NATIONAL INSTITUTE OF ARTHRITIS AND MUSCULOSKELETAL AND SKIN DISEASES

ANNUAL REPORTS

INTRAMURAL RESEARCH PROGRAMS

OCTOBER 1, 1989 TO OCTOBER 1, 1990

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PROJECT NUMBERS

NATIONAL INSTITUTE OF ARTHRITIS AND MUSCULOSKELETAL AND SKIN DISEASES

ACTIVE PROJECTS

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Laboratory of Physical Biology

National Institute of Arthritis and Musculoskeletal and Skin Diseases

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Muscle Contraction

Crossbridge motion.

A widely accepted model of muscle contraction assumes that force is generated by "rocking" subfragment 1, the head part of the myosin molecule, after it attaches to the actin filament. Many investigators have tried to produce this kind of motion by stretching rigor muscle fibers, but have not been able to detect it with either X-ray diffraction or various spectroscopic probes. Addition of ATP analogues which weaken the binding of subfragment 1 to actin was also without effect on the response to stretch. However, an X-ray signal was detected when muscle fibers in rigor were first crosslinked with EDC, a zero-length crosslinker, and then stretched. The time course of the effect could be accelerated by addition of ATP analogues (AMPPNP and PP_i) to the fibers and was fastest in the presence of ATP itself. The results indicate that a myosin head is capable of rocking on actin and that the EDC tethered myosin head is more flexibly attached to actin than the normally attached head (Iwamoto, Podolsky).

Crossbridge distribution in rigor muscle.

The pattern of crossbridge formation between the thick and thin filaments was studied in the rigor state by using low salt, high pH conditions to dissociate the thick filaments and monitoring the detailed structural changes by electronmicroscopy and X-ray diffraction. In the part of the A-band that contains only thick filaments (the H-zone), the filaments dissociated mainly into three subfilaments. In the part of the A-band where thick and thin filaments interdigitate and actomyosin crossbridges are present, the thick filaments open to form hollow rings and then numerous subfilaments. This pattern of dissociation indicates that the thick filaments are made up of three parallel subfilaments, and that the crossbridges from each subfilament are shared by at least three of the adjacent actin filaments. (Barbosa, Podolsky)

Regulation of skeletal muscle.

Several years ago two ways of artificially creating ATP-independent weakly-binding crossbridges were described. Weakly-

binding crossbridges created either with para-phenylenedimaleimide (pPDM) or with N-phenyl maleimide (NPM) treatment have binding strength and attachment and detachment rate constants virtually identical to normal myosin ATP crossbridges were used as a model for the normally-occurring weakly-binding crossbridges. Because of this very close similarity between pPDM- or NPM-treated crossbridges and normal relaxed crossbridges, the artificially created weakly-binding crossbridges were used as a model for normally-occurring weakly-binding. It was found that the rate constants and binding constants of the weakly-binding pPDM- and NPM-crossbridges were insensitive to changes in $[Ca^{2+}]$ over the physiological range. This is an important result with regard to the mechanism of muscle regulation in fibers because it provides evidence that the regulation of muscle activity resembles the regulation of the actomyosin ATPase in solution and not the mechanism postulated by the classic steric blocking model of muscle relaxation, which states that muscle regulation is due to the effect of Ca^{2+} on the binding strength of weakly-binding myosin. (Barnett, Schoenberg)

The role of calcium in contraction regulation was investigated in rabbit psoas muscle fibers. Equatorial X-ray diffraction and mechanical measurements were used to study the effects of calcium on the actin affinity of crossbridges in the presence of an ATP analog, ATPgS. Crossbridges in the presence of ATPgS but in the absence of calcium exhibited identical binding characteristics as observed in relaxed muscle. It was found that calcium in the presence of ATPgS only slightly increases the actin affinity, whereas the kinetics of crossbridge binding were significantly affected. The findings are contrary to the predictions of the steric blocking model. Hence steric blocking is not likely the regulation mechanism in these muscle fibers. (Yu, Brenner)

Regulation of smooth muscle.

Using Western blotting with an ^{125}I-conjugated second antibody, an extremely sensitive radio-assay for detecting the percentage of 20-kDalton myosin light chain phosphorylation in very small preparations of tracheal smooth muscle was worked out. Vasoactive intestinal peptide (VIP), which causes tracheal smooth muscle relaxation, also causes a decrease in the percentage of myosin light chain phosphorylation in fibers stimulated with substance P. It has also been shown that myosin light chain kinase, the main enzyme responsible for phosphorylating myosin light chain, is itself a phosphorylatable protein in vitro. Additionally, phosphorylation of myosin light chain kinase in vitro reduces its activity. By stimulating the smooth muscle after its ATP pool had been equilibrated with ^{32}P, it was shown that the kinase is indeed phosphorylated. Experiments are currently underway to examine whether the site phosphorylated is the same as the one shown to regulate myosin light chain kinase in vitro. (Moussavi, Qian, Schoenberg, Adelstein, Sellers)

Intermediate state for force generation.

It has been proposed that the actomyosin ATPase cycles (crossbridge cycles) include several energetically distinct states. The cycle can be divided into two groups of states characterized by the strength of binding between actin and myosin - the weak binding states and the strong binding states. Force generation probably involves a transition from the weak to the strong binding states. Evidence was found that crossbridges bound to actin in the weak binding states are essential precursors to force generation. Caldesmon, a regulatory protein found in smooth muscle, specifically inhibits binding of crossbridges in the weak binding states. When caldesmon is added to skinned rabbit psoas muscle fibers, both X-ray diffraction and mechanical binding states decrease as the concentration of caldesmon increases. Accompanying the inhibition, the active force level generated by the muscle is found to decrease. This is the first evidence showing that the weak binding states are essential intermediates for force generation in vertebrate muscle. (Yu, Chalovich)

Resting tension

In the course of previous work with normal and dystrophic human muscle it was noticed that the tension produced by stretching these relaxed fibers is approximately five times smaller than produced by stretching rabbit psoas muscle. Since proximal limb muscles (quadriceps and biceps) were used in the human muscle study, limb muscle of the rabbit were examined to see if they showed low levels of resting tension, similar to that found in the human fibers. Using a sensitive, quantitative electrophoretic technique, the individual rabbit soleus muscle cells, as well as the human fibers, were found to contain the same concentrations of major myofibrillar proteins as the rabbit psoas fibers. The five-fold difference in resting tension observed is therefore not due to different levels of elastic, tension bearing proteins, but indicates a qualitative difference within these proteins at the molecular level. (Horowits, Podolsky)

Cell Membrane

Calorimetry of critical bilayer assembly.

The critical bilayer assembly temperature T* can be found for aqueous phospholipid dispersions at the terminus of a second-order phase transition. This assertion is based on a thermodynamic analysis of the equilibrium surface film properties of phospholipid dispersions. While this analysis is rigorous, it is laborious and time-consuming. Using a newly designed, quasi-adiabatic calorimeter, in which the heat capacity is measured as a function of temperature, T* for dispersions of dimyristoylphosphatidylcholine (DMPC) have been measured. Surface chemical and calorimetric methods gave the same value of T* for this lipid. The calorime-

ter measurement has several notable advantages: it provides a precise measure of the heat of bilayer assembly and the critical temperature, and it allows T* to be measured within several days instead of the normal period of weeks required by the previous technique. (Gershfeld, Berger)

Membrane bilayer instability and pathogenesis of myelin disorders.

According to the critical state theory of bilayer assembly both the ambient temperature and the lipid composition of the membrane are critical conditions for membrane stability. If the critical conditions are no longer met the theory predicts that an accumulation of cerebroside sulfate in excess of the normal amount in myelin would lead to a lowering of T* and to demyelination. Myelin lipids were also examined for another demyelinating disorder, multiple sclerosis (MS), where the process is believed to occur by immune-mediated mechanisms and not by a membrane lipid defect. The critical bilayer temperature for these lipids is 37°C, consistent with the general belief that MS demyelination occurs by a non-lipid dependent mechanism. (Ginsburg, Gershfeld)

Platelet Aggregation

Evidence was found that the mechanism of decompression-inducible platelet aggregation (DIPA) is due to self-adhesion (aggregation) of platelets and their adhesion to the blood vessel wall in the small blood vessels in the web of the frog's foot. A similar stoppage of blood flow was observed in the ear of the mouse. Vascular occlusion could be prevented in the mouse as well as in the frog by the oral administration of the drug piracetam. The experimental findings confirmed the hypothesis that Acute Mountain Sickness is a vascular occlusive disease. (Murayama)

Radiation Inactivation Analysis

This technique continued to provide important data from a very wide range of biological systems.

Sodium/glucose cotransporter.

The intestinal brush border membrane sodium/glucose cotransporter is the best known eukaryotic system demonstrating ion-coupled transport, but structure-function relationships of the cotransporter in the native membrane have been lacking. Using radiation inactivation it was shown that the cotransporter functions in the membrane as a 290 kDa homotetramer comprised of four independently-activated 73 kDa subunits. These subunits work in concert and an individual subunit's activity can only be manifested by the interaction of all the other components. At the extracellular surface sodium binds to the cotransporter, which results in a conformational change that permits glucose to bind to the glucose-binding site. With both species bound, the cotrans-

porter undergoes a new conformational shift, placing sodium and glucose near the inner surface of the membrane. Sodium and glucose are then released to the cytoplasm, thereby triggering a cotransporter conformational shift to expose the binding sites once again to the extracellular surface of the membrane. The cotransporter thus again assumes the optimal conformation for binding extracellular sodium and glucose. (Kempner, Wright)

Cholesterol regulatory enzyme.

Cholesterol is required by all mammalian cells for the proper functioning of cellular membranes. Most cellular cholesterol is found esterified to long-chain fatty acids. The esterification is catalyzed by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT), one of a family of acyl-CoA acyltranferases. Because of its central role in cellular cholesterol metabolism, there has been great interest in the properties and regulation of ACAT but it has not been successfully purified from the endoplasmic reticulum. Clearly, the ACAT enzyme must have separate binding sites for cholesterol and for acetyl-CoA. Radiation inactivation of rat microsomes revealed a target size of 180 kDa. A related microsomal enzyme, Acetyl-CoA hydrolase (ACH) revealed a complex inactivation curve resembling those due to an enzyme-inhibitor complex, and leading to an enzyme target size determination of 45 kDa. Estimates of the size of the inhibitor in the range of 100-200 kDa. It was proposed that the ACAT enzyme was composed of two independently-inactivated regions, one of which binds cholesterol. The other region binds acetyl-CoA, and either transfers the acetyl group to cholesterol, or (in the absence of cholesterol) hydrolyzes the acetyl-CoA. The cholesterol-binding region of ACAT in effect becomes an inhibitor for the hydrolase activity. This model predicts that the hydrolase and acetyltransferase activities will depend on cholesterol concentration, and the increase in ACAT activity will be mirrored by an equal decrease in ACH activity. These predictions were confirmed experimentally. Conversely to the inhibitory action of cholesterol, the hydrolase activity can be considered a regulator for the ACAT function. Such regulation may be a general phenomenon which applies to other members of the acetyl-CoA transferase family and would be revealed by the presence of inherent ACH activity which is depressed by the other acceptors. (Kempner, Billheimer).

High Resolution Electron Microscopy

Work in this area was done in the Structural Biology Section of the Laboratory of Physical Biology. In a reorganization of NIAMS, this Section became the core of the new Laboratory of Structural Biology Research under which heading the report of activity can be found.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 27000-28 LPB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Mechanism of Muscular Contraction

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI:	Richard J. Podolsky	Chief	LPB/NIAMS
	Leepo C. Yu	Research Physicist	LPB/NIAMS
	Robert Horowits	Senior Staff Fellow	LPB/NIAMS
	Maria Barbosa	Senior Staff Fellow	LPB/NIAMS
	Hiroyuki Iwamoto	Visiting Associate	LPB/NIAMS

COOPERATING UNITS *(if any)*

Dr. Alasdair Steven, Section on Structural Biology, LPB; Dr. Ellis Kempner, Section on Macromolecular Biophysics, LPB; Dr. Marinos Dalakas, NINCDS; Dr. Brian Collett, Hamilton College, Clinton, NY

LAB/BRANCH

Laboratory of Physical Biology

SECTION

Section on Muscle Biophysics

INSTITUTE AND LOCATION

NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.25	4.25	

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☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Striated muscle cells consist of three sets of filaments: (1) thick, myosin containing filaments, which interdigitate with (2) thin, actin-containing filaments and (3) titin filaments, which center the thick filaments axially in the sarcomere and produce the resting tension. The resting tension is different in fibers taken from different muscles: the tension produced by individual rabbit soleus muscle fibers is about five times smaller than that produced by rabbit psoas muscle fibers. Sensitive quantitative electrophoretic measurements show that the concentration of the major myofibrillar proteins is the same in both types of fibers. Therefore the different tension levels in the soleus and the psoas fibers are due to a qualitative difference in the elastic properties of the titin molecules in the different muscle fibers.

A widely accepted model of muscle contraction postulates that force is generated by "rocking" the head part of the myosin molecule after it attaches to the actin filament. This model is supported by X-ray reflections from fibers put into the rigor state, cross-linked with EDC, and then strained by 1% in the presence and absence of ATP analogues. The 1,1 reflection decreases by about 10% at a rate that depends on the particular ATP analogue. The results suggest that an EDC tethered crossbridge becomes more orthogonal to the thin filament axis when strained.

The pattern of dissociation of thick filaments in rigor muscle fibers fixed for electronmicroscopy under low salt, high pH conditions shows that crossbridges from a given myosin subfilament can attach to at least three of the adjacent thin filaments.

Major Findings:

1. In the course of previous work with normal and dystrophic human muscle it was noticed that the tension produced by stretching these relaxed fibers is approximately five times smaller than that produced by stretching rabbit psoas muscle. Since proximal limb muscles (quadriceps and biceps) were used in the human muscle study, limb muscles of the rabbit were examined to see if they showed low levels of resting tension, similar to that found in the human fibers. Using a sensitive, quantitative electrophoretic technique, the individual rabbit soleus muscle cells, as well as the human fibers, were found to contain the same concentrations of major myofibrillar proteins as the rabbit psoas fibers. The five fold difference in resting tension observed is therefore not due to different levels of elastic, tension bearing proteins, but indicates a qualitative difference within these proteins at the molecular level.

2. Equatorial X-ray reflection intensities are sensitive to the rotational motion to the actomyosin crossbridge which is often assumed to play a major role in force production. Conversely, externally applied force would be expected to change the angle of the attached crossbridge. However, in rigor muscle, in which association constant of the crossbridges is large, little change was observed in equatorial reflections upon stretch. Therefore the present study was done in the presence of ATP analogues (PPi, AMPPNP) to reduce the association constant, while preventing the crossbridges from translocating by crosslinking the fiber with EDC. The bundles were repeatedly stretched and released by 1% of their length at 5 C while time-resolved X-ray patterns were collected using a laboratory X-ray source. Without crosslinking, the bundles showed little change in equatorial reflections upon stretch in any solution. With crosslinking (40% of the crossbridges are estimated to be crosslinked), the 1,1 reflection decreased by about 10% upon stretch in 4mM MgPPi. This effect was almost independent of the duration of stretch ranging from 100ms to 100s, and only with 50ms stretch, the 1,1 showed a smaller change. In 1mM MgAMPPNP, the amplitude of change in 1,1 increased with increasing duration of stretch from 1s to 100s reaching a maximum of 6%. In rigor, little change was observed with 5s stretch, but 5% decrease was observed with 20 or 100s stretch. In 4 mM MgATP, the decrease of 1,1 was 10% and independent of the duration of stretch. These results reflect the biochemical and mechanical rate constants in various ligands and suggest that the observed changes in X-ray pattern are related to crossbridge motion.

3. The pattern of crossbridge formation between the thick and thin filaments was studied in the rigor state by using low salt, high pH conditions to dissociate the thick filaments and monitoring the detailed structural changes by electronmicroscopy and X-ray

diffraction. In the part of the A-band that contains only thick filaments (the H-zone), the filaments dissociated mainly into three subfilaments. In the part of the A-band where thick and thin filaments interdigitate and actomyosin crossbridges are present, the thick filaments open to form hollow rings and then numerous subfilaments. This pattern of dissociation indicates that the thick filaments are made up of three parallel subfilaments, and that the crossbridges from each subfilament are shared by at least three of the adjacent actin filaments.

Project Description:

1. To work out the molecular mechanism of muscular contraction.
2. To understand the control processes for contractility.
3. To characterize the structures and processes responsible for the high degree of order seen in striated muscle fibers.

Methods Employed:

1. Analysis of the motion and the X-ray diffraction pattern of both intact muscle fibers and "skinned" fiber segments under chemically controlled conditions.

2. Conventional and cryoelectronmicroscopy of muscle fibers fixed under various conditions.

3. Analysis of mechanical and structural properties of muscle fibers following selective degradation of the megadalton muscle proteins (titin and nebulin) by high energy radiation and/or enzymatic digestion.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of molecular mechanism of muscular contraction, together with the chemistry of the activation process, is useful in the rational handling of musculoskeletal, cardiovascular and arthritic diseases.

Proposed Course of Project:

1. The study by X-ray diffraction of the influence of stress on crossbridge configuration in the rigor state will be continued. The possibility of modulating this effect by the use of myosin subfragment 1 ligands which weaken the binding between actin and myosin will be examined. At present, these experiments are being done using a laboratory X-ray source. In the future, it is planned to make use of a brighter source (e.g., the National Synchrotron Light Source at Brookhaven, NY) which would significantly shorten the amount of time needed for data collection.

2. The influence of biochemical state on the compliance of the titin filaments, which link the thick filaments to the Z discs, will be studied in skeletal muscle fibers.

3. The structural changes associated with muscle contraction will be studied by cryoelectronmicroscope and image processing, using techniques worked out by Trus et al. (1989).

Publications

Horowits R, Dalakas M, Podolsky, RJ. Single skinned muscle fibers in Duchenne muscular dystrophy generate normal force. Ann Neurol 1990;27:636-641.

Horowits R, Maruyama K, Podolsky RJ. Elastic behavior of connectin filaments during thick filament movement in activated skeletal muscle. J Cell Biol 1989;109:2169-2176.

Podolsky RJ, Horowits R, Tanaka H. Ordering mechanisms in striated muscle fibers. In Frontiers of Muscle Research, Ozawa, E ed, Elsevier Amsterdam in press.

Yu LC, Podolsky RJ. Equatorial X-ray diffraction studies of single skinned muscle fibers. In Molecular Mechanisms in Muscle Contraction. Squire, JM ed, Macmillan, London, 1990:265-286.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 27001-16 LPB

PERIOD COVERED
October 1, 1989 Through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation and Contractility of Skeletal and Smooth Muscle

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: M. Schoenberg, Medical Officer, LPB, NIAMS

V.A. Barnett, Staff Fellow, LPB, NIAMS
M. Moussavi, IRTA Fellow, LPB, NIAMS
S. Qian, IRTA Fellow, LPB, NIAMS

COOPERATING UNITS *(if any)*
Laboratory of Molecular Cardiology, NHLBI (R.S. Adelstein, J.R. Seller)

LAB/BRANCH
Laboratory of Physical Biology

SECTION

INSTITUTE AND LOCATION
NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3.5	3.5	0

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have been studying the regulation of both skeletal and smooth muscle. In skeletal muscle we did this by examining the calcium sensitivity of weakly binding crossbridges. Since it was very difficult to do this with the normally occurring weakly-binding myosin.ATP crossbridge, we instead found a way of creating ATP-insensitive weakly-binding crossbridges. This was accomplished by treating fibers with either para-phenylenedimaleimide (pPDM) or with N-phenyl maleimide (NPM). We found that these pPDM- or NPM-crossbridges, in the presence or absence of ATP, had binding strength and attachment and detachment rate constants virtually identical to normal myosin.ATP crossbridges. Unlike with myosin.ATP crossbridges, we were able to examine the calcium sensitivity of these artificially created crossbridges without concern about the effects of ATP hydrolysis. As postulated for myosin.ATP crossbridges based upon solution studies, we found that the binding strength and rate constants of the pPDM- and NPM-crossbridges in fibers are relatively insensitive to changes in calcium over the physiological range.

In smooth muscle we examined the molecular mechanism of action of two compounds which stimulate adenylate cyclase and are known to relax smooth muscle, vasoactive intestinal peptide (VIP) and isoproterenol. Results to date show that VIP, when it causes relaxation, also causes a reduction in the percentage phosphorylation of the 20 kDalton smooth muscle myosin light chain. In vitro, phosphorylation of myosin light chain kinase, the enzyme that phosphorylates the myosin light chains, reduces its activity. We are currently examining whether a similar mechanism of regulation might function in fibers. To date we have labeled the ATP pool of small samples of smooth muscle, isolated the kinase through immunoprecipitation, and have found that samples relaxed with VIP have increased phosphate incorporation. We are currently examining the site of this increased phosphorylation to see if it is the same as the site phosphorylated in vitro.

OBJECTIVES: To understand the molecular basis of muscle contraction.

METHODS AND MAJOR FINDINGS:

I) Regulation of skeletal muscle: Properties of weakly-binding crossbridges (Barnett)

A decade ago we reported the discovery of weakly-binding myosin·ATP crossbridges in normal relaxed muscle fibers and two years ago we reported two ways of artificially creating, in muscle fibers, ATP-independent weakly-binding crossbridges. In FY90 we compared the kinetic properties of these artificially created weakly-binding crossbridges with the physiologically occurring ones. We were surprised to learn that weakly-binding crossbridges created either with para-phenylenedimaleimide (pPDM) or with N-phenyl maleimide (NPM) treatment have binding strength and attachment and detachment rate constants virtually identical to normal myosin·ATP crossbridges. Because of this very close similarity between pPDM- or NPM-treated crossbridges and normal relaxed crossbridges, we decided to use the artificially created weakly-binding crossbridges as a model for the normally-occurring weakly-binding bridges, and to do experiments extremely difficult with the normally-occurring myosin·ATP crossbridge. This approach is similar to that of Drs. Jody Dantzig and Yale Goldman who used the ATP-γ-S crossbridge as a model for the normal weakly-binding crossbridge. Our approach is significantly better than theirs because the ATP-γ-S crossbridge, unlike the pPDM- and NPM-crossbridge, has kinetics that differ substantially from those of the normal weakly-binding crossbridge.

