (ALS) in the tongue and pharynx, but the diagnosis was delayed until the disease had extended to the musculature of the limbs and trunk, in which areas the neurologist's classical methods of electromyography and biopsy were familiarly applied. Employing our standard cinephotographic and cineradiographic routines and criteria, we are increasingly confident of identification of motor unit and of certain coordinative abnormalities, such as ALS, myasthenia, dysautonomia and tardive dystonia as these may be initially evidenced in the tongue, palate and the constrictor walls of the pharynx.

IV. Significance

Adaptations of selected diagnostic methods, including sensory evaluation, have enhanced the definition of sensorimotor disorders of the mouth, pharynx and larynx. 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.

V. Proposed Course

Clinical studies of subjects having the above categories of disorders will continue with current methods.

Our radiographic studies of sensorimotor pharyngeal impairments are currently under accumulation into a general volume, Radiography of the Pharynx, under authorship of James F. Bosma and Martin Donner, of the Department of Radiology, the Johns Hopkins Medical Center. Publication will be by Springer-Verlag.

Publications:

Bosma, J.F., Physiology of the Mouth
Chapter in Otolaryngology, Edition 2. Eds. Paparella, M.M. and Shumrick, D.A., Philadelphia, Saunders Co., 1980

Bosma, J.F. and Donner, M., Physiology of the Pharynx, ibid.

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 00212-04 CI

PERIOD COVERED
October 1, 1979 to September 30, 1980

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

Meiffenbach, James M.	Psychologist	CI	NIDR
Wolf, Robert O.	Dentist	CI	NIDR
Baum, Bruce J.	Dentist	LMA	NIA
Cowart, Beverly J.	Psychologist	CI	NIDR
Brown, Vivian J.	Student trainee	CI	NIDR
Taylor, Ronald	Student trainee	CI	NIDR

COOPERATING UNITS (if any)
Laboratory of Molecular Agency NIA
Surgery Branch, NCI

LAB/BRANCH
Clinical Investigations Branch

SECTION
Oral and Pharyngeal Development

INSTITUTE AND LOCATION
NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.50	PROFESSIONAL: 1.05	OTHER: 1.45

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 selection and refinement of appropriate psychophysical methods for the separate measurement of the various aspects of taste perception is a primary and continuing concern of this project. Normal variation in taste perception with chronological age is assessed by procedures which quantify not only the taste detection threshold but also the intensity and pleasantness of the subject's taste experience. Both naturally occurring anomalies and therapeutically induced changes in taste are investigated in parallel studies which emphasize the role of saliva in distortions of taste perception. Finally, the possibility that taste perception differences exist between individuals with and without caries experience is being investigated.

Project Description

I. Objectives

This project employs selected modern psychophysical methods to define the relation of taste perception to age, to experience, to saliva and to oral or systemic disease. Cases in which disorders of taste perception are the presenting symptom are also studied.

II. Methods

Currently, all testing is done with room temperature tastant fluids and an immediately prior rinse of double-distilled water. For threshold measurement, the "up-down" procedure for determining stimulus presentation order is combined with a forced-choice response format. Measures of the intensity and pleasantness of supra-threshold stimulation are obtained by direct scaling using cross modality matching.

III. Major Findings

Eleven pairs of matched caries-free and caries-experienced subjects demonstrated no difference in sensitive to sucrose when tested by a technique which negates the effect of saliva. The taste threshold differences reported by others probably depend upon the use of techniques which allow saliva to affect the results. These findings encourage the hypothesis that taste modifying factors in saliva should be identifiable by comparing thresholds obtained by saliva dependent and saliva independent tests.

Detection thresholds by two commonly used procedures applied to normal subjects showed large and significant differences for salt and small nonsignificant differences for sucrose. The salient difference between the procedures was that the one producing lower thresholds for salt required subjects to rinse before each sampling of taste fluid.