If one attempts to examine the Ca^{2+}-sensitivity of the normal myosin·ATP crossbridges, release of phosphate is accelerated and one is left with strongly-binding myosin·ADP crossbridges. To avoid this problem and still learn about the Ca^{2+}-sensitivity of weakly-binding crossbridges, in FY90 we examined the Ca^{2+}-sensitivity of the weakly-binding crossbridges created with pPDM and NPM treatment. We found that the rate constants and binding constants of the weakly-binding pPDM- and NPM-crossbridges were insensitive to changes in $[Ca^{2+}]$ over the physiological range. This is an important result with regard to the mechanism of muscle regulation in fibers because it provides additional suggestive evidence that the regulation of muscle activity resembles the regulation of the actomyosin ATPase in solution and not the mechanism postulated by the classic steric blocking model of muscle relaxation. That model states that muscle regulation is due to the effect of Ca^{2+} on the binding strength of weakly-binding myosin.

II) Regulation of smooth muscle (Moussavi, Qian)

In FY90 we continued to develop techniques required for examining the biochemical changes in small tissue samples. Using Western blotting with an ^{125}I-conjugated second antibody, we have developed an extremely sensitive radio-assay for detecting the percentage of 20-kDalton myosin light chain phosphorylation in very small preparations of tracheal smooth muscle. We are examining the mechanism of action of two muscle relaxants known to stimulate adenylic cyclase, vasoactive intestinal peptide (VIP) and isoproterenol. In experiments recently concluded we have documented that VIP, at the same time it causes tracheal smooth muscle relaxation, also causes a decrease in the

percentage of myosin light chain phosphorylation in fibers stimulated with substance P. Although experiments with isoproterenol are still ongoing, we are carrying the project a step further by examining how VIP regulates the amount of myosin light chain phosphorylation.

In vitro it has been shown that myosin light chain kinase, the main enzyme responsible for phosphorylating myosin light chain, is itself a phosphorylatable protein. Additionally, phosphorylation of myosin light chain kinase in vitro reduces its activity. To see if such a regulatory mechanism might be functioning in intact smooth muscle, we worked out a procedure for isolating the majority of myosin light chain kinase from small specimens of smooth muscle tissue. The procedure involved imunoprecipitation and isolated more the 90% of the cells' myosin light chain kinase. By stimulating the smooth muscle after its ATP pool had been equilibrated with ^{32}P, we were able to show that the kinase is indeed phosphorylated. Experiments are currently underway to digest the kinase with trypsin, do 2-D peptide mapping, and examine whether the site phosphorylated is the same as the one shown to regulate myosin light chain kinase in vitro. This work is done in close collaboration with Drs. Robert Adelstein and James Sellers of the Laboratory of Molecular Cardiology, NHLBI.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Experiments on the mechanism of muscular contraction are important in that they contribute to a sound understanding of how normal muscle works. This, in turn, is useful in understanding diseased muscle. Furthermore, the high degree of organization of the contractile proteins in skeletal muscle makes this a particularly useful system for elucidating contractile mechanisms, some of which may be common to the cellular and subcellular motions of other, less easily studied, cells. Understanding the regulation of smooth muscle of course, likely holds the answer to any number of important questions, one of them being the etiology of essential hypertension.

FUTURE COURSE: We hope to continue to study the behavior of weakly-binding crossbridges by designing experiments that exploit our ability to make ATP-independent weakly-binding crossbridges. Additionally, while we were attempting to produce weakly-binding crossbridges with pPDM, we discovered that pPDM, in addition to reacting with myosin, also reacts with a higher molecular weight protein in the fiber. We hope to follow this up, exploring whether this protein is titan, and also whether crosslinking of titan may underly the mechanism by which pPDM treatment causes an increase in fiber resting tension. Finally, we hope to continue to investigate the way in which VIP lowers myosin light chain phosphorylation when it causes smooth muscle relaxation.

PUBLICATIONS:

Corson MA, Sellers JR, Adelstein RA, Schoenberg M. Substance P contracts bovine tracheal smooth muscle via activation of myosin light chain kinase. Am J Physiol 1990; in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 AR 27003-31 LPB

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Biophysical Studies of Metabolic Activity and Control

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Ellis S. Kempner, Ph.D., Physicist and Chief,
Section on Macromolecular Biophysics LPB NIAMS
Jay Miller, Chemist

COOPERATING UNITS *(if any)*
Drs. C Steer (Univ. Minnesota), JC Osborne Jr (Beckman), B Stevens (Univ. Florida), E Wright (UCLA), J. Billheimer (DuPont)

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SECTION
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NIAMS, NIH, Bethesda Maryland 20892

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Target analysis of the inactivation of biological activity by ionizing radiation was applied to several major problems including the avian lectin receptor, the sodium/glucose cotransporter, and the cholesterol regulatory enzyme ACAT.

Objectives: 1) An understanding of the nature of active structures in vivo which are involved in biochemical processes, principally by means of the technique of inactivation by ionizing radiation.
2) Detailed knowledge of the molecular damage caused by ionizing radiation and of the mechanisms of the transfer of radiation energy throughout these structures.

Methods: 1) General biochemical techniques including enzyme reactions, fluorescence, and gel electrophoresis.
2) Ionizing radiation, usually high energy electrons from a linear accelerator, to expose samples under carefully controlled conditions.

Major findings:

The intestinal brush border membrane sodium/glucose cotransporter is the best known eukaryotic system demonstrating ion-coupled transport, but structure-function relationships of the cotransporter in the native membrane have been lacking. Using radiation inactivation it was shown that the cotransporter functions in the membrane as a 290 kDa homotetramer comprised of four independently-inactivated 73 kDa subunits. These subunits work in concert and an individual subunit's activity can only be manifested by the interaction of all the other components. At the extracellular surface sodium binds to the cotransporter, which results in a conformational change that permits glucose to bind to the glucose-binding site. With both species bound, the cotransporter undergoes a new conformational shift, placing sodium and glucose near the inner surface of the membrane. Sodium and glucose are then released to the cytoplasm, thereby triggering a cotransporter conformational shift to expose the binding sites once again to the extracellular surface of the membrane. The cotransporter thus again assumes the optimal conformation for binding extracellular sodium and glucose.

In mammalian liver, carbohydrate-specific receptors (composed of two or three polypeptide species) recognize and clear circulating glycoproteins. Avian liver does not recognize asialoglycoproteins, but does contain a receptor capable of binding N-acetylglucosamine-terminated glycoproteins. This receptor (composed of only one polypeptide species) shows remarkable amino acid sequence homology to the mammalian asialoglycoprotein receptor. Radiation inactivation revealed that the receptor in the avian plasma membrane functions as a trimer. Sedimentation equilibrium analysis shows that on extraction into detergent, the avian purified receptor forms a heterogeneous population of irreversible oligomers that exhibit binding activity proportional to size. This state of aggregation, as with the asialoglycoprotein receptor, may be indicative of membrane events that are involved in ligand internalization.

Cholesterol is required by all mammalian cells for the proper functioning of cellular membranes. Most cellular cholesterol is found esterified to long-chain fatty acids. The esterification is catalyzed by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT), one of a family of acyl-CoA acyltranferases which involve other acceptors such as retinol and carnitine. Because of its central role in cellular cholesterol metabolism, there has been great interest in the properties and regulation of ACAT but it has not been successfully purified from the endoplasmic reticulum. Clearly, the ACAT enzyme must have separate binding sites for cholesterol and for acetyl-CoA. Radiation inactivation of rat microsomes revealed a target size of 180 kDa. A related microsomal enzyme, Acetyl-CoA hydrolase (ACH) revealed a complex inactivation curve resembling those due to an enzyme-inhibitor complex, and leading to an enzyme target size determination of 45 kDa. Estimates of the size of the inhibitor are in the range of 100-200 kDa. It was proposed that the ACAT enzyme was composed of two

independently-inactivated regions, one of which binds cholesterol. The other region binds acetyl-CoA, and either transfers the acetyl group to cholesterol, or (in the absence of cholesterol) hydrolyzes the acetyl-CoA. The cholesterol-binding region of ACAT in effect becomes an inhibitor for the hydrolase activity. This model predicts that the hydrolase and acetyltransferase activities will depend on cholesterol concentration, and the increase in ACAT activity will be mirrored by an equal decrease in ACH activity. These predictions were confirmed experimentally. Conversely to the inhibitory action of cholesterol, the hydrolase activity can be considered a regulator for the ACAT function. Such regulation may be a general phenomenon which applies to other members of the acetyl-CoA transferase family and would be revealed by the presence of inherent ACH activity which is depressed by the other acceptors.

Future course:

Two lines of research will be continued. Fundamental studies of radiation include a)the nature of energy transfer between and along polymers; b)examination of radiolytic fragments from proteins of known structure; and c)examination of conformational changes in irradiated macromolecules. Applications of target analysis to important biochemical problems includes soluble glutathione S-transferases, ferrochelatase, and phospholipases.

Publications:

Bolger GT, Skolnick P, Kempner ES. Radiation inactivation reveals discrete cation binding sites that modulate dihydropyridine binding sites. Mol Pharmacol 1989;36:327-332.

Ozasa S, Kempner ES, Erickson SK. Functional size of acyl coenzyme a:diacylglycerol acyltransferase by radiation inactivation. J Lipid Res 1989;30:1759-1762.

Stevens BR, Fernandez A, Hirayama B, Wright EM, Kempner ES. Intestinal Brush Border Membrane Na^+,Glucose Cotransporter Functions in situ as a Homotetramer. Proc Nat'l Acad Sci USA 1990; 87:1456-1460.

Steer CJ, Osborne JC Jr, Kempner ES. Functional and Physical Molecular Size of the Chicken Hepatic Lectin Determined by Radiation Inactivation and Sedimentation Equilibrium Analysis. Jour Biol Chem 1990;265:3744-3749.

Billheimer JT, Cromley DA, Kempner ES. The Functional Size of Acyl-CoA:Cholesterol Acyltransferase and Acyl-CoA Hydrolase as Determined by Radiation Inactivation. Jour Biol Chem 1990;265:8632-8635.

Kempner ES, Osborne JC Jr, Reynolds L, Deems R, Dennis EA. Analysis of Lipases by Radiation Inactivation. Meth Enzymol (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 27004-21 LPB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

The Dynamic Properties of Cell Membranes and Related Systems

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Norman L. Gershfeld, Ph.D. Research Chemist LPB,NIAMS
Lionel Ginsberg, Ph.D., M.D. Visiting Scientist
William F. Stevens, Jr. Biological Lab Technician

COOPERATING UNITS *(if any)*

Cooperating Units
Dr. Robert Berger, LBC, NHLBI

LAB/BRANCH

Laboratory of Physical Biology

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Cell membrane bilayers have been reconstructed in vitro utilizing total lipid extracts from normal human neural tissue. In agreement with the critical state theory of membrane bilayer assembly (see previous annual report) these lipid extracts spontaneously formed purely unilamellar structures in aqueous dispersion, but only at a critical temperature, T*, equal to the physiological temperature of the tissue; for normal human myelin lipids T* was measured as 37±1°C. The critical state theory has also been applied to two demyelinating diseases to test whether the theory can distinguish among several possible mechanisms for the pathological condition. T* for the total lipid extracts of myelin from a patient with metachromatic leukodystrophy (MLD) was below 30°C, indicating that myelin lipid composition was inappropriate for normal bilayer assembly at the core temperature of this patient. Under these conditions, the critical state theory for bilayer stability predicts that a bilayer will spontaneously degenerate. This mechanism provides a direct link between the metabolic disorder---a defect in arylsulfatase activity that leads to accumulation of cerebroside sulfate in myelin---and the formation of pathological myelin. In contrast, total lipid extracts from pathological myelin in a patient with multiple sclerosis, currently believed to be an immune-mediated mechanism and not due to a lipid disturbance, gave a normal value for T* equal to 37°C. Thus, the critical bilayer theory provides a method for distinguishing when demyelination is caused by a lipid defect. Application of this theory and measurement of T* for detecting other lipid defect-dependent neurological disorders is currently in progress.

Objectives:

The principal goals of this project are to describe the processes of membrane assembly, and to identify the conditions which lead to breakdown of the normal membrane structure. Recent observations indicate that the assembly process for the membrane lipid bilayer is a critical phenomenon in which the ambient temperature of the cell (its physiological temperature) is a critical point; associated with each critical bilayer assembly temperature is a lipid composition that is characteristic of the cell and, like the temperature, exhibits critical properties. Current objectives have focussed on extending the concept that the most stable membrane is in the critical bilayer state, and on assessing the physiological consequences when the critical conditions of temperature or lipid composition for bilayer stability are violated.

Methods:

Substantial progress has been made in the measurement of the critical temperature and membrane lipid composition. Development of quantitative methods for total analysis of membrane lipids has been partially achieved. HPLC methods have been used to separate membrane phospholipids of brain tissue. Among the phospholipids the diglyceride composition of the phosphatidylethanolamine (PE) and phosphatidylcholine (PC) classes have been quantitated by conversion of the phospholipid to the dinitrobenzoyl diglyceride. The method entails enzymatic conversion of the phospholipid to the diglyceride using phospholipase C from B. cereus, followed by reaction with dinitrobenzoyl chloride in pyridine. For each phospholipid class upwards of 30 distinct diglycerides, each with a unique pair of fatty acids, has been identified and quantitated by HPLC.

The thermodynamic methods previously reported for measuring critical bilayer assembly temperatures T* have now been supplemented by a direct calorimetric method. The heat capacity of aqueous dispersions of phospholipids have been measured as a function of temperature with a newly developed calorimeter; as predicted, an anomoly in the heat capacity occurs at T*. A patent application for the calorimeter design is currently in preparation.

Major findings:

A) Calorimetry of critical bilayer assembly

The critical bilayer assembly temperature T*, in principle, may be found for aqueous phospholipid dispersions at the terminus of a second-order phase transition. This assertion is based on a thermodynamic analysis of the equilibrium surface film properties of phospholipid dispersions. While this analysis is rigorous, and provides a general approach for obtaining T*, it is laborious and time-consuming. Using a newly designed, quasi-adiabatic calorimeter, in which the heat capacity is measured as a function of temperature, T* for dispersions of dimyristoylphosphatidyl-choline (DMPC) have been measured. Previous studies, using surface chemical methods, gave T* for this lipid as 29°C compared to 28.9±0.01°C obtained with the quasi-adiabatic calorimeter. The calorimeter measurement has several notable advantages: it provides a precise measure of the heat of bilayer assembly and the critical temperature, and it allows T* to be measured within several days

instead of the normal period of weeks required by the previous technique.

B) Membrane bilayer instability and pathogenesis of myelin disorders

According to the critical state theory of bilayer assembly both the ambient temperature and the lipid composition of the membrane are critical conditions for membrane stability. If the critical conditions are no longer met the theory predicts that membrane bilayers will degenerate with potentially lethal consequences for the cell. This mechanism for membrane degeneration has been found to be relevant for assessing demyelination in patients with metachromatic leukodystrophy (MLD); patients with MLD exhibit a deficiency of arylsulfatase A which is manifested in the accumulation of cerebroside sulfate in myelin. We have measured T* for total lipid extracts from normal neural tissues and for myelin lipids from a patient with MLD; T* for the normal myelin was 37°C, while for the MLD myelin it was 30°C. This change in T* from the normal physiological temperature can be due only to a defect in the lipid composition, since the lipid preparations are protein-free. Under these conditions the critical state theory predicts that an accumulation of cerebroside sulfate in excess of the normal amount in myelin would lead to a lowering of T* and concomitantly demyelination. We have also examined the myelin lipids for another demyelinating disorder, multiple sclerosis (MS), where the process is believed to occur by immune-mediated mechanisms and not by a membrane lipid defect. The critical bilayer temperature for these lipids is 37°C, consistent with the general belief that MS demyelination occurs by a non-lipid dependent mechanism.

Publications:

Gershfeld NL. Spontaneous assembly of a phospholipid bilayer as a critical phenomenon: influence of temperature, composition, and physical state. J Phys Chem 1989;93:5256-5261.

Gershfeld NL. The critical unilamellar lipid state; a perspective for membrane bilayer assembly. Biochim Biophys Acta 1989;988:335-350.

Ginsberg L, Gilbert DL, Gershfeld NL. Membrane bilayer assembly in neural tissue of rat and squid as a critical phenomenon: influence of temperature and membrane proteins. J Membrane Biology (in press).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 27005-8 LPB

PERIOD COVERED

October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Aggregation of Human Platelets Induced by Decompression

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Makio Murayama Research Chemist LPB, NIAMS

COOPERATING UNITS *(if any)*

Dr. K. K. Kumaroo, Biochemist, U.S. Naval Research Institute, Bethesda, MD

LAB/BRANCH

Laboratory of Physical Biology

SECTION

Section on Macromolecular Biophysics

INSTITUTE AND LOCATION

NIAMS, NIH Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The main thrust of the laboratory is focussed on the influence of pressure on the blood coagulation system, including both hemostasis and thrombosis. The molecular mechanism of platelet interactions in DIPA (decompression-inducible platelet aggregation) has been investigated for the past several years and is still continuing. We have found that the molecular mechanism of DIPA can be explained by the interactions of oppositely charged amino acid residues, i.e., positively charged arginyl residues interact with negatively charged aspartyl residues. We found this to be the molecular mechanism in AMS (acute mountain sickness); vascular occlusion has been observed in the small blood vessels in the web of the frog's foot and in the ear of the mouse. Photomicrographs were made showing platelets adhering to each other and to the blood vessel wall. We found that AMS can be prevented in the frog andin the mouse by the drug piracetam, administered by stomach tube prior to simulated ascent. At the beginning of the project I had assumed that DIPA is accompanied by a volume increase and that there is a neutralization of positive and negative charges. The measurement of this increment is now being determined by using a specially constructed volumetric flask. The experimental findings of the past year confirmed our hypothesis that AMS is a vascular occlusive disease.

Major Findings:
During the past year we found that the mechanism of DIPA was due to self-adhesion (aggregation) of platelets and their adhesion to the blood vessel wall in the small blood vessels in the web of the frog's foot and we observed similar stoppage of blood flow in the ear of the mouse. We found that vascular occlusion could be prevented in the mouse as well as in the frog by the oral administration of the drug piracetam. The experimental findings confirmed our hypothesis that AMS is a vascular occlusive disease We found that the volume increase of PRP in DIPA is approximately 2.5 percent (v/v), i.e., 25 cc per liter of packed human platelets.

Project Description:
Objectives: The main objectives are to investigate the molecular mechanism underlying spontaneous aggregation of human platelets induced by decompression, DIPA (acronym for decompression-inducible platelet aggregation) and the molecular mechanisms to prevent and/or inhibit DIPA. DIPA is fibrinogen-dependent; any mechanism which can block fibrin(ogen) aggregation could also block the initial stages of human platelet aggregation. This can be done by neutral salts and also (at a lower concentration) by a molecule having a large electric dipole moment such as the drug piracetam, which blocks fibrin polymerization and inhibits DIPA. Other investigators have shown that the amino acid residues involved in the specific recognition site on the platelet membrane and in the binding site of fibrin (ogen) is the peptide sequence Arg.Gly.Asp.Ser (R.G.D.S) We showed that R.G.D.S causes 50% inhibition of DIPA at about 3 mM. Atomic scale models suggest that R.G.D.S is a sufficient and necessary sequence for the interaction of juxtaposed counter-charged amino acid residues during DIPA. therefore, it seems reasonable to assume that under compression (positive pressure), the dimer of R.G.D.S would dissociate, thereby decreasing the volume of the solution. There is a second mechanism in the volume decrease of the solution: the breaking of hydrogen bonds.

Methods Employed:
Platelet aggregation was investigated by microscopic examination, by the change in light absorbance at 380 nm of PRP (platelet-rich plasma), and also by the nephelometric method of Born. Further data were obtained at the U.S. Naval Med. Res. Inst. where a Chronolog Aggregometer was available. Currently we are using 32P as orthophosphate in PRP concentrate. The influence of pressure on phosphorylation is being studied by conventional SDS-PAGE and autoradiography of the dried gel slab. In order to investigate DIPA in vivo, mice were decompressed at 253 torr (pressure at the top of Mt. Everest). The mice were removed from the chamber and the microcirculation in the ear was observed microscopically. Clumps of platelets appeared to be lodged in the very small blood vessels, obstructing the flow of blood.

Significance to Biomedical Research and the Program of the Institute:

The spontaneous aggregation of platelets appears to be important in understanding how some other cells are aggregated. Platelets are cytoplasmic fragments of megakaryocytes whose only known function is to form the hemostatic plug. While normally helpful, there are not events in which spontaneous aggregation of platelets is not desirable: platelet aggregation due to reduced barometric pressure causes AMS (acute mountain sickness); also, platelet aggregation is induced by decompression in surfacing after deep sea diving (this problem is further complicated by the "bends"). Understanding the basic mechanism of DIPA should be of value in finding means to prevent aggregation of platelets. The mechanism of recompression of divers who suffer acute decompression sickness after deep sea diving can be investigated by in vitro methods. Compression can reverse and/or inhibit platelet aggregation. Cardiovascular problems appear to arise from DIPA. This appears to take place because of the paradoxical Bernoulli principle: where the velocity of the blood is high (as in arteriostenosis), pressure is low, thus the decompression. We also hypothesize that there is contact activation of platelets due to shear (there is a pressure gradient from the outer wall of the artery to its core). Relatively low hydrodynamic decompression, super imposed upon contact activation, can occur concomitantly at a bifurcation of an artery and can cause massive platelet aggregation as in a heart attack. We will continue to investigate the molecular mechanism of DIPA and ways of preventing undesirable platelet aggregation.

Proposed Course of Project:

It is important to confirm the basic assumption of DIPA: there is a volume increase of human PRP during DIPA. It was assumed that there is a volume increase when electrically charged groups interact, thus neutralizing the opposite charges. This produces concomitant relaxation of electrostriction, accompanied by randomization of the organized water molecules surrounding the charged residues. In this stage of our experiments, we will measure the volume increase during DIPA.

Publications:

Murayama M. DIPA (decompression-inducible platelet aggregation) and hemostasis: vascular occlusion and its prevention, in the web of the frog's foot. Thrombosis Res 1989;54:493-8.

Murayama M. AMS (acute mountain sickness), a vascular occlusive disease. Medical Hypothesis 1990; 31:189-95.

Murayama M. Decompression-induced hemostasis in mice: High altitude simulation in the Everest Chamber. Thrombosis Res 1990;57:813-6.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 27012-06 LPB

PERIOD COVERED
October 1, 1989 through September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structural and Mechanical Properties of Muscle Fibers

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Leepo C. Yu, Research Physicisit, LPB, NIAMS

Sengen Xu, Visiting Associate, LPB, NIAMS

COOPERATING UNITS *(if any)*
University of Ulm, FRG (Drs. B. Brenner and T. Kraft); East Carolina University Medical School (Dr. J. Chalovich); National Institute of Science and Technology (Dr. E. Prince).

LAB/BRANCH
Laboratory of Physical Biology

SECTION
Section on Muscle Biophysics

INSTITUTE AND LOCATION
NIAMS, NIH, Bethesda, Maryland

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.75	1.75	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither

☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have continued investigating mechanism of muscle contraction by correlating structural, mechanical and biochemical techniques applied to muscle fibers. In FY 1990, the following results are obtained:

(A) Precursors to force generation: It has been proposed that during muscle contraction, crossbridges cycle between weak- and strong-binding states, and that force generation occurs as the result of a transition from the weak to the strong binding states. We have now shown that if the attachment of crossbridges to actin is blocked in the weak binding states, active force level is inhibited. The results show that within the cyclic interactions of crossbridge with actin, the crossbridges are required first to be bound to actin in the weakly bound states before generating active force, i.e. force-generating states are accessible only from attached weak-binding precursors.

(B) Effect of calcium on the affinity of crossbridges to actin: According to the steric blocking model of calcium regulation in muscle, the affinity of crossbridges to actin is greatly affected by calcium. However, we have shown that in the presence of ATPgS, an ATP analog, calcium does not affect the affinity of crossbridge to actin. Rather, the kinetics of binding is affected. This finding is inconsistent with the steric blocking model.

(C) Stiffness of the crossbridges in the radial direction: One mechanical property of crossbridges, i.e. stiffness, in the direction perpendicular to the fiber axis is shown to depend on the state of crossbridges. The differences in radial stiffness probably reflects structure differences in the binding of crossbridges to actin. This finding also raises the question whether axial stiffness is the same for all crossbridge states, which is commonly assumed.

Project Description:

To understand the acto-myosin (crossbridge) interactions in muscle by studying the structural and mechanical properties of the crossbridges during various phases of contraction.

Methods Employed:

Low angle equatorial X-ray diffraction from single demembranated muscle cells under various interventions are obtained to study the structure of contractile proteins and their interactions during such interventions. These structural studies are correlated with mechanical properties of the muscle, such as force, velocity and stiffness, under the same conditions, so that the structural information obtained from diffraction studies are related to functions of muscle.

Major Findings:

(1) It has been proposed that the actomyosin ATPase cycles (crossbridge cycles) include several energetically distinct states. The cycle can be divided into two groups of states characterized by the strength of binding between actin and myosin - the weak binding states and the strong binding states. Force generation probably involves a transition from the weak to the strong binding states. There have been experimental evidence for the existence of crossbridges bound to actin in the weak binding states both in the relaxed and activated muscle fibers. However, the physiological significance of the weak binding states has been a subject of debate. We now have evidence that crossbridges bound to actin in the weak binding states are essential precursors to force generation.

Caldesmon, a regulatory protein found in smooth muscle, specifically inhibits binding of crossbridges in the weak binding states. When caldesmon is added to skinned striated muscle fibers, both X-ray diffraction and mechanical measurements indicate that the number of crossbridges bound to actin in the weak binding states decreases as the concentration of caldesmon increases. Accompanying the inhibition, the active force level generated by the muscle is found to decrease. This is the first evidence showing that the weak binding states are essential intermediates for force generation in vertebrate muscle.

(2) The role of calcium in contraction regulation was investigated. Until recently, a generally accepted model for calcium activation, the steric blocking model, states that in the relaxed state the crossbridges are blocked sterically from interacting with actin and calcium removes the blocking upon activation. In this hypothesis, calcium should greatly affect the affinity of crossbridge to actin. One of the difficulties in directly testing the role of calcium in muscle regulation is that activation renders binding studies technically difficult. We have used (in collaboration with B. Brenner) equatorial X-ray diffraction and mechanical measurements to study the effects of calcium on the actin affinity of crossbridges in the presence of an ATP analog, ATPgS. Crossbridges in the presence of ATPgS but in the absence of calcium exhibited identical binding characteristics as observed in relaxed muscle. It is found that calcium in the presence of ATPgs only slightly increases the actin affinity, whereas the kinetics of crossbridge binding are significantly affected. The findings are contrary to the predictions of the steric blocking model. Hence steric blocking is not likely the mechanism of muscle regulation.

(3) Elasticity of the crossbridges, for lack of contrary evidence, has been assumed to be the same for all crossbridge states. Mechanical measurements of axial stiffness cannot determine if this assumption is correct. We have shown that crossbridges exhibit elastic properties not only in the axial direction but also in the radial direction. We have further evidence that the radial elastic property of the crossbridge depends on the physiological state of the muscle and is affected by the attached ligand on myosin. Nucleotides and analogs such as ATPgS, PP_i, and ADP changed the radial elasticity of the crossbridges rather significantly. Such changes are not due to changing the number of crossbridges attached to the actin. Thus the assumption that the axial elasticity being the same may not be valid. Furthermore, the elastic property in the radial direction can be used as an effective indicator of conformational differences of crossbridge attachment in various states.

(4) Determining the phases of the equatorial X-ray diffraction reflections has been part of our continuing interest. In recording X-ray diffraction patterns, phase information is lost, but it is necessary for unambiguous reconstruction of electron density maps. In collaboration with Dr. Edward Prince we have initiated a systematic studying of phase selection based on the algorithm of maximum entropy, i.e. the distribution of electron density in a unit cell follows the principle of lowest contrast between lattice points. Following this algorithm, we have obtained a density map based on experimental data from relaxed muscle with 12 equatorial reflections at 75 Å spatial resolution. However, it is not yet clear whether maximum entropy is a valid principle in determining mass distribution in a well ordered system such as muscle. Further experimental evidence is needed to support the conclusion based on applying such an algorithm.

(5) At the suggestion of Scientific Counsellors (1989), a working two dimensional X-ray diffraction camera for muscle preparations has recently been set up to study the conformation of acto-myosin interaction along the contractile filaments. Although an imaging plate detector system has yet to be installed, we have already made observations on rigor muscles that have not been previously reported.

Future Plans:

(1) By introducing various interventions into muscle fibers, contraction mechanism will continue to be studied by combining equatorial X-ray diffraction, which is sensitive to crossbridge binding to actin, with mechanical studies, which is sensitive to kinetics of crossbridge interaction with actin.

(2) A leading edge X-ray recording technology, an imaging plate, will be added to the two dimensional camera in FY 1991, completing the experimental set up. We will combine the two dimensional diffraction data with quantitative modelling to obtain more detailed knowledge of crossbridge conformations in muscle cells.

Publications reported in press:

Yu LC, Podolsky RJ. Equatorial X-ray diffraction studies from single skinned muscle fibers. In: J. Squire, ed. Molecular Mechanisms in Muscular Contraction. London: Macmillan, 1989; 265-286.

ANNUAL REPORT

LABORATORY OF STRUCTURAL BIOLOGY RESEARCH

NATIONAL INSTITUTE OF ARTHRITIS, MUSCULOSKELETAL AND SKIN DISEASES

APRIL 1, 1990 THRU SEPTEMBER 30, 1990

Structure, Assembly, and Function of Biological Macromolecules

The Laboratory of Structural Biology Research formally came into being in April 1990. However, since this group had been functioning for many previous years as an independent research unit - the Section for Structural Biology in the Laboratory of Physical Biology - its activities are reviewed for the year as a whole, i.e. from October 1, 1989 thru September 30, 1990. Over the past year, several major studies involving viral proteins or viral assembly phenomena have been completed. The conformational change that accompanies maturation of the bacteriophage T4 capsid has been demonstrated to involve a substantial change in secondary structure, thus requiring that its major capsid protein must be at least partly re-folded. Accordingly, this conformational change is a much more radical event than the transitions previously characterized in such contexts as ligand-binding by allosteric enzymes. Cryo-electron microscopy and three-dimensional image reconstruction techniques have been used to define the molecular topography of the herpesvirus capsid, and the structure of the encapsidated DNA. Taken together, these observations indicate that, in certain major aspects, herpesvirus capsid assembly resembles the assembly pathways of the DNA phages. The oligomeric status, molecular weights, and carbohydrate contents of the surface glycoproteins gp120/gp160 of Human Immunodeficiency Virus, Type 1 have been determined from analysis of scanning transmission electron micrographs. In particular, these data appear to settle the oligomer issue, which has been controversial in the aftermath of earlier studies using conventional experimental approaches. Furthermore, progress has been in characterizing the protein channels that permeabilize the outer mitochondrial membrane; and in further developing the Laboratory's resources for high-resolution electron microscopy of frozen, hydrated specimens and digital image processing.

Conformational Basis of Phage T4 Capsid Maturation.

The T4 capsid is first assembled as a relatively fragile, DNA-free, particle, into which DNA is subsequently packaged. After packaging, the mature capsid is a considerably larger (18%) and much more resistant structure. The basis of this transition is a concerted conformational change undergone by the ~ 1000 molecules of gp23 that are arrayed in its icosahedral surface lattice. We have used laser Raman spectroscopy to measure the secondary structure contents of gp23 in both the precusor and the mature states, and have found that the α-helix content drops from 33% to 21%, whereas the β-sheet content increases from 34% to 46%. Two extreme scenarios may be considered, depending on whether or not the N-

terminal delta-domain of gp23 which is proteolytically removed upon maturation is primarily responsbile for the observed effects. In either case, the remaining molecule (gp23*) must undergo a significant conformational change, whereby at least 30, and possibly as many as 80, of its 456 residues switch from an α-helical to a β-sheet conformation (Steven, Greenstone, Bauer, Williams).

Herpesvirus Capsid Assembly.
The capsid of herpesvirus is first assembled as a DNA-free precursor particle in the cell nucleus. Subsequently, DNA is packaged, and the viral envelope acquired from the nuclear membrane. We have examined the structure of the capsid and its contents by cryo-electron microscopy and three-dimensional image processing of various populations of capsids purified by density gradient centrifugation. The hexon capsomers are hexamers of the 150 kDa major capsid protein, and form a 15nm thick shell, that is completely transversed by axial channels at the centers of each capsomer. On the outer surface, at the three-fold positions between hexons or between hexons and pentons, are density features ("triplexes") which we infer to be trimers of a minor capsid protein. Such proteins are present on phage capsids where they are known to play a stabilizing role. The DNA in fully packaged capsids is arranged in locally ordered, parallel, "liquid-crystalline" bundles of heteroduplexes with an average center-to-center spacing of 2.6nm. This configuration of packaged DNA closely resembles that previously visualized in phages T4 and lambda (Booy, Trus, Newcomb, Brown, Baker, Steven).

Supramolecular Structure of the HIV-1 Glycoprotein, gp120/gp160.
The surface glycoproteins of human immunodeficiency virus play important roles in the etiology of AIDS. Ideally one would like to have a high-resolution three-dimensional model of these molecules on which to base rational drug design and immunochemical strategies. However, although amino-acid sequences have been determined for many strains of HIV by cloning methods, very little information on three-dimensional molecular structure has been forthcoming. We have analyzed scanning transmission electron micrographs of gp120 (the mature ectodomain) and gp160 (the precursor molecule) purified from a recombinant vaccinia expression system. We find that gp160 is a dimer of 125 kDa subunits, each of which contains 91 kDa of protein and, on average, 34 kDa of carbohydrates. Thus, the nominal monomer molecular weight of 160 kDa is a substantial overestimate. Gp120 is a monomer containing 56 kDa of protein and 34 kDa of carbohydrates. Accordingly, gp41, the membrane-associated component has 35 kDa of protein (in close agrement with the sequence prediction) and, at most, vestigial amounts of carbohydrates. The gp160 dimer is an asymmetric dumbell, whose major domain is a dimer of gp120, and whose minor domain is a gp41 dimer (D Thomas, Trus, Kaczorek, Booy, Wall, Hainfeld, Steven).

Mitochondrial Outer Membrane Channel.
The outer mitochondrial membrane contains an intrinsic protein that forms voltage-dependent, anion-selective channels (VDAC). Earlier studies by others have shown that treatment of VDAC-enriched membrane fragements with phospholipase A2 induces the formation of crystalline patches. These crystals are suitable for structural studies to complement electrophysiological measurements of the protein's channel-forming activity in black lipid films. We have investigated freeze-dried crystals by electron microscopy, both with and

without platinum shadowing. Topographic reconstructions of shadowed crystals show that their two surfaces are very similar. Mass density measurements performed on unstained crystals indicate that the unit cell contains 32% lipid and 68% protein in the form of 6 copies of the 30 kDa VDAC subunit. Thus there is a one-to-one ratio between subunits, and the 6 channels per unit cell visualized in filtered electron micrographs, i.e. one monomer per channel. Taken together, these data indicate that little of the protein protrudes beyond the surface defined by the lipid bilayer, and that most of the protein is recessed relative to this plane, where it is involved in forming the walls of the ~2.5nm diameter channel (L Thomas, Colombini, Kocsis, Trus, Steven).

Mapping Epidermal Proteins In Situ by ImmunoLabelling.
We have followed up our earlier work on mapping the biosynthetic pathways of loricrin and filaggrin, two major proteins expressed by terminally differentiated epidermal keratinocytes. Over the past year, we have focussed on reconciling somewhat divergent results obtained with two different embedding media. With Lowicryl, we tended to achieve superior morphological preservation, but to encounter relatively high levels of non-specific binding of colloidal gold particles in the transitional layers between the granular layer and the cornified layers of cells. With LR white, on the other hand, the morphological preservation has been somewhat less good, but with much lower backgrounds of non-specific binding. We have now conducted a comprehensive quantitation of the densities of the gold particles present in the various compartments (cytoplasm, inter-cellular space; keratohyalin granules that contain loricrin (L-granules) or which contain profilaggrin (F-granules), etc). After subtraction of the resulting local background values, the labelling densities for the various compartments were equivalent for both embeddings, thus firmly substantiating our conclusions concerning the respective biosynthetic pathways of loricrin and filaggrin (Bisher, Roop, Steinert, Steven).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 27002-12 LSBR

PERIOD COVERED

OCTOBER 1, 1989 THROUGH SEPTEMBER 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Macromolecular Structure

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI: Alasdair C. Steven, Laboratory Chief, LSBR, NIAMS
Margaret E. Bisher, Microbiologist LSBR, NIAMS
Frank P. Booy, Visiting Scientist LSBR, NIAMS
James F. Conway, Visiting Fellow LSBR, NIAMS
Heather L. Greenstone, Biologist LSBR, NIAMS
Eva Kocsis, Visiting Fellow LSBR, NIAMS
Benes L. Trus, Guest Worker LSBR, NIAMS

COOPERATING UNITS *(if any)* Cooperating Units: Computer Syst. Lab., Div., Computer Resrch & Technology, NIH; Lab., Skin Biology, NIAMS, (Dr Steinert); Dept., Cell Biology, Univ., Rennes, France (Dr D Thomas); Dept of Biology, BNL (Drs J Wall, J Hainfeld); others as noted.

LAB/BRANCH

Laboratory of Structural Biology Research

SECTION

Section on Structural Biology

INSTITUTE AND LOCATION

NIAMS, NIH, Bethesda, Maryland 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
6.2	4.2	2

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The structures and mechanisms of assembly of biological macromolecular, complexes, and subcellular structures are studied, primarily by high-resolution electron microscopy and computer image processing. Two projects completed during the past year concern (1) characterization of a major conformational change in a viral capsid protein that occurs when the precursor capsid is transformed into the mature virion; and (2) determination of the molecular weights, oligomeric status, carbohydrate contents, and two-dimensional mass-map of the gp160/gp120 surface glycoprotein of human immunodeficiency virus, type 1 (HIV-1). 1) After polymerization of bacteriophage T4 precursor capsid is complete, its surface lattice undergoes a radical, cooperative, conformational change that results in an 18% increase in size, and striking morphological, functional, and immunological differences. We have investigated the conformational basis of this event by using laser Raman spectroscopy to determine the respective secondary structures of the precursor and mature states. Upon expansion, the alpha-helix content of the major capsid protein is reduced from 36% to 21%, and its beta-sheet content increases from 33% to 46%. We conclude that the conformational change involves a fundamental re-folding of a substantial portion of the protein. 2) Scanning transmission electron microscopy has been applied to preparations of gp160/gp120 of HIV-1 purified from a recombinant viral expression system. From these data, we have established that the precursor molecule, gp160, is a dimer of 125 kDa subunits, each of which comprises 91 kDa of protein together with, on average, 34 kDa of oligoasaccharides. Thus the nominal SDS-PAGE-derived molecular weight of 160 kDa is a major overestimate, presumably on account of its high carbohydrate contents. Gp120 and gp41, the maturation cleavage products of gp160, have monomer masses of 89 kDa and 36 kDa respectively, indicating that gp120 contains virtually all the carbohydrates present on gp160. Moreover, gp160 monomers dimerize primarily through interactions between their gp41 moieties.

Project Description:
Statement of Progress:
A. DEVELOPMENT OF EXPERIMENTAL FACILITIES:
Computer Image Processing:
(i) (Collaboration with Mr W Gandler, CSL/DCRT). We have effected an interface between our recently installed Zeiss EM902 electron microscope and our Microvax II minicomputer so that electron images may now be captured directly in digital form. For certain classes of image, this innovation will allow users to circumvent the laborious alternative of first recording photographic negatives and then scanning them on a microdensitometer. The new digitization system has some residual drawbacks arising from uneven illumination, geometrical distortions, and localized blemishes in the recording optics. The first can be largely corrected by applying a "shading correction", and future work will aim to improve the system in other respects. (ii) Three-dimensional Reconstruction of Icosahedral Viruses. A sophisticated set of computer programs specifically developed for the reconstruction of three-dimensional density maps of icosahedral particles from two-dimensional electron microscope images has been implemented. These programs, which use the "common lines" formalism of Fourier analysis were made available to us by Dr T S Baker, Dept. of Biology, Purdue University. To complement these programs, additional software has been written by us to facilitate the analysis. The programs have been tested and are now in routine use.
B-1 THE STRUCTURES OF VIRUSES AND VIRUS-RELATED PARTICLES:
(a) Conformational Basis of Bacteriophage T4 Capsid Maturation. (collaborator: R W Williams, Uniformed Services University of the Health Sciences). The expansion transformation that accompanies maturation of the bacteriophage T4 prohead involves major changes in the physical, chemical, and immunological properties of the capsid, and is preceded in vivo by proteolytic cleavage of its major protein, gp23 (56 kDa), to gp23* (49kDa). The respective secondary structures of gp23 in the unexpanded state, and of gp23* in the expanded state of the surface lattice were determined from the laser Raman spectra of polyheads. These tubular polymorphic variants of the capsid contain none of the minor capsid proteins whose presence would impede interpretation of the data in terms of the conformations of gp23/gp23*. Similar measurements were also made on uncleaved polyheads that had been expanded in vitro. We find that expansion is accompanied by a substantial change in secondary structure, whereby at least 30, and possibly as many as 80, of the 456 residues in gp23* are converted from α-helix to β-sheet. It follows that this conformational change involves refolding of a substantial part of the polypeptide chain. Furthermore, its β-sheet content is sufficient for a domain with the "jelly-roll" fold of antiparallel β-sheets, previously detected in the capsid proteins of many other icosahedral viruses.
(b) Organization of Encapsidated DNA in Herpes Simplex Virus, Type 1: (collaborators: Mr W Newcomb and Dr J Brown, U. Va., Charlottesville). We have used cryo-electron microscopy and digital image reconstruction techniques to analyze the three-dimensional structures of purified nucleocapsids of HSV-1. Thus we compared A-capsids which are devoid of DNA with C-capsids which contain fully packaged DNA. Micrographs of C-capsids show sets of fine, often curvilinear, striations ("fingerprints") with a spacing of 2.4nm - 2.8nm, which are not visible on A-capsids. Their visibility is considerably enhanced when the contribution of the capsid is computationally filtered away, exposing the packaged DNA. The latter does not exhibit icosahedral ordering but instead is a uniformly dense ball, consisting of locally ordered parallel packings of DNA heteroduplexes. The ball of DNA extends radially flush against the inner surface of the icosahedral (T=16) capsid shell. Variations in its appearance among particles that are viewed in different orientations suggests that the DNA is not randomly wound, but is at least partially

oriented. Overall, the organization of encapsidated DNA in HSV-1 resembles that previously visualized in bacteriophages T4 and lambda.
(c) Oligomeric Structure of the Env Glycoprotein of HIV-1.
(collaborators : Dr D J Thomas, University of Rennes, France; Drs J Wall & J Hainfeld, Brookhaven National Laboratory). Dark-field scanning transmission electron microscopy (STEM) was used to effect mass measurements of several hundreds of individual molecules of both gp160 and gp120. The results lead to rigorous conclusions concerning the oligomeric status, molecular weights, and carbohydrate contents of the env glycoproteins which have been a matter of controversy in earlier studies employing conventional biochemical procedures. Taking into account cloning-derived information on their protein sequences, the STEM mass data reveal that, in its isolated state, gp160 is primarily a dimer of 125 kDa subunits, each of which contains 91 kDa of protein and 34 kDa of carbohydrates. In contrast, gp120 was found to be a monomer of 89 kDa, consisting of 54 kDa of protein and 35 kDa of carbohydrates. Thus gp41 should have a monomer mass of 36 kDa and no significant amount of glycosylation. As visualized both in unstained STEM micrographs and in negatively stain, the predominant morphological type is an asymmetric dumbell, whose two domains have masses of 175 kDa and 75 kDa respectively. We infer that the small domain is a gp41 dimer and the large domain, a dimer of gp120.
B-2 STRUCTURE AND ASSEMBLY OF BIOLOGICAL MEMBRANES
(a) The Voltage-Dependent Anion-Selective Channel of Mitochondrial Outer Membranes (Collaboration with L Thomas & M Colombini, University of Maryland). VDAC is known from earlier work to form relatively wide-bore (3nm) channels which are compressed into crystalline arrays when isolated membranes are treated with phospholipase A2. We have used electron microscopy to analyze frozen-dried crystals, both with and without platinum shadowing, to investigate their surface topography and their proteo-lipid contents. Computed surface reconstructions indicate that the respective topographies of the two surfaces are very similar, and the protein component is, for the most part, recessed relative to the outer surfaces of the lipid bilayer. These findings correlate with functional data as to side-symmetric gating properties and ligand-binding. The basic structural unit is determined to be a non-equivalent trimer of VDAC, containing three channels, with two such trimers per unit cell of the p2 crystal lattice.