Detection thresholds for sucrose sodium chloride, citric acid and quinine sulfate have been obtained from more than seventy subjects in the initial year of a collaborative investigation of the development of taste perception with the National Institute of Aging (N. .). These data were obtained from subjects who range in age from the early twenties to the late eighties. The thresholds obtained for sucrose and for sodium chloride exhibit excellent agreement with the data obtained in previous developmental studies when thresholds for these two substances were obtained from separate samples. The data base for the current study consists not only of thresholds for all four basic qualities but also of direct scaling of intensity for the four qualities.

Using cross modality matching to linear extension of a tape measure we have obtained reliable direct scaling for taste intensity and pleasantness from child subjects as young as 4.5 years of age. Appropriate comparisons with traditional magnitude estimation techniques have been made with adult subjects who can be tested with both techniques. This procedure will be used in a doctoral study with subjects of various ages.

IV. Significance

The human taste system 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. Variations in taste with systemic disease suggest that they may serve as diagnostic indicators.

Saliva plays a major role in taste function. Saliva contents, whether glandular excretory product or exudate, modify the taste experience. Taste threshold testing can assess the taste experience either with saliva or without saliva. The latter assess the base function of the taste apparatus; the former its in vivo performance.

V. Proposed Course

Our collaboration with NIA investigators at the Gerontology Research Center in Baltimore on longitudinal studies of taste and aging will continue.

Age related changes in the relation of suprathreshold stimulus concentration to the intensity and pleasantness of taste experience are being studied cross-sectionally. Kindergarten age children are being tested. Additional groups of elementary and high school age subjects and two adult samples will be tested.

Studies of the relation of taste to caries status will be enlarged to include direct scaling of supra threshold pleasantness and intensity. As before, all four qualities will be studied. Studies evaluating the reliability and efficiency of clinical assessment procedures will continue. A greater number of patients with taste abnormalities will be accepted for evaluation.

Studies in collaboration with the Surgery Branch of the National Cancer Institute will explore threshold and supra threshold taste function in individuals being maintained by parientral procedures and thus deprived of the normal oral sensory experience of feeding.

Studies to identify further salivary modification of taste according to qaulity are being designed. They include studies of the metallic taste generated by elevated (> 100 millivolts) potentials between restorations of dissimilar metals.

Publications

Wolf, R.O. and Weiffenbach, J.M.: Saliva and taste disorders. Proc. of 28th Inter. Congress of Physiological Sciences, 1980, Akademiai Kiado, Publishing House of the Academy of Science, Budapest, Hungary, (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 00217-02 CI

PERIOD COVERED

October 1, 1979 to September 30, 1980

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

Wolf, Robert O.	Dentist	NIDR CI
Hubbard, Van Saxton	Physician	NIAMDD
Baum, Bruce J.	Dentist & Biochemist	NIA LMA
Mannix, Margaret	Nurse	NIDR LOM
Kuhn, Gerard A.	Technician	NIDR CI
Nivera, Brenda	Technician	NIDR CI
Papadopoulos, Nicholas	Biochemist	CA CR

COOPERATING UNITS (if any)

Laboratory of Molecular Aging, NIA
Georgetown University of Dental School, Washington, D.C.

LAB/BRANCH

Clinical Investigations Branch

SECTION

Oral and Pharyngeal Development

INSTITUTE AND LOCATION

NIDR, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	.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)

This project is concerned with mechanisms of production and control of extrinsic (i.e., saliva) and intrinsic (e.g., serum salivary isoamylase) salivary gland product. Human (primarily parotid) saliva chemical constituents and mechanisms are evaluated as related to health and disease. Parotid salivary flow rate, protein content and enzymes (particularly lysozyme and amylase) are evaluated in normals and selected disorders such as Sjögren's syndrome and aphthous stomatitis. The intrinsic secretion of salivary isoamylase in serum of cystic fibrosis of the pancreas and Sjögren's syndrome are being studied. Diagnostic application and an understanding of the mechanisms of hyperamylasemia are being sought.