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Annual Report of the Arthritis and Rheumatism Branch
National Institute of Arthritis and Musculoskeletal and Skin Diseases
October 1, 1989 through September 30, 1990

The Branch's professional activities include both disease-related and basic investigations. The disease related studies involve both laboratory studies -frequently using animal models- and clinical studies, on the etiology, pathogenesis and treatment of a variety of inflammatory / autoimmmune disorders. In addition the Orthopedic Research Section studies normal and pathological development of bone particularly as it relates to healing of fractures. The Branch also supports a collaborative effort in conjunction with the National Institute of Mental Health, under Dr. Ester Sternberg, on basic mechanisms in arthritis. In addition the Branch supported a post-doctoral Fellow conducting research in the Biomechanics Laboratory of the NIH Clinical Center.

The clinical program is directed by five senior physicians who serve as the Principal Investigators on the experimental protocols. During the past year there were approximately 750 in-patient admissions of which about 250 were to the newly-established "Day Hospital" unit. About 2000 outpatient visits were recorded. In addition the Branch responded to some 50 requests for rheumatology consultations for patients involved in research studies of other Institutes.

The clinical and laboratory program also serves to train young physicians with a strong emphasis on research training in rheumatology.

I. Disease Related Studies

A. Arthritis and Related Diseases

1. Studies in animals (48, 79)*

Studies are continuing on the arthritis induced in LEW/N rats injected with bacterial cell walls -an excellent model for chronic arthritis, such as rheumatoid arthritis (RA), in humans. Continuing their studies on the nature of the mediators present during inflammation, Dr. Ronald Wilder and colleagues have found evidence implicating heparin binding growth factor-1 (HBGF-1): Intense expression of phosphotyrosine, particularly in endothelial cells was observed to develop coincident with

*Numbers in parentheses refer to project number

expression of HBGF-1. Sustained expression was found to be thymus-dependent. Little expression was found in arthritis-resistant F344/N rats.

These workers have also found enhanced expression of cyclooxygenase mRNA and protein in the joints of the Lew/N but not of the resistant F344/N rats.

In collaborative studies with Dr. Esther Sternberg and with colleagues in the National Institute of Mental Health, these workers had noted that the susceptible LEW/N animals exhibited hyporesponsiveness of the hypothalamic/pituitary/adrenocortical (HPA) axis. In a continuation of these studies they have studied the responsiveness of plasma ACTH and corticosterone to cholinergic muscarinic receptor agonists, alpha-1 adrenergic receptor agonists, and type 2 serotonin receptor agonists. In all cases the LEW/N rats showed blunted responses. They have attempted to localize the defect using hypothalamic explants. LEW/N explants released reduced amounts of corticotropin releasing hormone. No decrease in the number of receptors for any of the agonists was observed. The explants also show reduced enkephalin gene expression therefore suggesting some global defect in second messenger generation or effects.

Other findings include hyporesponsiveness of LEW/N animals to a variety of behavioral stressors. Initial breeding studies suggest that the hyporesponsiveness may be regulated by a limited number of genes, and that their effect is present throughout the lifespan of the animals.

2. Studies in humans (66)

A characteristic of RA is tumor-like proliferation of the synovium. Continuing their studies on biopsy specimens from humans with RA, Wilder and colleagues have recently obtained additional evidence that platelet-derived growth factor (PDGF) particularly PDGF-B like peptide play a role in regulating the fibroblast-like cells in the diseased synovium. Its production paralleled expression of acidic fibroblast growth factor and HBGF-1. Continuation of studies on the regulation of collagenase and transin/stromelysin implicate a cis element and the transactivating factors, c-jun and c-fos, which together comprise the AP-1 element. Retinoid suppression is mediated through inhibition of c-fos transcription. IL-1 regulation of stromelysin involves an inhibitory cAMP-dependent and a stimulatory protein kinase C-dependent pathway.

B. Myositis and related diseases

1. Studies in animals (75)

Dr. Paul Plotz and his colleagues have continued their studies on the pathogenesis of EMC-221A virus-induced inflammation in mice. They have recently found that nude mice have lower anti-viral antibody titers, longer viral persistance, and much more severe myocarditis and central nervous system disease. Viral persistance was detected using in situ hybridization and the polymerase chain reaction (PCR).

2. Studies in humans

a. Pathogenesis/etiology (74): Over 250 patients have been studied using a variety of immunological, clinical and epidemiological observations. The data suggest that there are subsets of patients that differ with respect to rapidity of onset, clinical manifestations and their responsiveness to therapy. Continued searches are being made by the techniques of in situ hybridization and PCR for five different viruses in biopsy specimens from 50 different patients. Evidence has been obtained that the antibody responses in these patients is antigen driven. To aid in following antibody responses, attempts are being made to produce histidyl-tRNA synthetase - the antigen to which a prominent antibody in this disease (anti-Jo) is directed- in bacterial expression systems .

b. Diagnosis (80): Magnetic resonance imaging (MRI) is being assessed as a diagnostic tool in myositis patients. MRI was found to be more sensitive than biopsy in detecting inflammation. Profound abnormalities of Pi and creatine phosphate have been observed in inclusion body myositis and tryptophan-induced eosinophilic-myalgia syndrome with lesser abnormalities in polymyositis and dermatomyositis.

c. Therapy (76): Trials have been undertaken using 1)controlled double-blind trial of plasmapheresis and leukapheresis, 2) intravenous methotrexate with leucovorin rescue, and 3) a combination of methotrexate and azathioprine. So far 39 patients were studied in the apheresis trial and the results are now being evaluated. The i.v. methotrexate and combination therapy trials were stopped after 12 patients showed poor outcomes.

d. Eosinophilia Myalgia Syndrome (79): Dr. Ester Sternberg has studied nine patients with this syndrome as a result of taking L-tryptophan. She and her colleagues have found evidence that the

patients have an activated indoleamine 2,3-dioxygenase (IDO) and suggest that inflammatory factors may have triggered this response. Six of nine patients were taking drugs that have been shown to suppress the HPA axis (above) in animal studies. They postulate that a contaminant in L-tryptophan (currently thought to be the etiologic agent rather than tryptophan itself) together with intrinsic or drug-induced HPA hyporesponsiveness may cause the syndrome.

C. Lupus and related diseases

1. Animal studies

a. Pathogenesis of murine lupus (20): i) Dr. Alfred Steinberg and his colleagues are continuing to explore the role of endogenous retrovirus in autoimmune prone mice. In particular they are studying the expression of the 8.4kb MCF-related RNA. One subgroup (Mpmv) are characteristic of autoimmune-prone strains whereas another (Pmv) is not. The latter are however induced by mitogens. Certain genotypes that promote autoimmunity when bred onto non-autoimmune backgrounds, increase Pmv transcripts but do not induce Mpmv and so act like mitogens. These studies suggest that Mpmv represents the action of a background gene for autoimmunity and does not result from the disease itself

ii) Ig mRNA was studied by in situ hybridization in peritoneal and splenic B cells. Three distinct groups were identified: cells with low expression (characteristic of resting B cells), high (characteristic of plasma cells) and intermediate (characteristic of a subset of peritoneal B cells). Northern analysis showed that the majority of the mRNA in these cells is characteristic of mIg rather than secreted Ig.

iii) The role of cytokines is being explored. Mice are injected with anti-CD3, anti-IgD, with or without a variety of antigens and mRNA for cytokines. It was found that anti-CD3 induced rapid production of mRNA for cytokines characteristic of TH1 and TH2 CD4+ helper cells. These results differ from those seen with in vitro stimulation where cytokine mRNA formation is much slower.

iv) Following up on prior studies of the CD44 (Ly24) gene product, PCR was used to prepare a construct for transfection into Ly24- lines. Whereas the untransfected cells grew in clumps, the transfected cell were more dispersed-phenotypes consistent with endogenously - and + cells. This suggests that CD44 expression is important for lymphocyte motility.

b. Therapeutic studies (22): Repetition of an earlier

study, has confirmed that anti-Il-4 antibody injected into MRL-lpr/lpr mice reduces lymphadenopathy. NZB autoimmune mice injected from 2 weeks of age for 5 weeks with anti-IL-5 showed no beneficial effects. In particular, the abnormally elevated levels of IgM were not reduced.

2.Studies in humans

a.Pathogenesis (23)

Human endogenous retroviral mRNA has been discovered in leukocytes from patients with a variety of auto-immune disorders but in normals as well. Additional studies with more specific probes have been conducted but none of these detected differences between normals and patients with lupus. Additional probes will be sought for testing in the future.

b.Therapy (40)

Twenty-three additional patients with diffuse lupus nephritis have been assessed after 1 year of various cytotoxic drug protocol. After 1 yr, none of the three protocols have produced statistically significant changes as measured by progression to renal failure. However, it is noteworthy that a prior trial failed to discover a major beneficial effect until patients had been followed for 5 or more yrs.

D. Other Inflammatory Diseases (83)

Familial Mediterranean Fever is a rheumatic disease caused by a single autosomal recessive gene. The location and nature of the gene and its potential product are unknown. Dr. Daniel Kastner is attempting to identify the chromosomal location and eventually the gene itself. Gene mapping studies are being performed on specimens from 55 Israeli families in which more than one individual suffers from the disease. Cell lines from 347 individuals were established using transformation with Epstein-Barr virus. To date, he and his colleagues have excluded about 10% of the genome, and have increasing evidence for a linked marker.

E. Bone Metabolism and Fracture Healing (77)

1. Studies of TGF-β1 and TGF-β2 revealed high levels of both in both human and rat fracture calluses. Antibodies to TGF-β2 stained only osteoblasts in human fracture callus. TGF-β1 stimulated chondrocyte differentiation and a 3-fold increase in chondocyte-gene expression of culture periosteal cells.This is consistent with the hypothesis that the periosteum is a source of chondrocyte precursors.

2. Animal and in vitro culture models are being used to determine the role of various cytokines, a) in cartilage repair after laceration and b) in adhesion formation in chickens following flexor tendon injury . Various probes for type I and type II collagen, for TGF-β,

acidic FGF, and PDGF are being used to follow changes in these components during healing. The effect of exogenous administration of some of these factors on the healing is also being assessed, and some substantial changes have already been noted in rates of cell proliferation.

3. The effect of estrogen administration on fracture repair in ovarectomized female rats have noted major increases in strength of the healed fracture. Histological evaluation suggest this is principally due to increased endochondral bone formation.

II Basic studies

A common mechanism by which cells of the immune system are stimulated is by way of aggregation of cell surface receptors. Metzger's group is continuing its studies on one such system: the IgE-mast cell system. In particular, these workers are attempting to identify the molecules through which the receptor for IgE activates the cells. The current principal strategy is to identify functionally critical regions on the receptor by genetic engineering. During the past year further mutant cDNAs for each of the subunits were prepared and tested for their ability to be expressed. The most striking result is the relatively modest influence on assembly and expression of those parts of the receptor thought to protrude into the cytoplasm. On the other hand, the transmembrane regions appear to be critical. These mutants have also given new insight into the arrangement of the subunits of the receptor. Truncation of the cytoplasmic tails also leads to rather small effects on the immobilization and internalization of the receptors, phenomena thought to reflect interaction of the receptors with cytoskeletal structures or coated pits, following aggregation. This is a surprising result, because it had been thought that membrane proteins interact with such components via their own cytoplasmic domains. Finally, parallel studies examine the role of different regions of the receptor in initiating the early cellular response using a mast cell-like lines that lack receptors. Studies on transfection of the wild-type receptors have been completed but accumulating a sufficient number of stable cells lines expressing the mutant receptor has been difficult. New cell lines are being examined as well alternative ways of identifying and/or selecting for suitable clones investigated.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41020-23 ARB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Pathogenesis of Autoimmunity In Mice With SLE-like Illness

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*
P.I. Alfred D. Steinberg, Chief Cellular Immunology Section, ARB, NIAMS
Mark Gourley, Arthur M. Krieg, and Dorothy Scott, Medical Staff Fellows, Cellular Immunology Section, ARB, NIAMS
Leslie King, IRTA Fellow, Cellular Immunology Section, ARB, NIAMS
Clara Pelfrey, IRTA Fellow, Cellular Immunology Section, ARB, NIAMS
Wendy Kisch, Biologist, Cellular Immunology Section, ARB, NIAMS
Tina McIntyre, Biologist, Cellular Immunology Section, ARB, NIAMS
Antonela Svectic, Visiting Fellow, Cellular Immunology Section, ARB, NIAMS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Arthritis and Rheumatism

SECTION
Cellular Immunogy

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7.54	5.54	2.0

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have been studying expression of 8.4 kb endogenous MCF related RNA which is found in thymuses of autoimmune-prone but not other mouse strains. The lupus-associated expression is limited to the Mpmv sub-set of MCF endogenous retroviral sequences. In contrast, the Pmv subset expression is associated with mitogenic stimulation.

CD 5^+ B cells, thought to be important in autoantibody secretion, are present in increase percentages in the peritoneal cavities of mice of many strains. Using in situ hybridization, we have found that peritoneal B cells produce levels of Ig mRNA intermediate between those of resting B cells and plasma cells. However, the mRNA was of the membrane rather than the secreted form. Moreover, splenic B cells produced much more Ig in short-term culture than did peritoneal B cells. Thus, cells may be stimulated in the peritoneal cavity but appear to leave prior to Ig secretion.

Project Description:

Objectives

Several strains of mice spontaneously develop autoimmune diseases resembling human systemic lupus erythematosus. Early in life they are immunologically hyperactive, become relatively resistant to tolerance induction, and gradually develop antinuclear and other autoantibodies followed by clinical disease. We believe that a detailed understanding of the genetic, cellular and biochemical basis for disease in these mice will contribute to the understanding of a variety of immune mediated diseases.

Methods Employed

1. Studies of peripheral lymphoid cells: Assays for antibodies to RNA, DNA and other ligands using the ammonium sulfate precipitation or the ELISA technique. Assay of antibody forming cells to a number of antigens by a modified ELISA assay. Immunization of animals with a variety of antigens with and without protein carriers or adjuvants. Studies of $CD5^+$ B cells, including peritoneal cells. Transfer of spleen, bone marrow, peritoneal, and thymus cells into recipients to evaluate the functional properties of the different cell types. Separation of lymphoid cells using monoclonal antibodies and either C-mediated cytotoxicity or magnetic beads. Evaluation of subpopulations of T cells and B cells using the fluorescence activated cell sorter.

2. Studies of thymocyte differentiation in vitro using a variety of cytokines to stimulate either proliferation or differentiation. Special emphasis is placed on $CD4^-$, $CD8^-$ thymocytes, the precursors to the other cells. Normal thymocytes from fetal and adult animals are studied prior to studies of autoimmune animals.

3. Molecular studies of RNAs: Northern blot analyses. Isolation of poly A^+ mRNA from lymphoid organs or cell subsets or cell clones and probing. Nuclear run-off to determine whether increased gene expression is due to increased specific mRNA production. In situ hybridization using riboprobes. Separate isolation of nuclear and cytoplasmic RNA to determine partition of different messages.

4. Southern blot analyses.

5. Culture of spleen cells with deoxyribonucleotides anti-sense to known retroviral sequences.

6. Gene cloning and sequencing using standard techniques.

7. Transfection of genes into cell lines.

8. Production of transgenic mice.

Major Findings

1. We have previously found that lupus -prone mouse strains, NZB, BXSB, MRL-+/+, MRL-lpr/lpr, and (NZB x NZW) F1, all have full-length, 8.4 kb, MCF retroviral transcripts in their thymuses whereas non-auroimmune mice do not. Probes able to distinguish sub-groups of MCF viruses on the basis of env and LTR differences indicate that Mpmv type 8.4 MCF transcripts are characteristic of autoimmune-prone strains whereas Pmv type transcripts are not; the Pmv 8.4 kb transcripts are, however, induced by mitogens. The gld/gld and lpr/lpr geneotypes on non-autoimmune backgrounds increase Pmv 8.4 kb RNA but do not induce Mpmv 8.4 kb mRNA and so act like mitogens. The 8.4 kb Mpmv expression was present from birth in several autoimmune-prone strains and was not altered by genes which accelerate or retard disease. These studies suggest that Mpmv 8.4 kb mRNA represents the action of a background gene for autoimmunity and does not result from disease itself.

2. Using in situ hybridization, Ig mRNA levels were studied in peritoneal and splenic B cells. Ig mRNA production fell into 3 distinct groups - low (characteristic of resting B cells), high (characteristic of plasma cells), and intermediate (characteristic of a subset of peritoneal B cells). Intermediate Ig mRNA was T cell dependent in that congenic nu/nu mice did not express any. The intermediate Ig mRNA containing cells wre primarily CD 5^+ , CD11b $^+$, and IgM^{bright} . In contrast, conventional B cells, CD 5^-, CD11b$^-$, IgM^{dull+} produced negligible levels of Ig mRNA. The peritoneal population that is CD 5^-, CD11b$^+$, IgM^{bright} contained levels of Ig mRNA intermediate between those of conventional B cells and CD 5^+ peritoneal B cells. Northern analysis showed that the majority of Ig mRNA expressed in the peritoneum is of the membrane rather than the secreted form. Consistent with the Northern data, short term culture revealed much less Ig production by peritoneal than splenic B cells. These studies describe novel Ig mRNA expression by peritoneal B cells and emphasize that within the peritoneal cavity, B cells do not tend to become antibody secreting cells.

3. Normal and autoimmune mice have been injected with anti-CD 3, anti-IgD, and/or a variety of antigens and splenic mRNA for several cytokines measured. Anti-CD 3 induced rapid production of message for cytokines characteristic of both TH 1 and Th 2 type CD 4+ helper cells. These results suggest that, in contrast to many in vitro studies, in vivo, both types of cytokine messages may be rapidly induced.

4. The murine CD 44 gene product (the Ly 24 or pgp-1 marker) is found on the cell surface of a subpopulation of double negative thymocytes and on memory T cells. In collaboration with Dr. J.T. August's lab, we cloned and sequenced the murine CD 44 gene as reported last year. Using PCR, we have fashioned a construct for transfection into Ly 24^- lines. CD 4- , CD 8-, CD 44- thymoma cell lines grew in clumps whereas daughter CD 44+ cell lines were more dispersed. Transfection of the negative lines with CD 44 led to decreased formation of large clumps. This work is consistent with our prior studies of CD 44 positive and negative thymocyte populations which had suggested that CD 44 expression is important for lymphocyte motility.

Significance to Bio-medical Research and the Program of the Institute

The diseases of mice occur spontaneously and are a close approximation of the comparable human diseases. The cellular and genetic bases of disease are becoming increasingly better understood. Such insights provide a firm basis for the investigation of the human diseases as well as strategies for immune intervention.

Our thymocyte experiments continue to provide insights into early thymocyte development, insights which can now be applied to autoimmune strains. T cell receptor expression on progenitor thymocytes appears to include critical exposure to cytokines. Therefore, in autoimmunity, regulation of T cell development could be defective at this stage and/or intervention could be applied at this stage.

Expression of a unique retroviral transcript in association with murine lupus provides a possible clue to early events in, or even pathogenesis of, lupus. In addition, evidence that endogenous retroviral sequences may play a role in normal lymphocyte activation provides a possible pathway for abnormal lymphocyte activation in autoimmune diseases.

The B cell studies further dissect biological compartments as well as cell types responsible for autoantibody production.

Proposed Course

The role of cytokines in thymocyte development will be explored further in autoimmune-prone strains. Unfortunately, these studies are technically difficult because of the small percentage of thymocytes which are CD 4-, CD 8- (1-4%).

The role of retroviral expression will be further dissected in normal and autoimmune-prone mice. RNA turnover and studies of RNA binding to splicing proteins should better define the abnormal retroviral gene expression observed in autoimmune thymuses. We have obtained from an NZB genomic library a full-length MCF retrovirus. This will be studied and used in transfection studies. Studies similar to those done in mice will also be carried out with human RNA and DNA using human retroviral probes.

Transfection of the CD 44 gene into CD 44^- cell lines and evaluation of their biology is being carried out. Further studies should begin to elucidate the functions of this important gene. Production of transgenic mice carrying the CD 44 gene would further contribute to that effort.

Z01 AR 41020-23 ARB

Publications:

1. Krieg AM, Khan AS and Steinberg AD: Expression of an endogenous retroviral transcript is associated with murine lupus. Arthritits Rheur 32:322-329, 1989.

2. Krieg AM, Gause WC, Gourley MF and Steinberg AD. A role for endogenous retroviral sequences in the regulation of lymphocyte activation. J Immunol 143:2448-2451, 1989.

3. Kastner DL, McIntyre TM, Mallett CP, Hartman AB and Steinberg AD. Direct quantitative In situ hybridization studies of Ig VH utilization: A comparison between unstimulated B cells from autoimmune and normal mice. J Immunol 143:2761-2767, 1989.

4. Steinberg AD, Klinman DM and Scott DE. From animal models to human pathology: facts and fantasies. pp 2-5 in, Proceedings of the International League Against Rheumatism XVII (1989) Congress, Rio de Janeiro, Brazil, September, 1989, edited by WH Chahade, RD Giorgi, EM Hirose-Pastor, EI Sato, Companhia Melhoramentos de Sao Paulo, Sao Paulo, Brazil, 1989.

5. Steinberg AD, Laskin CA, and Scott DE. Sex hormones and autoimmunity pp 170-173 in, Proceedings of the International League Against Rheumatism XVII (1989) Congress, Rio de Jaeiro, Brazil, September, 1989, edited by WH Chahade, RD Giorgi, EM Hirose-Pastor, EI Sato, Companhia Melhoramentos de Sao Paulo, Sao Paulo, Brazil, 1989.

6. Steinberg AD, Klinman DM, McIntyre TM and Kastner DL. Cellular and Molecular basis of SLE. I. Genesis of autoantibodies. Proceedings of the Second International Congress of SLE, 1989, Singapore, edited by ML Boey, Professional Postgraduate Services, International Publishers, Singapore, 1989, pp 15-18.

7. Steinberg AD, Gause WC, Scott DE, Gourley M, Takashi T and Krieg AM. Cellular and Molecular Basis of SLE II. Thymus, T cells, and Retroviruses. Proceedings of the Second International Congress of SLE, 1989, Singapore, edited by ML Boey, Professional Postgraduate Services, International Publishers, Singapore, 1989, pp 19-22.

8. Krieg AM and Steinberg AD. Retroviruses and autoimmunity. Journal of Autoimmunity 3:137-166, 1990.

9. Klinman DM, Eisenberg RA and Steinberg AD. Development of the autoimmune B cell repertoire in MRL-lpr/lpr mice. J Immunol 144:506-511, 1990.

10. Wolffe EJ, Gause WC, Pelfrey C, Holland SM, Steinberg AD and August JT. The cDNA sequence of mouse PgP-1 and momology to human CD44 cell surface antigen and proteoplycan core/link proteins.
J Biol Chem 265:341-347, 1990.

11. Gourley MF, Krieg AM and Steinberg AD. Preferential nuclear compartmentalization of endogenous MCF-related retroviral transcripts.
J Exp Med 171:1443-1452, 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41022-18 ARB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Therapeutic Studies in Murine Lupus

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. Alfred D Steinberg, Chief, Cellular Immunology Section, ARB, NIAMS
Arthur M Krieg, Medical Staff Fellow, Cellular Immunology Section, ARB, NIAMS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Arthritis and Rheumatism

SECTION
Cellular Immunology Section

INSTITUTE AND LOCATION
NIAMS - Building 10, Room 9N218, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.04	.04	

CHECK APPROPRIATE BOX(ES)

- ☐ (a) Human subjects
  - ☐ (a1) Minors
  - ☐ (a2) Interviews
- ☐ (b) Human tissues
- ☒ (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Spontaneously autoimmune mouse strains provide good models for potential new therapies for humans with autoimmune diseases. We have repeated a prior study and have administered anti-IL 4 antibody to MRL-lpr/lpr mice from birth and again observed retarded from the development of lymphadenopathy. Anti-IL5 given to NZB mice from 2 weeks of age had no beneficial effect on IgM production.

GPO 914-918

Project Description:

Objectives

Several mouse strains and crosses spontaneously develop autoimmune immune-mediated pathology resembling that of human systemic lupus and other rheumatic autoimmune diseases, including Sjogren's syndrome and rheumatoid arthritis. (NZB x NZW)F_1 mice spontaneously develop antibodies to nucleic acids and die of immune complex glomerulonephritis. NZB mice develop Coomb's positive hemolytic anemia, membranous nephrosis, lymphadenopathy, antibodies to DNA and glomerulonephritis. MRL-lpr/lpr mice develop massive lymphadenopathy, arthritis, arteritis, nephritis and produce very large amounts of IgG antibodies. BXSB mice and their hybrids with NZW and NZB mice develop degenerative coronary artery disease in addition to glomerulonephritis. These mice are excellent models for studying different therapeutic programs. The overall objective is to develop potentially promising new therapies which ultimately might be beneficial in the treatment of patients.

Methods Employed

Comparison of different therapeutic regimens on the natural history of mice with lupus by study of autoantibodies, proteinuria, renal and coronary pathology, lymphadenopathy, and survival.

Major Findings

1. In a prior study, we demonstrated beneficial effects of anti-IL 4 antibody administered to MRL-lpr/lpr mice. This study has been repeated and again reduced lymphadenopathy was observed.

2. Anti-IL 5 administered to NZB mice for 5 weeks from 2 weeks of age had no obvious effect. In particular, there was no reduction in serum IgM concentrations.

Proposed course:

Ultimately we would like to extend therapeutic mouse studies to humans; however, the agents so far tested are not of sufficient promise to supplant currently used human therapies. We are trying to obtain more useful reagents for that purpose.

Publications:

1. Steinberg AD. Idiotype regulation of autoantibody production. pp. 21–44 in Recent Advances in Autoimmunity and Tumor Immunology ed. F Dammacco, Edi-Ermes Publisher, Milan (Italy), 1988.

2. Steinberg AD, Scott DE, Gourley MF, and Klinman DM. Autoimmunity. In, Proceedings of The Fourth International Inflammation Research Meeting (October, 1988), 1989.

3. Steinberg AD, Klinman DM and Scott DE. From animal models to human pathology: facts and fantasies. pp. 2-5 in, Proceedings of the International League Against Rheumatism XVII (1989) Congress, Rio de Janeiro, Brazil, September, 1989, edited by WH Chahade, RD Giorgi, EM Hirose-Pastor, EI Sato, Companhia Melhoramentos de Sao Paulo, Sao Paulo, Brazil, 1989.

4. Steinberg AD, Klinman DM, Krieg AM, Seldin MF, Kastner DL. Approach to the use of Antigen Non-specific Immunosuppression in Systemic Lupus Erythematosus and Other Rheumatic Autoimmune Diseases. ed. JF Bach, Academic Press, San Diego, 1989.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41023-16 ARB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies of Patients with Immune-mediated Diseases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. Alfred D. Steinberg, Chief, Cellular Immunology Section, ARB, NIAMS
Arthur Krieg, Medical Staff Fellow, Cellular Immunology Section, ARB, NIAMS
Daniel Kastner, Medical Staff Fellow, Cellular Immunology Section, ARB, NIAMS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Arthritis and Rheumatism

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
.47	.47	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

mRNA hybridizing with probes specific for human endogenous retroviruses has been detected in leukocytes from patients with autoimmune diseases and normal individuals. We have used additional probes not available to us last year; however, we still do not have probes comparable to those used in the murine studies. Additional studies need to be performed with more specific subclass probes to determine whether or not there is disease specific expression.

Project Description:

Objectives

To understand the pathogenesis of human immune mediated diseases. This will include analyses of genetic, immune, and environmental factors which might contribute to disease. Special emphasis will be placed upon genetic factors. We will try to capitalize upon advances in murine studies.

Methods Employed

1. Study of patients and families with SLE, polymyositis, rheumatoid arthritis, angioimmunoblastic lymphadenopathy and familial Medeterranian fever (FMF) with regard to immune functions.

2. Northern analyses of endogenous retroviral expression.

3. Map and then clone gene(s) associated with disease.

4. ELISA spot assay to enumerate antigen-specific antibody secreting B cells at the single cell level.

Major Findings.

1. DNA has been obtained from patients with FMF and family members. Multiple Southern blots have been made, and the process of mapping the gene for FMF has begun.

2. We have previously found that peripheral blood cells from patients with several autoimmune rheumatic diseases were found to contain endogenous human retroviral RNA. Such RNA was also found in some normal individuals. We have now studied additional probes and additional individuals. Unfortunately, we have not yet found a probe that distinguishes autoimmune and normal expression. To date, the probes available are not comparable to - as subtype specific as - those used in murine studies. The latter studies have demonstrated a need for very specific endogenous retroviral probes for the detection of differences in expression between lupus and normals. We are still trying to obtain such probes for the human studies.

3. The ELISA spot assay is suitable for comparing SLE patients and normal with regard to Ig secreting cells.

Significance to Bio-medical Research and the Program of the Institute

We are trying to establish the basis of diseases at a molecular level and determine possible genetic basis of diseases.

Proposed Course

Dr. Daniel Kastner has been the prime mover of the FMF project. He has now moved to his own laboratory and will be the principal investigator of that project. The retroviral studies will be expanded upon when newer probes become available to us. With the newer probes we will attempt to determine if there is a retrovirus or endogenous retroviral "gene" uniquely associated with lupus. If a positive result is obtained, we will clone, sequence, and study it.

Publications:

1. Steinberg AD, Gause WC, Scott DE, Gourley M, Takaski T, and Krieg AM. Cellular and Molecular Basis of SLE II. Thymus, T cells, and Retroviruses. Proceedings of the Second International Congress of SLE, 1989, Singapore, edited by ML Boey, Professional Postgraduate Services, International Publishers, Singapore. 1989. pp. 19-22.

2. Krieg AM and Steinberg AD. Retroviruses and Autoimmunity. Journal of Autoimmunity 3:137-166, 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41025-19 ARB

PERIOD COVERED
October 1, 1189 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on the cell surface receptor for IgE

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	H. Metzger	Chief, Sec. Chem. Immunol.	ARB/NIAMS
	S. Mao	Visiting Fellow	ARB/NIAMS
	L. Miller	Staff Fellow	ARB/NIAMS
	G. Alber	Visiting Fellow	ARB/NIAMS
	N. Varin-Blank	Sp. Volunteer	ARB/NIAMS
	M. Edidin	Dept. Biology, Johns Hopkins	
	J. Rivera	Biologist	ARB/NIAMS
	G. Poy	Biologist	ARB/NIAMS

COOPERATING UNITS *(if any)*
None

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Chemical Immunology

INSTITUTE AND LOCATION
NIAMS, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
7	6	1

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The high affinity receptor for IgE on mast cells and basophiils plays a central role in immediate hypersensitivity reactions. Crosslinking of bound IgE by polyvalent antigen leads to aggregation of the receptors and cellular secretion of both preformed and newly synthesized mediators of inflammation. Although a large number of biochemical and morphological events have been observed to follow closely aggregation of the receptors, the molecular mechanism by which aggregation of the receptors generates these responses - a principal interest of our laboratory is still largely undefined. During the past year we have continued to employ mutated receptors in order to analyze which portions of the receptor participate in its principal functions We continued to employ a three tiered strategy: 1) Construction of mutants and testing for their expression; 2) Identification of a suitable host cell which itself lacks receptors but which possesses the molecular machinery to respond to aggregation of transfected receptors in a manner characteristic of mast cells; 3) Assessment of which portions of the receptor are required to activate the molecules responsible for the cellular activation. Our new results show: 1) Expression of receptors is relatively tolerant of even drastic modifications of the cytoplasmic but not of the transmembrane domains. Some structural insights into the arrangement of the receptor subunits was obtained. 2) Some functional characteristics of the monomeric and aggregated receptor can be observed on easily transfectable COS-7 cells. 3) Other functional sequelae of receptor aggregation while not observed on the COS cells, but which we previously showed can be observed on a receptor-less mast cell line were characterized further. 4) Although the latter cell line will also express mutant receptors, accumulating a panel of such mutant transfectants may prove intolerably slow unless different approaches for selecting such mutant transfectants can be developed.

Project Description:

Objectives:

IgE is a unique class of immunoglobulin that plays a predominant role in the pathophysiologic events associated with a variety of allergic phenomena. The IgE mediates its functions by binding to plasma membrane receptors on mast cells and related cells. The binding itself is not known to produce changes; however, if the cell bound IgE becomes aggregated (e.g. by binding to a multivalent antigen), rapid degranulation of the cell ensues. It is now known that the critical initiating event is aggregation of the receptor to which the IgE is bound. Our objective is to understand the immediate molecular sequelae of this aggregation since this may have implications for therapeutic manipulation of this system as well as other antibody-mediated cell perturbations.

Methods Employed

The principal new method employed during this year's work was fluorescence photobleaching and recovery (FPR) in order to examine the translational diffusion of receptors in situ.

Major Findings

1) Preparation and expression of mutant receptors. We extended our previous studies so that each of the five cytoplasmic extensions of the receptor was truncated and examined in all combinations. Each was expressed on the surface of COS cells (as determined by a rosetting assay) but in general the more drastically modified receptors were least efficiently expressed. A mutant in which the single cytoplasmic loop of the β chain is cleaved is expressed almost normally, but an attempt to isolate the intact receptor from such cells was unsuccessful - presumably because of the increased lability of the subunit interactions. Nevertheless, this β chain mutant should prove useful in exploring the interactions between the subunits. We were more successful with mutants of the γ chain in which the effect of converting one or both cysteine residues was examined. Combined with biosynthetic studies, our results unambiguously showed that it is cysteine 7 of the processed γ chain which forms the disulfide bond in the dimer of γ chains. This made it possible to define the radial disposition of the residues in the transmembrane domain of the γ chain and revealed a pattern previously seen with other transmembrane segments: The residues that form the interface between the transmembrane domains are considerably more polar than those that are presumptively exposed to the lipid bilayer. The latter residues are also more subject to evolutionary changes than those at the interface.

Consistent with this pattern, when relatively modest changes in the polar residues of the γ and other subunits were genetically engineered, surface expression was much less efficient. We surmise that such changes interfere with the assembly of the receptor making it more vulnerable to degradative pathways that compete with the pathway towards surface expression.

2) Studies of receptor movements in transfected COS cells. We transfected COS cells with wild-type or various combinations of mutated subunits and examined several properties of the receptors: their rate of lateral diffusion and the mobile fraction, both before and after aggregation, and their internalization after crosslinking. Studies in other systems have implicated the cytolasmic domains of various membrane proteins as affecting such properties. IgE receptors with one or more truncated cytoplasmic domains were examined. Surprisingly the properties of such mutants were very similar to those of the wild-type. We conclude that to the extent the particular properties we studied involve interactions with other cellular proteins, such interactions do not involve the cytoplasmic extensions of the receptor We noted however that the rate of internalization of the transfected receptors on COS after crosslinking was relatively slow compared to the internalization of either endogenous or transfected receptors on mast cell lines. There is evidence that the rapid internalization on such cells is via coated pits. It is possible that the ability of mutated receptors to interact with coated pit components will be found to be impaired.

3) Receptor activation of transfected mast cells. We completed studies of a receptor-less mast cell line (P815) which exhibits some of the characteristic responses after aggregation of the IgE receptors with which it had been transfected. Although the response was not as robust as seen with normal mast cells or a mast cell line with endogenous receptors, this was also true when the cells were stimulated via the thrombin receptor or non-specifically with ionophore. Thus the reduced responsiveness appears to reflect a general property of the cells rather than a defect in the transfected receptors.

These cells also possess one or more endogenous Fc_γ receptors. When aggregated, these receptors are also able to initiate cellular responses such as a rise in cytoplasmic Ca^{2+}. These receptors are thought to consist of either an α-like chain alone ($Fc_\gamma RII$) or an α-like chain associated with a γ chain ($Fc_\gamma RIII$), but in any case no β chain. This raises the question of what the special role the β chain of the IgE receptor plays.

Work has begun on preparing a panel of P815 transfectants containing mutant receptors. Although we have had success with two of these, our overall experience has been frustrating. Thus, while such transfectants initially show a small or occasionally modest percentage of cells expressing receptors, we have usually been unsuccessful in obtaining stable cloned lines of cells all of which are receptor positive. We tried using a panning technique and although this is capable of selecting receptor-positive cells, many of these appear to bear only small numbers of receptors.

Significance to Biomedical Research

It appears that using mutational analysis will provide us a powerful tool by which to identify regions of the IgE receptor which are critical for a variety of cellular responses that the receptor initiates. Although so

far we have principally identified regions that are not essential for the functions being examined, such results are in fact the most easily interpreted. (When a mutation leads to a loss of function there is always the concern that the effect is not due to the direct involvement of the region in question, but results instead from an indirect effect on the receptor's structure.) Once critical regions are defined this information can be used to try to identify critical "post-receptor" macromolecules with which the receptor interacts. Interfering with such interactions can then be studied as a means by which to interfere with receptor function in allergic reactions.

Proposed Course

1) Studies on receptor movements. We will begin studies on transfected P815 cells (and possibly other cells [below]) in which the endocytosis of the aggregated occurs at a rate similar to that observed in other systems in which a coated pit mechanism prevails. If this proves to be the case with wild-type receptors, we will then begin examining cells transfected with mutant receptors.

We also plan to study another phenomenon: the adherence of aggregated receptors to cytoskeletal structures. Although there is evidence that such an interaction may play a role in deactivating rather than activating the receptor, it will nevertheless be interesting to see whether we can define exactly which subunits of the receptor may be directly involved in these interactions.

2) Studies on functional transfectants. We plan to pursue our attempts to utilize the P815 line for studying the effect of receptor mutations on transmembrane signalling. New approaches will be used by which we can select and/or identify the small number of transfectants expressing substantial numbers of receptors.

We also plan to study other receptor-less cell lines which for a variety of reasons can be expected to be responsive to transfected receptors.

Publications

1. Kinet J-P and Metzger H. Genes, structure and actions of the high affinity Fc receptor for IgE. In: Metzger H, ed. Fc receptors and the action of antibodies. Wash DC: Am Soc of Microbiology, 1990;239-59.

2. Metzger H. General aspects of antibody action. In: Metzger H. ed. Fc receptors and the action of antibodies. Wash DC: Am Soc of Microbiology, 1990;7-11.

3. Miller L, Alber G, Varin-Blank N, Ludowyke R, Metzger H. Transmembrane signalling in P815 mastocytoma cells by transfected IgE receptors. J Biol Chem 1990;265:12444-52.

4. Varin-Blank N, Metzger H. Surface expression of mutated subunits of the high affinity mast cell receptor for IgE, in press J Biol Chem.

5. Blank U, Ra C, Miller L, Metzger H, KinetJ-P. Complete structure and expression in transfected cells of high affinity IgE receptor. Nature 1989;337:187-9.

6. Metzger H, Kinet J-P, Blank U, Dreskin SC, Miller L. The receptor with high affinity for IgE. Allergol 1989;12:S120-3.
(previously listed as in press).

7. Metzger H, Kinet JP, Blank Un, Miller L, Ra C, Rivera J, White KN. The mast cell receptor for immunoglobulin E: Prospects for therapy. Allergy Clin Immunol 1989;1:43-5.
(previously listed as in press).

8. Miller L, Blank U, Metzger H, Kinet JP. Expression of high-affinity binding of human immunoglobulin E by transfected cells. Science 1989;244:334-7.

9. Dreskin SC, Pribluda V, Metzger H. IgE receptor-mediated hydrolysis of phosphoinositides by cytoplasts from rat basophilic leukemia cells. J Immunol 1989;142:4407-4415.

10. Kinet JP, Metzger H, Alber G, Blank U, Huppi K, Jouvin M-H, Kuster H, Miller L, Ra C, Rivera J, Varin-Blank N. The high affinity receptor for immunoglobulin E: New developments. In: Melchers F, ed. Progress in Immunology VII. Berlin: Springer-Verlag, 1989;716-23.

11. Metzger H, Blank U, Kinet JP, Kochan J, Ra C, Rivera J. Emerging picture of the receptor with high affinity for IgE. Intl Arch Allergy Appl Immunol 1989;88:14-7.

12. Metzger H, Kinet JP, Blank U, Miller L, Ra C. The receptor with high affinity for IgE. In: Chadwick D, Evered D, Whelan J, eds. IgE, Mast cells, and the allergic response. Chichester: John Wiley and Sons, 1989;93-101.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41040-18 ARB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT (80 characters or less. Title must fit on one line between the borders.)
Study of Various Cytotoxic Drug Programs in Diffuse Lupus Nephritis

PRINCIPAL INVESTIGATOR (List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)

P.I. Alfred D. Steinberg, Chief, Cellular Immunology Section, ARB, NIAMS
Mark Gourley, Medical Staff Fellow, Cellular Immunology Section, ARB, NIAMS
Dorothy Scott, Medical Staff Fellow, Cellular Immunology Section, ARB, NIAMS
Susan C. Steinberg, Special Volunteer, Cellular Immunology Section, ARB, NIAMS

COOPERATING UNITS (if any)

James E. Balow, Senior Investigator, NIDDK
Howard A. Austin, Attending Nephrologist, Clinical Center Foreign: NONE

LAB/BRANCH
Arthritis and Rheumatism

SECTION
Cellular Immunology

INSTITUTE AND LOCATION
NIAMS - Building 10, Room 9N218, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.16	2.16	

CHECK APPROPRIATE BOX(ES)

☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK (Use standard unreduced type. Do not exceed the space provided.)

New patients have been randomized to receive (i) monthly IV-cyclophosphamide, (ii) monthly IV methylprednisolone, or (iii) both. The study is now closed to new entrants. Rate of progression to renal failure, requirements for re-treatment, and drug toxicities are being assessed. Since last year's report, we have now followed and additional 23 patients for at least one year. Clear-cut differences have yet to emerge for the entire group of patients completing 1 year of study. [Since it took > 5 years for differences to occur in prior studies, we believe that a longer follow-up is necessary for even trends to emerge].

Project Description:

Objectives

The Arthritis and Rheumatism Branch has a major interest in the management of the nephritis of systemic lupus erythematosus (SLE). Renal failure is the most feared complication in the disease. The renal damage is largely in the glomeruli. It has been suggested that drugs capable of altering the antibody response, "immunosuppressives", would reduce the formation of antibody and thus of antigen-antibody complexes. The objective of this study is to assess the efficacy and toxicity of various immunosuppressive drug programs in SLE nephritis.

Methods Employed

Patients with systemic lupus erythematosus and active nephritis have been entered into several trials. All patients are repeatedly reevaluated with a tests of renal inflammation and renal function, serological studies, and urinalyses. Treatment failures can be withdrawn according to protocol. Long-term follow-up of patients previously admitted continues. Our latest protocol involved randomizing patients to receive monthly one of the following: (i) bolus cyclophosphamide, (ii) bolus methylprednisolone, or (iii) both. Eighty patients have now been recruited and the study is closed to new patients.