1. Project Description:

Objectives: Associating intrinsic and extrinsic salivary secretion and secreted variables with disease and physiological condition is the overall objective of the project. Subobjectives can be listed as (i) determining the diagnostic value of parotid saliva lysozyme concentration in Sjögren's syndrome, (ii) determine the relationship of parotid salivary concentration as related to the ulcerative stages of apthous stomatitis, (iii) determine the extent and importance of the ratio of bound to unbound parotid salivary lysozyme in a normal population, (iv) determine on a large aging population if a polyacylamide gel electrophoresis (PAGE) fast moving isoamylase is associated with the elderly, (v) determine if there is evidence for a serum amylase compensitory mechanism operating in cystic fibrosis of the pancreas keeping total serum amylase level up by an increase in salivary amylase as pancreatic amylase decreases with concurrent decreased pancreatic function, (vi) determine if the serum pancreatic salivary isoamylase ratio is a useful and efficient diagnostic tool for determining pancreatic insufficiency.

Methods Employed: Parotid salivary lysozymes have been studied in patients with Sjögren's syndrome and longitudinally in patients with apthous ulcers to determine the patterns of variation. The ratio of bound to unbound lysozyme is also being studied in a normal population. Radial diffusion of lysozyme in agar-gel containing the substrate Micrococcus luteus has been the basic quantitative assay for these studies. The bound lysozyme is obtained by acidification, which releases more lysozyme, followed by centrifugation and neutralization of the sample. For Sjögren's syndrome the lysozyme values of single saliva collections from diagnosed cases were compared with the lysozyme values obtained from normal subjects' samples. The quantitative relation of parotid lysozyme to apthous stomatitis within subject has been studied by collecting five consecutive two minute samples at several time points within at least one complete cycle of ulcers and no ulcers.

The amylases are being studied associated with cystic fibrosis of the pancreas and during aging. The total amylase is being quantitated by the blue starch method and the serum pancreatic to salivary ratio is obtained by PAGE isoenzyme separation with subsequent amylase activity determined in serial slices of the gel. The salivary isoamylases are separated by PAGE and visualized as zymograms for between sample comparison. Serum, whole saliva and parotid saliva are being collected before and after meals from cystic fibrosis patients and from age and sex matched normal volunteers. This is to assess the dynamics of serum salivary to serum pancreatic amylase ratios as a first step to authenticate a postulated serum salivary amylase compensitory mechanism for the loss of serum pancreatic amylase in cystic fibrosis patients with pancreatic insufficiency. In further cooperation with NIAMDD, studies of the accuracy and usefulness of the serum pancreatic and salivary amylase ratio obtained by PAGE as compared to a new and a standard pancreatic function test are in progress.

Parotid salivary isoamylases are being studied in cooperation with NIA at the Baltimore City Hospital within a stratified aging population in an effort to identify faster migrating isoamylases which are reported to increase during aging (Chilla, R. et al, 1977). Total parotid salivary amylase values will be correlated with all other data associated with the population.

Major Findings: A longitudinal study of parotid saliva lysozyme concentration within several apthous stomatitis patients sampled at varied times through several cycles of ulcerations has demonstrated no consistency between ulceration and parotid saliva lysozyme level flow rate or total parotid saliva protein. In another study an increased lysozyme concentration distinguished primary Sjögren's syndrome patients from normal control patients and patients with Sjogren's syndrome secondary to hyperlipoproteinemia. In the study of bound and unbound parotid saliva lysozyme in a normal population (n = 21) bound to unbound ratio has varied from 0.21 to 1.20 without a predictable association with flow rate or total parotid saliva protein.