Major findings

1. We have completed a long-term analysis of patients randomized many years ago to different treatments. With regard to progression to renal failure, there is a statistically significant difference between patients randomized to receive intravenous cyclophosphamide and randomized to receive corticosteroids only. This was true of the entire group (which was not true of the prior paper from our group on this subject) as well as the group at "high risk" for renal failure.

2. Short-term follow-up (at one year) of patients in the current study has not provided evidence for clear-cut differences between the groups. Renal failure has been observed in a few patients, including those randomized to receive cyclophosphamide. Re-treatment has been required for many patients in all three groups. The average changes in urine sediment, proteinuria, and serum creatinine are not different among the three groups at one year. Four patients receiving both IV-CY and IV-MP have become allergic to the IV-MP, and two patients receiving IV-MP also have become allergic to the drug. Some of these have successfully switched to oral medication.

Significance to Bio-Medical Research and the Program of the Institute

These studies are relied upon by physicians concerned with SLE all over the world. We have been one of very few units able to speak from the hard data base of controlled studies.

Proposed Course

We will continue to follow these patients on their assigned programs.

Publications:

1. Steinberg AD, Scott DE, Gourley MF and Klinman DM. Autoimmunity. In, Proceedings of The Fourth International Inflammation Research Meeting (October, 1988), 1989.

2. Steinberg AD, Lindahl M, Gourley M, Scott D and Steinberg SC. Approach to lupus nephritis based upon randomized trials. Proceedings of the Second International Congress of SLE, 1989, Singapore, edited by ML Boey, Professional Postgraduate Services, International Publishers, Singapore, 1989, pp 206-209.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41048-11 ARB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Bacterial cell wall-induced arthritis and hepatic granuloma formation in the rat

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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Developmental Endocrinology Branch, NICHD
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LAB/BRANCH: Arthritis and Rheumatism Branch

SECTION: Connective Tissue Diseases

INSTITUTE AND LOCATION
NIAMS, NIH, Bethesda, MD 20892

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3.5	3.5	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Streptococcal cell wall arthritis in LEW/N rats closely resembles rheumatoid arthritis in humans. In addition to other growth factors, recent data have implicated heparin binding growth factor (HBGF-1) in the disease process.

We have previously presented data indicating that a defective hypothalamic-pituitary-adrenal (HPA) axis response is associated with the extreme susceptibility to arthritis in LEW/N rats. During the past year, we have extended our studies of neuroendocrine regulatory mechanisms. In addition to the profound defect in responsiveness to IL-1 and streptococcal cell walls, LEW/N rats, in marked contrast to arthritis-resistant F344/N rats, are deficient in their responses to a variety of neurotransmitters as well. Receptor affinities and concentrations were the similar. A data considered, our current results suggest that the defect in arthritis-prone LEW/N involves a defect in the mechanisms that transduce the activation of the gene encoding corticotropin releasing hormone or CRH. These observations provide new insights into the role of the stress response and the development of human autoimmune diseases.

GPO 914-918

Project Description:

Background and Objectives

Bacterial cell wall-induced arthritis is an experimental animal model of chronic proliferative and erosive synovitis, closely resembling rheumatoid arthritis, that is induced by systemic administration of biodegradation resistant bacterial cell wall fragments in aqueous solution. The development of disease is under the control of genetic factors. Previoulsly published data from our laboratories have shown that arthritis-susceptible LEW/N rats have a profound defect in the HPA axis response to various inflammatory stressors, whereas arthritis-resistant F344/N rats have robust responses. Studies in progress have the following goals:

A. To characterize the host molecular genetic factors that regulate susceptibility to develop arthritis.

B. To better define pathophysiologic processes by characterizing differences between high and low responder inbred rat strains.

C. To define mechanisms leading to tissue injury and destruction.

D. To develop techniques to modulate the process.

Major New Findings:

1. Cytokines/Growth Factors: Studies of the roles of various factors have continued.

   a. Synovial neovascularization is a characteristic feature of streptococcal cell wall arthritis. We have recently published data providing evidence that polypeptide, acidic fibroblast growth factor, or heparin binding growth factor-1, was involved. In our most recent studies we have examined arthritic joints for evidence of HBGF-1 effects. For example, we have found intense expression of phosphotyrosine in situ, particularly in endothelial cells which developed coincident with HBGF-1 expression. Sustained synovial expression of HBGF-1 and phosphotyprosine was thymus-dependent. Expression was minimal in arthritis-resistant F344/N rats.

   b. The expression of cyclooxygenase in situ in arthritic joints is being examined. Enhanced expression of both mRNA and protein has been demonstrated. Like other inflammatory mediators, expression was also thymus-dependent and minimal in arthritis-resistant F344/N rats.

2. Studies of neuroendocrine regulatory mechanisms in the disease process have continued. New findings include:

a. To evaluate the mechanism and specificity of the hypothalamic-pituitary-adrenal (HPA) axis defect in arthritis-prone LEW/N rats, we examined the ability of three major excitatory neurotransmitter systems to activate the HPA axis in LEW/N and F344/N rats. The responsiveness of plasma ACTH and corticosterone to cholinergic muscarinic receptor agonists, alpha-1 adrenergic receptor agonists, and serotonin type 2 receptor agonists was significantly blunted or absent in LEW/N versus arthritis-resistant F344/N rats. To localize the defect to the hypothalamus, we evaluated the ability of explanted hypothalami from the two strains for secretion of corticotropin releasing hormone (CRH) in vitro in response to the various agonists. LEW/N hypothalami released less CRH in all cases, and the dose-response curves were shifted to the right or abolished, suggesting markedly decreased sensitivity of the CRH neurons to the neurotransmitters. The binding affinity and number of muscarinic, alpha-1/alpha-2, or serotonin type 2 receptors in LEW/N and F344/N hypothalamic and cerbral cortex were virtually identical. These data indicated that the HPA axis of arthritis-prone LEW/N rats is hyporesponsive to a variety of neurotransmitter stimuli, as well as stimuli such as interleukin-1 and streptococcal cell walls, and suggest that the defects may relate to a global defect in the mechanisms transducing activation of the CRH gene in the hypothalamus. Since a similar defect has also been noted for enkephalin gene expression, abnormalities in second messenger gneration or effects are suspected.

b. Preliminary data has been obtained indicating the LEW/N and F344/N rats differ dramatically in their HPA axis response not only to inflammatory mediators and neurotransmitters, but also to a wide variety of behavorial stressors.

c. Breeding studies of LEW/N and F344/N rats have been initiated with the goal of defining the genetic mechanisms underlying the HPA defect in LEW/N rats. Preliminary data suggest that the defective phenotype is codominant and segregates in the F2 generation as if it is regulated by a limited number of genes.

d. Studies of the ontogeny of the HPA defect have been initiated. Preliminary data indicate that HPA axis responses are blunted throughout the lifespan of the LEW/N rat. In contrast, a blunted response is observed only during the first 21 days after birth in F344/N rats.

Significance to Biomedical Research and Program of the Institute

Peptidoglycan-containing bacterial cell wall fragments have received increasing attention in recent years as possible pathogenetic agents in some cases of chronic synovitis. This experimental animal model provides a powerful tool to explore the pathogenesis of chronic proliferative and erosive arthritis. We have defined parameters of both the host (cell wall distribution, inflammatory mechanisms, role of T-lymphocytes and various cytokines, neuroendocrine mechanisms, etc.) and the bacteria (type, resistance to biodegradation, fragment size) which influence the development of arthritis. Our recent studies on this model have provided an entirely new concept for the inherited basis of autoimmune/inflammatory diseases. Our recent data suggest that autoimmune/inflammatory diseases may result from defective activation of counterregulatory stress pathways involving the hypothalamic-pituitary-adrenal axis. These observations suggest a multitude of new research opportunities in autoimmune/inflammatory in humans.

Proposed Course:

The role of the hypothalamic-pituitary axis in regulating disease susceptibility will continue to be a major focus of additional research. In particular, delineation of the molecular genetic basis for the defect in LEW rats will continue to be vigorously pursued. Studies already begun on HBGF-1, cyclooxygenase, and phosphotyrosine will be completed.

Publications

1. Wilder RL, Lafyatis RT, Case JP, Yocum DE, Kumkumian GK, Remmers EF. Cytokines in rheumatoid arthritis and streptococcal cell wall arthritis in the rat, In Therapeutic Approaches to Inflammatory Diseases, Editors A.J. Lewis, N.S. Doherty, and N.R. Ackerman, Elsevier Science Publishing Co., Inc. 1989, pp. 27-32.

2. Wilder RL, Lafyatis R, Yocum DE, Case JP, Kumkumian GK, Remmers EF. Mechanisms of bone and cartilage destruction in rheumatoid arthritis: Lessons from the streptococcal cell wall arthritis model in LEW/N rats. Clin Exp Rheumatol, 7/S-3:123-127, 1989.

3. Lafyatis R, Thompson NL, Remmers EF, Flanders KC, Roche NS, Seong-J K, Case JP, Sporn MB, Roberts AB, Wilder RL. TGF-beta production by synovial tissues from rheumatoid patients and streptococcal cell wall arthritic rats: Studies on secretion by synovial fibroblast-like cells and immunohistologic localization. J Immunol 143:1142-1148, 1989.

4. Case JP, Sano H, Lafyatis R, Remmers EF, Kumkumian GK, Wilder RL. Transin/stromelysin expression in the synovium of rats with experimental erosive arthritis. In situ localization and kinetics of expression of the transformation-associated metalloproteinase transin/stromelysin in euthymic and athymic LEW.N rats. J Clin Invest 84:1731-1740, 1989.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41066-08 ARB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Characterization of Synovial Tissues from Patients with RA and Related Conditions

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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J. Case	Medical Staff Fellow	ARB/NIAMS
H. Sano	Visiting Fellow	ARB/NIAMS

COOPERATING UNITS *(if any)*
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Holland Laboratories, American Red Cross

LAB/BRANCH
Arthritis and Rheumatism Branch,

SECTION
Connective Tissue Disease Section

INSTITUTE AND LOCATION
NIAMS, NIH, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.50	1.50	

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The single feature most characteristic of rheumatoid arthritis is tumorlike proliferation of the synovium. Transformed-appearing fibroblastlike cells and new blood vessels are the major proliferating cell populations. During the past year, we have obtained additional evidence that platelet-derived growth factor, particularly a PDGF-B-like polypeptide, plays a role in regulating the growth and function of the fibroblast-like cells in diseased synovium. We have shown that its production correlates with expression of acidic fibroblast growth factor, or heparin binding growth factor-1, in rheumatoid synovial tissues. These growth factors probably synergistically stimulate the tumorlike proliferation of rheumatoid synovium.

We have continued studies of the regulation of collagenase and transin/stromelysin transcription by interleukin-1 in rheumatoid synovial fibroblasts. We have provided evidence that collagenase transcription is regulated through a cis regulatory element, the TRE, and the transactivating factors, c-jun and cfos, which together comprise the AP-1 element. Retinoid suppression of collagenase transcription is mediated through inhibition of c-fos transcription. We have shown that IL-1 regulation of stromelysin involves two antagonistic pathways, an inhibitory, cAMP-dependent and stimulatory, protein kinase C-dependent pathway.
Project Description:

Objectives:

The inner surface of the capsule of diarthrodial joints is lined by a delicate vascular connective tissue known as the synovial membrane. It consists of one or two layers of synovial lining cells (synoviocytes) overlaying an areolar or fibrous connective tissue. In conditions such as rheumatoid arthritis (RA), the surface layer of the membrane is markedly thickened and the deeper areolar tissue of the membrane is filled by infiltrating T lymphocytes, macrophages and plasma cells, producing a picture typical of chronic inflammation. Massive tumorlike proliferation of the synovial connective tissue cell populations are also characteristic. The objectives of this study are to further characterize this inflammatory tissue by study of tissue biopsy specimens and to delineate the molecular mechanisms underlying its pathological development.

Current objectives include:

1. In situ characterization of the cells in rheumatoid synovium. We are analyzing frozen and paraffin embedded sections of synovial tissue with a battery of well-characterized antibodies. Particular emphasis, currently, is demonstrating the presence and sites of production of various growth factors in synovium.

2. In vitro culture studies. We are examining the effects of cytokines such as platelet-derived growth factor, transforming growth factor beta, intereukin 1, fibroblast growth factors on the growth and function of rheumatoid synovial fibroblast-like cells. Particular emphasis is currently on the regulation of collagenase and transin expression.

Major New Findings

1. PDGF and Rheumatoid Synovitis: The production of PDGF-related polypeptides by rheumatoid synovium has been examined further. In situ immunohistochemical staining with recently developed polyclonal anti-PDGF B antibodies further confirm the high level expression of these polypeptides in synovial macrophages. Metabolic labeling studies have shown that the dominant polypeptide is similar in size to the major form of macrophage derived PDGF-B (about 15kd). It is also similar to the recently described PDGF-B-like polypeptide found in wound fluids. In contrast, PDGF-A chain expression appears to be substantially less prominent. The data provide additional evidence that macrophage-derived PDGF-B-like polypeptides play a role in the hyperplasia of rheumatoid synovial connective tissues.

2. IL-1 Regulation of Collagenase Transcription: Since collagenase production by rheumatoid synovial fibroblasts plays a major role in cartilage and bone destruction in rheumatoid arthritis, we have been studying the molecular mechanisms involved in IL-1 stimulation of collagenase, as well as the mechanisms by which retinoids inhibit. By examining deletion mutants of the 5'-flanking region of the collagenase gene ligated to a chloramphenical acetyltransferase (CAT) reporter gene, we showed that the TRE, a cis

regulatory element in the collagenase gene promoter region, mediates both the IL-1 mediated stimulation and retinoid inhibition. We showed that IL-1 transiently induces the protooncogenes, c-jun and c-fos, and that retinoids inhibit c-fos, but not c-jun, expression. c-Jun and c-Fos have been previously shown by others to dimerize to form the active transactivating factor AP-1. These results are consistent with the view that retinoids inhibit collagenase gene transcription, in part through inhbition of c-fos and generation of AP-1. These results were confirmed using c-fos promoter CAT constructs in transfection experiments with rheumatoid synovial fibroblasts.

3. IL-1 Regulation of Transin/Stromelysin Transcription: IL-1 is a potent activator of the metalloproteinase, transin/stromelysin, in rheumatoid synovial fibroblasts, but the transduction mechanisms are controversial. cAMP has been regarded as an important second messenger, but we and others have shown that cAMP inhibits stromelysin transcription. In addition to stimulating stromelysin transcription, IL-1 induced prostaglandin E2 production in rheumatoid synovial fibroblasts. We have shown that IL-1 stimulates a time-dependent accumulation of cAMP in quiescent rheumatoid synovial fibroblasts, an effect that is blocked by the prostaglandin synthase inhibitor, indomethacin. The cAMP agonists forskolin, IMBX, and prostaglandin E2 suppressed the induction stromelysin. Conversely, indomethacin super-induced IL-1-elicited stromelysin transcription. These results were duplicated with cells transfected with the stromelysin promoter ligated to a CAT reporter construct. Moreover, 2',5'-dideoxyadenosine, an inhibitor of adenylate cyclase, also augmented the IL-1 induction of stromelysin mRNA. In contrast, staurosporine, a specific inhibitor of protein kinase C, blocked the IL-1 induction of transin/stromelysin transcription. We have concluded that IL-1 stimulates at least two transduction pathways in rheumatoid synovial fibroblasts, and that these have antagonistic effects of the regulation of transin/stromelysin transcription.

Significance to Biomedical Research and Program of the Institute

An understanding of the primary mechanisms by which joint destruction is produced in diseases such as rheumatoid arthritis, requires knowledge about the types of cells in the inflammed tissues, their differentiation state, the mechanisms regulating their activity, etc. Our studies address these questions and provide insight into pathogenetic mechanisms. New therapeutic approaches are suggested from these studies.

Proposed Course

Studies are continuing with particular emphasis in defining the roles of platelet derived growth factor, acidic fibroblast growth factors, interleukin-1 and transforming growth factor-beta on regulating synovial mesenchymal cell growth and function. In particular, the regulation of transin/stromelysin, collagenase and cyclooxyenase is being pursued. These meditors appear to play an essential role in extracellular matrix resorption. An understanding of these processes is highly relevent to rheumatoid arthritis.

Publications

1. Wilder RL, Lafyatis RT, Case JP, Yocum DE, Kumkumian GK, Remmers EF. Cytokines in rheumatoid arthritis and streptococcal cell wall arthritis in the rat, In Therapeutic Control of Inflammatory Diseases, Editors AJ. Lewis, N.S. Doherty, and N.R. Ackerman, Elsevier Science Publishing Co., Inc., 1989, pp. 27-32.

2. Kumkumian G, Lafyatis R, Remmers E, Case J, Wilder RL. Synergistic effects of IL-1 and PDGF on rheumatoid synovial fibroblast growth, and prostaglandin and collagenase production. J Immunol 143:833-837, 1989.

3. Lafyatis R, Thompson NL, Remmers EF, Flanders KC, Roche NS, Seong-J K, Case JP, Sporn MB, Roberts AB, Wilder RL. TGF-beta production by synovial tissues from rheumatoid patients and streptococcal cell wall arthritic rats: Studies on secretion by synovial fibroblast-like cells and immunohistologic localization. J Immunol 143:1142-1148, 1989.

4. Case JP, Lafyatis R, Remmers EF, Kumkumian GK, Wilder RL. Transin/stromelysin expression in rheumatoid synovium. A transformation-associated metalloproteinase secreted by phenotypically invasive synoviocytes. Amer J Pathol 135:1055-1064, 1989.

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6. Remmers EF, Lafyatis R, Kumkumian GK, Case JP, Roberts AB, Sporn MB, Wilder RL. Cytokines and growth regulation of synoviocytes from patients with rheumatoid arthritis and rats with streptococcal cell wall arthritis. Growth Factors 2:179-188, 1990.

7. Sano H, Forough R, Maier JAM, Case JP, Jackson A, Engleka K, Maciag T, Wilder RL. Detection of high levels of heparin binding growth factor-1 (acidic FGF) in inflammatory arthritis joints. J Cell Biol 110:1417-1426, 1990.

8. Kaplan H, Weinblatt ME, Wilder RL. Advances in arthritis drug therapy. Patient Care 23:31-39, 1989.

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13. Lafyatis R, Kim S-J, Angel P, Roberts AB, Sporn MB, Karin M, Wilder RL. Il-1 stimulates and all-trams-retinoic acid inbits collagenase gene expression through its 5' AP-1 binding site. Molec Endocrinol, in press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41074-03 ARB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Studies on etiology and pathogenesis of idiopathic inflammatory myopathy in humans

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Paul H. Plotz, M.D.	Sr. Investigator	ARB/NIAMS
	F.W. Miller, M.D., Ph.D.	Expert	ARB/NIAMS
	Richard Leff, M.D.	Sr. Staff Fellow	ARB/NIAMS
	Lori Love, M.D., Ph.D.	Expert	ARB/NIAMS
	David Fraser	Clinical Associate	ARB/NIAMS
	M. Dalakas		NINDS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Diseases Section

INSTITUTE AND LOCATION
NIAMD, Bethesda, Md 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.1	2.1	

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☒ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Idiopathic inflammatory myopathy (poly-dermatomyositis) is an inflammatory disease of muscle in which characteristic autoantibodies occur. By applying a broad range of immunological, clinical, and epidemiological observations to a large (expanded in the past year to over 250) group of patients, we have developed evidence for subsets of patients which strongly suggest that the disease can be divided into more meaningful groups with different etiology, pathogenesis and response to therapy. Studies on the seasonal and geographic distribution of disease sub-types are well underway. Laboratory studies have shown that the major autoantibody, anti-Jo-1, which defines a clinically important subgroup of patients, appears to be an antigen-driven secondary response directed to a conformational, not a linear, epitope of histidyl tRNA synthetase. The discovery of affinity-maturation in the early response to this antigen powerfully implicates the enzyme itself as the driving antigen.

Project Description:

Objectives:

1) By a study of a variety of parameters of disease, to uncover subsets of patients who may have a common etiology or pathogenesis.

2) To understand the relationship between the presence of a particular autoantibody and the disease in which it is found.

3) To seek direct evidence for a viral cause of human myositis by probing tissues for viral nucleic acid by in situ hybridization.

Methods:

1) Routine clinical observations have been supplemented by extensive evaluation of heart and lung function and by careful epidemiologic data. The observations have been entered into a computer and are being analyzed by programs developed in part by members of our group.

2) Studies on anti-Jo-1 antibodies have involved development of a sensitive, antigen-specific, sub-class specific ELISA assay and of a method for detection of isoelectrically-focussed antibodies. The Pepscan system was used to synthesize all possible hexapeptides of histidyl-tRNA synthetase to search for linear epitopes recognized by anti-Jo-1 antisera. The cloned HRS dDNA has been placed in several vectors in an attempt to product pure antigen for further studies.

3) In situ hybridization, worked out in animal studies, is being adapted to human biopsy specimens, to seek viral nucleic acid.

4) HLA antigens in patients haven been studied by routine methods. Cell lines have been established from a large number of patients. HLA types are being studied by PCR methods.

Major Findings:

1) With computer-assisted analysis, we have established that serologically and pathologically-defined subgroups of patients with IIM differ with respect to rapidity of onset, season of onset, clinical manifestations, and, most important, response to therapy. Some of these observations have been published and several manuscripts are in preparation.

2) We have recently established affinity maturation of the antibodies to HRS in a patient observed early in disease, showing that HRS itself, rather than a molecular mimic drives this response. We have found also that a subset of patients with anti-Jo-1 antibodies make antibodies to tRNA HIS simultaneously. Attempts - so far, none successful - have been made to express cloned cDNA of HRS in bacterial expression systems.

Significance to Bio-Medical Research and the Program of the Institute

The recognition of subsets may clarify the etiology and pathogenesis of this disease family and shed light on a number of closely related rheumatologic diseases. Establishing that an autoantibody response is antigen-driven and related to disease activity enforces the view that specific etiologic agents should be sought.

Future Course:

We will continue to gather patient clinical, serologic, and epidemiologic data and use the computer to help analyze them. In situ studies on putative positive tissue will continue as will the development of a PCR adapted to fixed biopsy specimens. The epitope analysis of Jo-1 will continue using cloned protein. The analysis of about 50 human human biopsy specimens for 5 candidate viruses in underway by PCR and ISH.

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Plotz PH, Dalakas M, Leff RL, Love LA, Miller FW, Cronin ME. Current concepts in the idiopathic inflammatory myopathies: polymyositis dermatomyositis, and related disorders, Ann Int Med 1989;14;143-57.

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Miller FW, Love LA, Barbieri SA, Balow JE, Plotz PH. Lymphocyte activation markers in idiopathic myositis: Changes with disease activity and differences among clinical and autoantibody subgroups. Clin Exp Immunology, in press, 1990.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41075-03 ARB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Picornavirus-induced chronic inflammation

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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	Lori Love, M.D., Ph.D.	Expert	ARB/NIAMS
	Richard Leff, M.D.	Sr. Staff Fellow	ARB/NIAMS
	Frederick Miller,M.D., Ph.D.	Expert	ARB/NIAMS

COOPERATING UNITS *(if any)*

none

LAB/BRANCH
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Connective Tissues Diseases Section

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
1.1	1.1	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

We have continued to study the pathogenesis of EMC-221A-virus initiated inflammation in mice. We are currently assaying tissues from mice by the more sensitive PCR technique to extend earlier observations made by in situ hybridization. We have extended studies on the relationship between viral persistence and pathogenesis by experiments with nude mice.

Project Description

Objectives:

To understand the pathogenesis of a chronic inflammation initiated by a short-lived virus in an attempt to understand how an infectious agent can initiate a self-sustaining, apparently autoimmune illness.

Methods:

We track virus by direct hybridization of tissue extracts and of tissue sections (in situ hybridization) using radioactive RNA probes. We have developed PCR for detecting single-stranded viral RNA. PCR has been used to expand a cDNA of the viral genome. Studies have been performed on T-cell deficient nude mice to further probe the relationship between viral persistence and pathogenesis.

Major Findings:

Nude mice, which have an impaired immune response due to T cell deficiency, have lower anti-viral antibody titres, much longer viral persistence, much more severe myocarditis and more severe central nervous system disease than congenic normal mice. Thus, continued inflammation appears to correlate with the presence of persistent viral genome in this model of apparently autoimmune inflammation.

Significance to Bio-Medical Research and the Program of the Institute:

Understanding the transition from acute viral infection to chronic, apparently self-sustaining inflammation should shed light on how a virus could initiate a human autoimmune disease.

Future Course:

To seek viral genome in infected animals by increasingly sensitive methods, particularly PCR, and to seek viral protein by sensitive immunologic techniques. This work depends in part on sequencing the genome of EMC-221A, a project which is underway. We will attempt to make infectious clones of EMC-221A so that the exact structures critical for infection and persistence can be recognized.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 AR 41076-03 ARB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Therapeutic trials in idiopathic inflammatory myopathies

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

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M. Dalakas		NINDS
S. Leitman		CC Blood Bank
J. Hicks		CC Rehabilitation

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TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2	2	

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☐ (a1) Minors

☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Severe idiopathic inflammatory myopathies (polymyositis, dermatomyositis, and related diseases) that respond poorly to corticosteroids are difficult to treat. We have undertaken three therapeutic trials for the treatment of such patients: a) a controlled double-blind trial of phasmapheresis and leukapheresis; b) a controlled, crossover trial of intravenous methotrexate with leucovorin rescue and a combination of methotrexate and azathioprine; c) an open trial of i.v. cyclophosphamide (completed).

The trial of apheresis has just been closed after 39 patients have completed it. Analysis of the results is underway. The trial of i.v. methotrexate and combination therapy continues, although patients with inclusion body myositis are no longer being entered because of poor results in the first 12 patients randomized.

Project Description

Objectives:

1. To determine whether plasmapheresis or lymphopheresis improves the course of severe polymyositis or dermatomyositis.

3. To determine whether methotrexate given intravenously or in combination with azathioprine improves the course of patients unresponsive to apheresis and/or conventional therapy.

Methods

a) Patients with PM or DM resistant to steroids or in whom severe steroid side-effects are present are randomized to 4 weeks (12 procedures) of phasmapheresis, lymphapheresis, or a sham procedure. Careful testing is done to determine the effect of therapy.

b) Patients who fail apheresis, are ineligible for apheresis, or who have inclusion body myositis are randomized to receive intravenous methotrexate or a combination of oral methotrexate and azathioprine. Those who have deteriorated at three months or who have failed to improve by six months are crossed over to the other therapy.

Major Findings:

1) The apheresis trial has been closed after entry of 39 evaluable patients. Analysis of the results is underway.

2) After 12 patients with IBM entered the trial of i.v. methotrexate and combination therapy, it was decided to stop because of poor outcomes with both therapies in the amjority of patients.

3) Patients with polymyositis and dermatomyosisis will continue to enter the trial.

4) The trial of i.v. cyclophosphamide is complete and published.

Significance to Bio-Medical Research and the Program of the Institute

This is an attempt to improve the therapy of this very serious illness and to allow better study of its pathogenesis.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41077-03 ARB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Regulation of gene expression in normal and impaired fracture healing

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Mark E. Bolander, M.D.	Senior Staff Fellow	ARB/NIAMS
	Toshi Izuma, M.D., Ph.D.	Visiting Fellow	ARB/NIAMS
	Sean Scully, M.D., Ph.D.	IRTA Fellow	ARB/NIAMS
	Tracy Ballock M.D.	Clinical Associate	ARB/NIAMS
	Ahlke Heydemann	Biologist	ARB/NIAMS

COOPERATING UNITS *(if any)*
Dept. of Orthopaedic Surgery, Georgetown University
Laboratory of Chemoprevention, NCI

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Orthopaedic Research Section

INSTITUTE AND LOCATION
NIAMS, NIH Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
4.25	3.25	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☒ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Investigations in the Orthopaedic Research Unit have focused on defining the events that regulate gene expression during repair. New projects include the development of a model to study cartilage repair after injury or in degenerative and traumatic osteoarthritis, and investigations of the role of growth factors in the development of tendon adhesions. Studies of TGF-beta demonstrate that this factor is an important regulator of cell proliferation, differentiation and protein synthesis during fracture healing. TGF-beta stimulates changes in chondrocyte development and gene expression associated with chondrocyte maturation in the fracture callus in vitro. TGF-beta stimulated mesenchymal cell proliferation and differentiation into both osteoblasts and chondrocytes with subsequent synthesis of cartilage and bone matrix respectively in vivo. TGF-beta 1, but not TGF-beta 2, is synthesized at high levels in both human and rat fracture calluses. TGF-beta stimulates chondrocyte differentiation and an increase in chondrocyte-specific gene expression in cultured periosteal cells. Studies of cartilage explant cultures demonstrate that, in the absence of inflammatory and synovial influences, serum stimulates cartilage chondrocytes to proliferate, synthesize new matrix, and initiate a repair response. This repair response is duplicated by the administration of basis FGF, but not TGF-beta. Antibodies to TGF-beta, and PDGF stained the tendon and tendon sheath after laceration in a chicken model. Both TGF-beta and aFGF stimulated a biphasic increase in tenocyte proliferation. In contrast, PDGF stimulated a 3 to 5-fold increase in cell proliferation. TGF-beta stimulated a 6-fold increase in matrix synthesis by proline incorporation.

OBJECTIVES:

To conduct basic investigation at the cellular and molecular levels into the pathophysiology of bone formation, remodeling, and repair.

METHODS AND MAJOR FINDINGS:

•The role of Transforming growth factor-ß in the regulation of fracture repair.

TGF-ß 1, but not TGF-ß2, is synthesized at high levels in both human and rat fracture calluses. Twenty-four hours after fracture there was a 125% increased in expression of TGF-ß2 in rats that returned to baseline levels by the fourth day after fracture. Immunohistology localized TGF-ß2 to the hematoma for up to 3 days after fracture. TGF-ß2 antibodies stained only osteoblasts in the human fracture callus. Consistent with the hypothesis that periosteum is a source of undifferentiated precursor cells for chondrocytes in the fracture callus, TGF-ß1 stimulated chondrocyte differentiation and a 3-fold increase in chondrocyte-specific gene expression in cultured periosteal cells.

• A model for studying cartilage repair after laceration.

Studies of cartilage explant cultures using a bovine model demonstrate that serum, in the absence of inflammatory and synovial influences, stimulates cartilage chondrocytes to proliferate, synthesize new matrix, and initiate a repair response. Immunostaining demonstrates the presence on cartilage-specific collagen, proteoglycan and chondrotin sulfates in the healing region. In situ hybridization demonstrates the presence of mRNA for cartilage-specific type II collagen, but no mRNA for type I collagen, in cells in the healing ares. This repair response is duplicated by the administration of basic FGF, which stimulates both cell proliferation and matrix synthesis, but not TGF-ß, which inhibits cell proliferation.

• A model for studying adhesion formation following flexor tendon injury.

Antibodies to TGF-ß, aFGF, and PDGF stained the tendon and tendon sheath after laceration in a chicken flexor tendon injury model. Both TGF-ß and aFGF stimulated a biphasic increase in tenocyte proliferation. In contrast, PDGF-BB stimulated a 3 to 5-fold increase in cell proliferation. TGF-ß stimulated a 6-fold increase in matrix synthesis as demonstrated by proline incorporation.

• Fracture healing in osteoporosis.

Studies of fracture repair in ovarectomized female rate have demonstrated a dose-dependent increase in fracture strength after estrogen treatment. Thirty days of treatment with estrogen (50 ug/kg/day) resulted in a significant increase in tensile strength (30%), stiffness (50%) and ultimate strength (35%) compared to fractures from untreated controls ($p<0.04$). Histological evaluation suggested that the difference in strength was due to increased endochondral bone formation.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

The morbidity and mortality associated with nonunion of fractures, especially in the elderly population, are significant. Although little is known about the pathophysiolosy of impaired fracture healing, investigations at the cellular and molecular levels into the pathophysiology of bone repair are adding to our understanding of, and potentially our ability to treat, these significant problems.

Previous studies have failed to demonstrate a healing response in intact cartilage matrix. The demonstration of cell proliferation, migration, and matrix synthesis in intact cartilage allows further studies of the potential for cartilage to repair lesions of the articular surface.

Recovery of function after a Zone II flexor tendon injury is dependent upon healing of the tendon and the prevention of adhesions. Restrictive adhesions may form as a result of excess growth factor localized to the epitenon. Localization of growth factors in the epitenon and an increase in collagen synthesis in response to these growth factors suggest the identification of a mechanism for adhesion formation. A greater understanding of this mechanism may allow the inhibition of adhesions without interfering with the process of tendon repair.

FUTURE COURSE:

Recombinant DNA techniques have continued to show great potential in our initial investigations of normal and impaired fracture repair. Additional studies are increasing our understanding of regulation in normal and impaired healing conditions, and should suggest possible treatment options.

The laboratory is currently developing a variation of the polymerase chain reaction termed, "competitive PCR", which will allow us to improve the quantitative analysis of changes in mRNA for growth factors during fracture repair. Using this method we should be able to calculate absolute mRNA levels for these proteins.

FUTURE COURSE:

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The laboratory is currently developing a variation of the polymerase chain reaction termed, "competitive PCR", which will allow us to improve the quantitative analysis of changes in mRNA for estrogen receptor during fracture healing. Using this method we should be able to calculate absolute mRNA levels for estrogen receptor or other proteins of interest.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41079-02 ARB

PERIOD COVERED: October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*

Role of the Central Nervous System in Susceptibility to Arthritis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. - Esther M. Sternberg, Visiting Scientist, ARB, NIAMS; Guest Researcher, CNB, NIMH
Ronald L. Wilder, Senior Investigator, ARB, NIAMS
Philip W. Gold, Chief, CNE, DIRP, NIMH
George P. Chrousos, Senior Investigator, DEB, NICHD
Aldo Calogeros, Guest Researcher, CNE, DIRP, NIMH
Harvey Whitfield, Medical Officer (Research), CNE, DIRP, NIMH
John Glowa, Research Psychologist, CNE, DIRP, NIMH
Mark Smith, Medical Officer (Research), CNE, DIRP, NIMH
Sofia Aksentijevich, IRTA Fellow, NIAMS; Guest Researcher, CNB, NIMH
Piotr Zelazowski, Visiting Fellow, NIAMS; Guest Researcher, CNB, NIMH
Leslie Crofford, Medical Staff Fellow, ARB, NIAMS
Craig Smith, Chemist, CNB, NIMH and Rachel Caspi, Senior Investigator, NEI

COOPERATING UNITS *(if any)*

CNE; DIRP; NIMH; DEB; NICHD; LCB; NIMH; NEI

LAB/BRANCH: ARB, NIAMS/CNB, NIMH

SECTION: Inter-Institute Unit on NeuroEndocrine Immunology and Behavior

INSTITUTE AND LOCATION: NIAMS and NIMH

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.20	2.00	.20

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

I. An animal model for susceptibility to inflammatory disease and abnormal behavioral responses to stress, related to a defect in regulation of hypothalamic-pituitary-adrenal responses to inflammatory and stress mediators.

Summary, Project Description, and Directions of Research:

A negative feedback loop exists between the immune and central nervous systems, in which immune/pro-inflammatory mediators signal the hypothalamic corticotropin releasing hormone (CRH) neuron to promote pituitary-adrenal activation and, hence, glucocortioicd mediated restraint of the immune response. We have previously found that increased susceptibility to streptococcal cell wall (SCW)-induced arthritis in the Lewis (LEW/N) rat is related to a defect in the central component of this negative feedback loop, resulting in deficient CRH responses to challenge with a variety of inflammatory mediators, including streptococcal cell wall peptidoglycan polysaccharide (SCW), interleukin-1 alpha (IL-1 alpha) , or the serotonin agonist quipazine. The relative arthritis resistance of F344/N rats compared to LEW/N rats is related to their intact HPA axis responses to these same inflammatory mediators. Physiologic replacement doses of corticosteroids (dexamethasone) in LEW/N rats significantly suppressed the severity of the arthritis in this arthritis susceptible strain, while interruption of the axis in F344/N rats with the corticosteroid receptor antagonist RU486 was associated with development of severe systemic inflammation and arthritis in response to SCW in this otherwise inflammatory disease resistant strain. Interruption of the F344/N HPA axis at the level of serotonin stimulation, with the serotonin antagonist LY53857, was also associated with development of arthritis. Thus, whether present on a genetic basis, as in LEW/N rats, or on a pharmacologic basis, as in RU486 treated F344/N rats, we have shown that interruptions of the HPA axis are associated with susceptibility to inflammatory disease. The deficient

ACTH and corticosterone responses in LEW/N rats are secondary to a profound defect in regulation of CRH biosynthesis and secretion rather than to a defect in the CRH gene.

Over the past year our work has focussed on:

(1) defining the biochemical, functional, neuroanatomical and molecular extent of the defect in LEW/N rats which renders them susceptible to inflammatory disease;
(2) defining the range of stimuli to which LEW/N rats express defective HPA axis responses (including behavioral stresses).
(3) defining the genetic mode of transmission of the phenotypes of arthritis susceptibility and HPA axis function in LEW/N x F344/N F1 and F2 offspring, in conjunction with probing LEW/N and F344/N parent rats for RFLP's of candidate genes.
(4) defining the ontogeny of CRH expression and responses and regulation in LEW/N and F344/N rats.

Studies in progress and new findings:

(1) Biochemical, neuroendocrine and neuroanatomical extent of the central nervous system defect associated with susceptibility to arthritis and inflammatory disease in the LEW/N rat. Since our previous studies have indicated a defect in regulation of CRH biosynthesis and secretion, studies currently under way focus on evaluation of CRH regulatory mechanisms:

•LEW/N rats, compared to F344/N, rats exhibit:
- •profound defects in hypothalamic CRH secretion in response to the neurotransmitters NE, ACh, 5-HT and quipazine (5-HT agonist),
- •corresponding profound blunting of plasma ACTH and corticosterone in response to i.p. arecholine (muscarinic ACh agonist).

•Alpha adrenergic, serotonergic and muscarinic cholinergic binding affinity and receptor numbers (Kd and Bmax) are identical in LEW/N rats and F344/N rats.

These studies indicate a broad defect in CRH responses to a variety of neurotransmitters, and suggest that the defect in CRH regulation is at the level of a common regulatory pathway for all these stimuli. This could include second messenger systems such as cAMP or components in the cAMP pathway, or glucocorticoid-receptor related mechanisms.

(2) Functional and receptor binding studies of glucocorticoid feedback in LEW/N and F344/N rats:
Compared to F344/N rats, LEW/N rats exhibit:
- • decreased basal secretion of ACTH from pituitary cells and pituitary explants in culture.
- •lower absolute ACTH secretion from pituitary cells and pituitary explants in culture.in response to CRH stimulation, but no difference in percent ACTH secretion when corrected for lower baseline.
- •greater sensitivity to corticosterone suppression of ACTH secretion by pituitary cells in culture.

- •significantly fewer Type 1 and somewhat fewer Type 2 glucocorticoid receptors than F344/N rats.
- •significantly higher affinity Type 1 glucocorticoid receptors (mineralocorticoid, MR) than F344/N rats.

Taken together, these data indicate that LEW/N rats may express a high affinity Type 1 glucocorticoid receptor which could act to inappropriately excessively negatively regulate CRH. Although at this time it is not clear whether this difference in GR and MR binding and function is primary or secondary in LEW/N rats, it could account for the differences in susceptibility to inflammatory disease and the differences in responses to behavioral responses to stresses in the two strains.

(3) Behavioral characteristics of SCW-arthritis susceptible LEW/N rats and SCW-arthritis resistant F344/N rats:

We have found behavioral differences as well as differences in neuroendocrine responses to behavioral stressors in LEW/N and F344/N rats, consistent with their relative hypo- and hyper-CRH responsiveness:

•LEW/N rats exposed to a variety of behavioral stresses, including swim stress, restraint or ether stress exhibit profoundly blunted plasma ACTH and corticosterone responses to these stresses compared to F344/N rats

•In open field studies LEW/N and F344/N rats show differences in behavior consistent with their relative deficient or increased CRH responses to the new environment of the open field.

•Behavioral assessment of LEW/N and F344/N rats after intra-cerebroventricular (i.c.v.) administration of CRH indicate that LEW/N rats are more sensitive to the behavioral effects of low dose CRH than are F344/N rats. This is consistent with chronic under-secretion of CRH in LEW/N rats. These studies are currently under way in collaboration with Dr. John Glowa (NIMH).

(4) Ontogeny of HPA axis responses and CRH, GR and MR gene expression expression in LEW/N and F344/N rats.

In functional, stimulation studies in LEW/N and F344/N rats at days 3, 7 and 14 post-natal, we have found that:

• in LEW/N rats, plasma corticosterone, hypothalamic paraventricular nucleus CRH and hippocampal MR mRNA expression levels remain at "stress un-responsive period" levels, while these measures of HPA axis responsiveness increase to their adult levels in HSD and F344/N rats by post-natal day 14.

This data indicates that the CRH regulatory defect in LEW/N rats is present early in life, may reflect a developmental defect involving factors important in governing the development of and evolution out of the normal post-natal "stress un-responsive period".

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO THE PROGRAM OF THE INSTITUTE:

SIGNIFICANCE TO BIOMEDICAL RESEARCH: Taken together, our data provide compelling evidence for a general principle underlying susceptibility to inflammatory and autoimmune disease: that the defective HPA axis response to inflammatory and immune mediators is a critical element in susceptibility to inflammatory/autoimmune disease. Thus the appropriate immune response genes allow the organism to recognize and react to antigenic or pro-inflammatory triggers with an appropriate immune/inflammatory response, while an intact HPA axis modulates the intensity of that immune/inflammatory response. The corresponding defective behavioral and HPA axis responses to behavioral stressors of LEW/N rats suggest that both susceptibility to inflammatory disease and non-adaptive behavioral responses to stress

may be related to the same underlying neuroendocrine defect. The ontogeny studies we have performed provide the basis for suggesting that these non-adaptive responses may occur very early in development in affected strains.

The studies outlined here are designed to determine the molecular mechanism of the HPA axis defect which leads to susceptibility to arthritis and defective behavioral responses to stress in LEW/N rats. If the principle also applies to susceptibility to autoimmune/inflammatory and behavioral disorders in humans, it will provide new avenues for identifying individuals at risk for development of inflammatory disease as well as potential new therapeutic approaches to autoimmune/inflammatory and associated affective disorders. This model also makes feasible identification of the molecular and genetic defect(s) in the central nervous system which are associated with susceptibility to autoimmune/inflammatory diseases. For the first time it provides a controllable experimental system to examine the interactions between the immune and the central nervous systems at a molecular level and to elucidate at a molecular level the relationship between behavior and susceptibility to inflammatory disease.

SIGNIFICANCE TO THE PROGRAM OF THE INSTITUTE: The work outlined here accomplishes the fundamental goals of the NIMH, in that it addresses one of the most basic problems in the field of mental health research: the elucidation of the relationship of stress and the stress response to susceptibility to medical diseases such as immune/inflammatory diseases. The Inter-Institute Unit between NIMH and NIAMS which has been developed as a result of this work, is ideally suited to accomplish these goals, through the collaborative efforts of scientists from a broad range of disciplines. It provides the basis for the formal establishment of links between the NIAMS and other Institutes whose mandates involve elucidation of susceptibility to diseases covered by this paradigm. The ontogeny work, if applicable to humans, suggests that susceptibility to autoimmune/inflammatory diseases and associated affective disorders may have an early developmental component, and thus these disorders should be addressed in childhood.

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2. L-Tryptophan related Eosinophilia Myalgia Syndrome:

We have observed a syndrome in 9 patients taking L-tryptophan (L-TRP) (1), identical to the one described by one of us (Dr. Esther Sternberg, 2) in 1980 in a patient taking L-5-hydroxytryptophan (L-5HTP) and carbidopa, and closely resembling the tryptophan related eosinophil myalgia syndrome reported now in epidemic form in over 1500 patients across the United States. This syndrome tends to occur in patients who use tryptophan to correct sleep disturbances in the context of depressive disorder. The patients present with fever, eosinophilia, myalgias, myositis, scleroderma-like skin fibrosis and fasciitis. The precise environmental trigger in the eosinophilia-myalgia syndrome (EMS) is not known, although epidemiological data suggest that a contaminant of the L-TRP preparation may be implicated in the etiology of the syndrome.