The assessment of the exocrine pancreatic function in cystic fibrosis patients using the serum amylase pancreatic salivary ratio as compared with a new orally administered specific substrate for chymotrypsin (BTP) has shown that both tests in a controlled study will indicate pancreatic insufficiency in cystic fibrosis patients previously documented to have pancreatic insufficiency. In the study of more than 200 parotid saliva samples from the NIA aging population we now have the impression that aged persons do not demonstrate a greater number of faster migrating parotid saliva isoamylase than has been observed in the younger groups. We have observed in a carefully controlled study an increase in both slower and faster migrating parotid saliva isoamylases as a function of storage time.

Significance to Biomedical Research and the Program of the Institute: In primary Sjögren's syndrome the levels of parotid salivary lysozyme are higher than in normal subjects or in those patients who have Sjögren's syndrome secondary to hyperlipoproteinemia. We postulate that the source of lysozyme is from the parotid inflammatory infiltrate and that the assessment of parotid salivary lysozyme may be a diagnostic adjunct for Sjögren's syndrome.

The failure of the longitudinal apthous stomatitis study to demonstrate a parallel increase and decrease of parotid salivary lysozyme level is at variance with a previous impression that lysozyme level was lower during ulceration. Bound and unbound lysozyme may be indirectly significant in disease. Lysozyme is known to bind with other substances including glycoproteins. The variation of the binding substance then may be of significance as reflected by the lysozyme level.

Davidson and co-workers (1978) have implied that cystic fibrosis patients have a compensatory mechanism which increases the level of serum salivary amylase as the pancreatic level decreases. We are studying the dynamics of serum salivary amylase production in parallel with amylase found in the saliva (i.e., simultaneous parotid endocrine exocrine amylase production). If such a clue can be established it will be an indication of parotid-pancreas communication or axis which is suspected but not clearly established.

The oral physiological data base of the extensive longitudinal studies of the NAI normative population at Baltimore is increased by the inclusion of the salivary amylase parameters in that some revealing associations are now much more likely to be discovered.

The diagnostic value of the serum salivary pancreatic ratio obtained by PAGE in cystic fibrosis of the pancreas is being tested. A blood test of this nature would be clinically useful because of difficulty in obtaining duodenal fluid by intubation for pancreatic enzyme testing which is difficult in some infants and small children. A new and second test utilizing a test compound of tyrosine bound to PABA (N-benzoyl-L-tyrosyl-p-amino-benzoic acid) is given orally. Normal pancreatic metabolism splits the PABA tyrosine bond and PABA is found in the urine. No PABA is found in the urine of patients with inadequate pancreatic function. The PAGE serum salivary pancreatic ratio is the third test in an NIAMDD study to determine the appropriateness of the three tests. These studies are not yet complete.

Proposed Course: The temporal dynamic of lysozyme in apthous stomatitis parotid saliva are under study as will be the lysozyme bound and unbound ratio in the parotid and labial gland saliva. The dynamics of blood lysozyme will next be studied as will the etiology (gene products or complexes) of the isoenzymes of lysozymes previously reported.

The results of parotid amylase analysis will be continued, if meaningful correlations are found in the NAI normative aging population.

Animal studies of the production dynamics of the salivary isoamylases and lysozyme are being initiated.

The results of the three compared pancreatic function tests will dictate the continuance of this amylase study.

2. Publications:

Moutsopoulos, H.M., Klippel, J.H., Pavlidis, N., Wolf, R.O., Sweet, J.B., Chu, F.C., Steinberg, A.D. and Tarpley, T.M.: Correlative histologic and serologic findings of sicca syndrome in patients with systemic lupus erythematosus. Arthritis and Rheumatism. 23(1):16-40, 1980.

Moutsopoulos, H.M., Karsh, J., Wolf, R.O., Tarpley, T.M., Tylanda, A. and Papadopoulis, N.M.: Lysozyme determination in parotid saliva from patients with Sjögren's syndrome. Amer. J. Med., (In press).

*U.S. GOVERNMENT PRINTING OFFICE: 1981-0-341-132/3503

New Book 6-6-81

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