(1) In our first study in humans, published in the New England Journal of Medicine, in collaboration with Dr. Melvyn Heyes (NIMH) we found:

•elevation of plasma kynurenine and plasma quinolinic acid, and normal tryptophan kinetics after tryptophan loading in patients compared to controls;

•this biochemical data suggests a pattern of tryptophan metabolites consistent with activation of the enzyme indoleamine 2,3-dioxygenase (IDO)

• Since IDO can be activated by inflammatory agents, these findings are consistent with initiation of the syndrome by an inflammatory trigger.

• 6 of the 9 patients we studied were also receiving drugs which we have shown suppress the hypothalamic-pituitary-adrenal (HPA) axis (6, and section ???), or had intrinsic suppression of the HPA axis.

• In light of these observations and the association we have recently described between HPA axis hypo-responsiveness and susceptibility to inflammatory disease in an animal model (7,8), it is conceivable that patients with L-TRP EMS developed the syndrome because of a confluence of factors:

•exposure to an environmental inflammatory trigger (which could be the contaminant(s) in the L-TRP preparation)
•together with simultaneous intrinsic or pharmacologic suppression of the HPA axis by drugs such as benzodiazepines.
•the well known effects of tryptophan and its metabolites on fibroblast proliferation, vascular permeability, vasospasm and neurotoxicity, could also contribute to the pathogenesis of the syndrome.

(2) In our second study, in Lewis (LEW/N) rats we have found that case-associated L-TRP, but not pure non-case associated L-TRP or vehicle control, (preparations provided to us blind by the CDC and FDA),was associated with:

•development of many of the specific pathologic changes in muscle and fascia characteristic of EMS, in the absence of development of eosinophilia.
•suppression of plasma corticosterone.
•no difference in L-TRP metabolism between groups.

This is the fist clear-cut evidence that contaminated L-TRP triggers the syndrome. This animal model now provides the means to:

•identify the role of specific purified contaminants in triggering the syndrome.
•identify host factors pre-disposing certain individuals to develop the syndrome.
•identify the role of the HPA axis in susceptibility to the syndrome.
•define the role of eosinophils in amplifying the syndrome.
•evaluate new approaches to treatment of the syndrome.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND TO THE PROGRAM OF THE INSTITUTE:

SIGNIFICANCE TO BIOMEDICAL RESEARCH: This work takes full circle my initial observation and description of the syndrome published in the New England Journal of Medicine in 1980, which is recognized by the scientific community to be the first description of

this syndrome in the scientific literature. The current series of studies define the full clinical spectrum of the EMS syndrome; define L-TRP biochemistry in the syndrome and show that the difference in L-TRP metabolism in EMS patients is secondary to inflammation or to an inflammatory stimulus rather than to an inborn error of metabolism; it suggests that an important host factor in susceptibility to the syndrome is a suppressed HPA axis; it establishes the role of contaminant(s) as the etiological trigger in the syndrome; it establishes an animal model in which to further delineate the complex cellular and biochemical mechanisms active in the pathogenesis of the syndrome; it establishes an animal model which further supports the role of the importance of HPA axis suppression in development of the syndrome; it provides an animal model in which specific contaminants can be tested and chemical structures capable of inducing this and similar syndromes can be defined; it provides an animal model which can be used for testing new approaches to therapy of the syndrome.

SIGNIFICANCE TO THE PROGRAM OF THE INSTITUTE: The recent epidemic of EMS has been deemed by the FDA and CDC as a national health crisis, not only because of the numbers of patients affected (now estimated between 5,000 and 10,000), and the numbers of deaths (26), but also because of the impact of this occurrence on the use of recombinant technology in pharmaceutical production in general, since production of L-TRP and possibly of the contaminant(s), involved recombinant technology. We have, in a period of 9 months progressed from public recognition of the syndrome, in November 1989, to resolution of many of the etiologic and pathogenetic factors in EMS and establishment of an animal model for EMS by July 1990. This was accomplished by a rapid and concerted joint collaborative effort involving the NIMH, NIH, FDA and CDC. As a result, the Institute has gained wordwide recognition for rapidly resolving a major health crisis, and for rapidly providing an animal model which can now be used to further elucidate pathogenetic mechanisms and treatment modalities in this and related syndromes. Since this syndrome is a model for idiopathic inflammatory fibrosing syndromes, particularly eosinophilic fasciitis and scleroderma-like syndromes, this work represents a major contribution to elucidation of the pathogenesis of these syndromes, and as such accomplishes a major mandate of NIAMS.

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DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41080-02 ARB

PERIOD COVERED
September 30, 1990 to October 1, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
The use of MRI to detect inflammation in muscle of patients with myositis

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Paul H. Plotz, M.D.	Sr. Investigator	ARB/NIAMS
	David Fraser, M.D.	Clin. Associate	ARB/NIAMS
	M. Dalakas		NINDS

COOPERATING UNITS *(if any)*

LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Connective Tissue Diseases Section

INSTITUTE AND LOCATION
NIAMS, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	

CHECK APPROPRIATE BOX(ES)

- [x] (a) Human subjects
  - [ ] (a1) Minors
  - [ ] (a2) Interviews
- [ ] (b) Human tissues
- [ ] (c) Neither

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

In an attempt to provide an improved assessment of muscle inflammation in patients with myositis, we have evaluated MRI. In a group of myositis patients, muscle biopsy, laboratory tests, and the "STIR" image of the thighs were compared to an assessment of clinical disease activity. MRI was more sensitive than biopsy in detecting inflammation in these patients. The extent of muscle inflammatory changes could be assessed in patients since the entire muscle group is imaged. Magnetic resonance spectroscopy allows measurement of the metabolic state of muscle cells. Profound abnormalities of inorganic phosphate/creatine phosphate have been found in inclusion body myositis and in the tryptophan-induced eosinophilia-myalgia syndrome, and lesser abnormalities in polymyositis and dermatomyositis.

Project Description

Objectives:

To assess the extent of muscle inflammation in patients with inflammatory muscle disease.

Methods:

STIR (inversion recovery) images were performed on the thighs of patients with myositis. Biopsies, clinical charts, laboratory tests and MRI results were scored and compared by standard statistical methods. Magnetic respnance spectroscopy was performed on the thighs of patients with a variety of muscle diseases.

Major Findings:

In a group of 38 patients studied so far, MRI scores correlated well the clinical disease activity. They were positive in 80% of patients thought to be clinically active, compared to 61% of positive biopsies. Thus, some patients had positive MRI with negative biopsy done at about the same time. Furthermore, the extent of inflammation and atrophy over the entire thigh could be observed. Over 50 normal volunteers and patients with a variety of muscle diseases have been studied by magnetic resonance spectroscopy. In two diseases - inclusion body myositis and eosinophilia-myalgia syndrome - major metabolic abnormalities are found by alternation of the inorganic phosphate to creatine phosphate ratios.

No abnormalities of STIR or MRS have been found in patients treated with corticosteroids for other conditions.

Significance to Bio-Medical Research and the Program of the Institute:

A method which can assess the extent of inflammation in myositis without involving a surgical procedure and which can be repeated would greatly assist planning therapy in inflammatory muscle disease.

Future Course:

We will continue to gather data on myositis patients and patients with other diseases.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41081-02 ARB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structure and Function of the Fc Receptor

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. Jean-Pierre Kinet, M.D. Visiting Scientist ARB/NIAMS

COOPERATING UNITS *(if any)*

LAB/BRANCH

SECTION

INSTITUTE AND LOCATION

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☐ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

This project has been discontinued - Dr. Kinet has moved to NIAID. The number of his Annual Report in NIAID is: Z01 AI 00594-01 ODIR

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41083-01 ARB

PERIOD COVERED
April 1, 1990 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Genetics of Familial Mediterranean Fever

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I. Daniel L Kastner, Senior Investigator, Cellular Immunology Section, ARB/NIAMS
Ivona Aksentijevich, IRTA Fellow, Cellular Immunology Section, ARB/NIAMS
Luis Gruberg, Visiting Fellow, Cellular Immunology Section, ARB/NIAMS

COOPERATING UNITS *(if any)*
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LAB/BRANCH
Arthritis and Rheumatism Branch

SECTION
Cellular Immunology Section

INSTITUTE AND LOCATION
NIAMS - Building 6, Room 114, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
2.80	2.80	

CHECK APPROPRIATE BOX(ES)

☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Familial Mediterranean Fever (FMF) is a rheumatic disease caused by a single autosomal recessive gene. Patients with this disorder experience acute attacks of fever, arthritis, abdominal pain, and/or pleurisy; some develop amyloidosis as a long-term complication. The biochemical lesion in FMF, as well as the chromosomal location of the FMF gene, is presently unknown. This project is designed to find the chromosomal location of the FMF gene, and ultimately the gene itself. We have identified 55 Israeli families in which more than one individual suffers from FMF. Blood samples have been obtained from 347 individuals from these families, and Epstein-Barr virus-transformed lymphoblastoid cell lives have been established for each individual. Using cell line DNA from a particularly informative subset of these families, initial mapping studies have been undertaken by probing Southern blots with highly polymorphic DNA markers.

To date we have excluded approximately 10% of the human genome as a potential site for the FMF gene. Large areas of exclusion have been established on chromosomes 1 and 9; smaller exclusionary area have been established on chromosomes 4 and 22. More importantly, we have found a DNA marker which shows modest evidence for linkage to the FMF gene (LOD=1.3 for Theta=0.1). From the data obtained thus far, the odds in favor of linkage to this marker are 20:1; a 1000:1 ratio is required for proof of linkage. Additional studies are in progress to determine whether this marker is in fact linked to the FMF gene.

Project Description

Objectives

The overall objective of these studies is to find the gene which causes the human rheumatic disease Familial Mediterranean Fever. Because there are no biochemical clues to the genetic basis of this disease, we are taking a "reverse genetic" approach to identifying the gene. Such a strategy has been used successfully to identify the genes for several human diseases, including cystic fibrosis and neurofibromatosis. The approach involves the identification of a gene based on its location within the human genome. Location is determined by genetic linkage studies within families of affected individuals.

The first objective of this project was therefore to identify a large panel of FMF families, and to obtain DNA samples from individuals in such families. The second objective is to establish the chromosomal location of the FMF gene through linkage analysis. The third objective is to establish a high resolution genetic map (i.e. 1 cM) and an even higher resolution physical map (i.e. 100 kb) for the relevant area of the genome. Such a map will allow substantial narrowing of the area of interest. The final objective is to screen candidate genes within the genetic area of interest for structural or functional alterations in FMF patients.

Methods Employed

FMF patients have been identified at the Clinical Center of the National Institutes of Health, and at the Sheba Medical Center in Tel Aviv, Israel. Peripheral blood lymphocytes were isolated from venous blood samples from these patients and selected family members. Cell lines for 347 individuals were established using Epstein-Barr virus transformation. Genomic DNA has been isolated from about one half of the cell lines by standard methods. All cell lines have also been stored in liquid mitrogen for future use.

Linkage studies have been performed by probing Southern blots of FMF family DNA with a panel of highly polymorphic DNA markers spanning the human genome. Over 200 such probes have been collected in our laboratory. Data analysis has been accomplished with the LINKAGE package of programs on a VAX computer maintained by the National Cancer Institute in Frederick, Maryland.

High resolution genetic mapping of the chromosomal region of interest will be performed using DNA probes from a chromosome-specific library and a panel of highly informative Mormon families from Utah (provided by Centre d'Etude du Polymorphisme Humain). Physical maps for the region will be constructed by the method of "chromosome jumping" and pulsed field gel electrophoresis.

Candidate genes in the region of interest will be studied by Southern hybridization to detect large scale insertion or deletion events. Standard sequencing methods will be used to study more limited structural polymorphisms. Northern hybridization will be used to detect differential tissue - or individual-specific expression of candidate genes.

Major Findings

The major accomplishments of this project to date are:

1) establishment of a large panel of cell lines from FMF families;

2) definite exclusion of approximately 10% of the human genome as a potential site for the FMF locus;

3) identification of a DNA marker possibly linked to the FMF locus.

Significance to Biomedical Research and Program of the Institute

Identification of the gene for FMF will provide insight into the genetic and biochemical mechanisms of rheumatic disease. This may also allow for the more rational design of pharmacologic agents for this and other rheumatic diseases. Successful characterization of this single-gene disorder will also establish technologies which can be applied in the Institute to rheumatic diseases with a more complex pattern of inheritance, such as systemic lupus and rheumatoid arthritis.

Proposed Course

During the next several months we will determine whether the marker we have identified as possibly linked to the FMF gene is in fact linked. This will be accomplished by studying other markers in the same chromosomal area as the original marker, and by extending our studies to additional families from our panel. If we are able to prove linkage (LOD > 3.0), we will proceed to the fine mapping phase of the project. Otherwise, we will continue to scan the genome until a linked marker is identified.

ANNUAL REPORT

LABORATORY OF SKIN BIOLOGY

NATIONAL INSTITUTE OF ARTHRITIS AND MUSCULOSKELETAL AND SKIN DISEASES

JANUARY 1 1990 THROUGH SEPTEMBER 30, 1990

The Laboratory of Skin Biology was newly formulated during the course of fiscal year 1990 from a research group formerly located within the Dermatology Branch, DCBDC, NCI. The Laboratory of Skin Biology conducts basic research on the structure, function and gene expression of several of the major differentiation products of normal human epidermis, using biophysical, electron microscopic, biochemical, molecular biological and genetic approaches. A major emphasis of this work is directed toward an understanding the possible role(s) of these proteins in keratinizing disorders of the epidermis.

Keratin Intermediate Filaments

Objectives

Terminally differentiating mammalian epidermal cells express large amounts of two intermediate filament (IF) chains, keratins 1 and 10, which are members of the class of IF proteins expressed in the cytoskeletons of virtually all eukaryote cells. These keratins, like all IF chains, have α-helical rod domains of precisely conserved secondary structures, but they have unusual end domains enriched in glycines which presumably define the function of these filaments in cells. This work is directed toward an understanding of the structure and function of the keratin IFs as well as their interactions with other components of the cytoskeleton and cell periphery.

Major Findings

1. Using limited proteolytic digestion- procedures, it was shown that mouse epidermal keratin 1/10 IFs consist of a two-chain coiled-coil dimer molecule consisting of a type I (keratin 10) - type II (keratin 1) heterodimer (Steinert).

2. A synthetic 38-mer polypeptide corresponding to the 1A portion of the rod domain of the type III vimentin IF chain was shown to form a stable two-stranded dimer in solution and is thus now useful for attempts at crystallization for X-ray

crystallography and two-dimensional solution NMR structural studies (Steinert and Mack).

3. Solid state NMR studies on ^{2}H-lysine-labeled keratin IFs reveals that the lysines are rather flexible, confirming and extending earlier views of the flexuous nature of IFs when visualized in vivo and in vitro (Mack).

4. The end domains of keratins 1 and 10 represent one of several types of fibrous and globular proteins that contain glycine-enriched sequences, configured as tandem quasi-repeating peptides, which are thought to fold into a 'glycine-loop' conformation, predicated on the recurrent aromatic residues (Steinert, Mack, Korge, Gan and Steven).

5. A 13.5 kbp fragment bearing the human keratin chain was found to have different glycine-rich sequences from previously published information for this chain. By PCR analysis, it is clear that keratin 10 chains are highly polymorphic with respect to their glycine loop sequences in different individuals within the human population (Korge and Gan).

6. This same genomic fragment for keratin 10 was used for the successful production of transgenic mice, created to study the expression of this gene (Steinert).

7. Certain sequences on the carboxyl-termini of desmoplakin, a major protein component of desmosomes, was shown to be highly homologous to rod domain sequences of keratin IFs, suggesting a possible mode of interaction between the keratinIFs with the desmosomal surface in epithelial cells (Steinert, Parry and Green).

Profilaggrin

Objectives

Filaggrins specifically interact with the keratins IFs of epidermal cells to form highly organized 'macrofibril' structures in vitro. In vivo, it is thought that the proteins are responsible for the proper alignment of the keratin IF during the terminal stages of epidermal differentiation and the formation of a flattened, dead, cornified cell. The filaggrins are initially expressed as large polyprotein precursors (profilaggrins) that are incapable of aggregating IFs, and are subsequently proteolytically processed into individual functional filaggrin molecules by excision of a short hydrophobic 'linker' sequence. The inappropriate expression of profilaggrin has been implicated in several important keratinizing disorders of human epidermis. Therefore, work in this area is directed toward an understanding of the structure, function and expression of this gene system in normal and abnormally keratinizing epidermis.

Major Findings

1. A partial clone encoding mouse profilaggrin has been characterized. It contained up to 24 individual filaggrin repeats that have essentially identical sequences, but there are at least two different 'linker' sequences adjoining

adjacent repeats in the mouse population. These may be important in the subsequent processing of the profilagrrin into indivudal functional filaggrin molecules (Rothnagel and Steinert).

2. By use of cDNA clones and genomic clones, it was shown that the human profilaggrin gen in contrast to mouse filaggrin, the exact sequences of neighboring repeats can vary by as much as 40%. However, the linker sequence has been precisely conserved. Further, the gene system consists of at least three allelic size variants, representing 10, 11 or 12 repeats, that segregate in kindred families by normal Mendelian genetic mechanisms. The genes contain unusual pro-protein sequences at the amino- and carboxyl-termini of profilaggrin that may have important roles in processing (Steinert, Idler, Markova, Gan and McBride).

3. Structurally, human filaggrin is a highly folded molecule with little or no ordered secondary or higher-order structures in vitro. This is as expected for a protein that acts as a matrix. Interestingly, despite the sequence variations, a common structural pattern is always maintained, with positively charged residues usually occupying positions at the apices of the numerous protein β-turns (Steinert).

4. Profilaggrin "minigenes" containing 0 or 3 filaggrin repeats only have been successfully used for the production of transgenic mice, which are now being bred to obtain homozygous animals for further characterization (Gan).

5. Macrofibrils formed in vitro from unlabeled mouse filaggrin and ^{13}C-glycine- and ^{2}H-lysine-labeled mouse keratin IF have been constructed to study the mode of interaction of filaggrin. The molecular dynamics of the glycine-labeled IFs was unchanged, but the lysine-labeled IF became much more constrained. These data establish that filaggrin interacts with the rod domains of the IF, rather than the glycine-rich end domains, probably by simple ionic interactions of the positively charged residues on the protein turns of filaggrin with negative charges commonly found on the rod domains (Mack and Steinert).

Loricrin

Objectives

During terminal differentiation, epidermal cells deposit a layer of protein on the inner surface of the plasma membrane, termed the cell envelope, which contributes to the barrier function of the epidermis. The cell envelope becomes highly insoluble due to crosslinking by transglutaminases that from isodidpeptide bonds and by disulfide bonds. While several putative protein components of the cell envelope have been described, none seems to be a mjor component because of major differences in amino acid compositions and likely properties. However, we have recently described a new protein, loricrin, which is the major component of the cell envelope of epidermal cells, as well as of many other stratified squamous epithelia. We have initiated a major study of this system because very little is known about the structure and function of the cell envelope and its possible role in pathology.

Major Findings

1. A full-length cDNA clone was isolated encoding human loricrin. It is a glycine-rich protein containing the glycine loop motif. Human loricrin is considerably smaller than its mouse counterpart, due to deletions of numerous glycine loop sequences, and because the loops are smaller. However, certain glutamine and glutamine + lysine sequences have been conserved between the two species (Steinert and Hohl).

2. We have shown that human loricrin is highly insoluble due to the presence of disulfide bonds, as well as isodipeptide bonds, that form crosslinks between recognizable loricrin peptide sequences. This is the first demonstration of the presence of these crosslinks involving any protein component of the cell envelope (Steinert and Lichti).

3. The human loricrin gene contains a single intron in the 5'-untranslated region. The gene has been located to chromosome region 1q21, apparently very near two other major epidermal proteins, profilaggrin and involucrin (another putative cell envelope component). Interestingly, a number of epidermal disorders also map near this region (Steinert and McBride).

4. Immunogold techniques have been used to show that loricrin is initally deposited in the granular layer of the epidermis in small round keratohyalin granules (L-granules) before being dispersed and ultimately concentrated at the cell periphery. These granules are morphologically quite distinct from larger irregularly-shaped granules containing profilaggrin (F-granules) that ultimately become tightly associated with the keratin filaments (Steven and Steinert).

Transglutaminases

Objectives

The formation of the cornified cell envelope involves the crosslinking of the protein components by isodipeptide N^{ε}-(γ-glutamyl)lysine bonds that are catalyzed by the action of transglutaminases. Comparatively little is known about the numbers, activities, specificities and functions of these enzymes during epidermal differentiation, or their possible roles in pathology.

Major Findings

1. By Northern blot analyses, there are at least three different transglutaminases expressed in normal human epidermis. These apparently correspond to: a ubiquitous tissue type II or type C activity; a membrane-bound type I or type K activity, originally thought to be found only in cultured keratinocytes, but now known to be present in many different cell types; and a glycine-rich zymogen activity recently located only in differentiating keratinocytes, termed the E type activity. These encode mRNAs of 3.7, 2.9 and about 3.3 kb, respectively (H. Kim, Idler, Chung and Steinert).

2. A full-length cDNA clone encoding the K enzyme has been isolated and characterized. It encodes a protein of 89.3kDa, has sequences containing likely membrane-binding regions, a conserved active site region characteristic of transglutaminases and properties expected for the partially purified enzyme (H. Kim, I. Kim, Han, Idler and Steinert).

3. PCR fragments containing mouse and human transglutaminase E sequences have been isolated using synthetic oligonucleotides designed from the known amino acid sequences of peptides of the purified guinea pig enzyme (I. Kim, Han, Gorman and Chung).

4. Peptides corresponding to the amino- and carboxyl-terminal ends of human loricrin have been used as substrates for the three enzyme activities now known to be present in the epidermis. The Km value for the E enzyme is much lower than for the other enzymes, suggesting a preference in vitro and possibly in vivo, of the E enzyme for loricrin as substrate (H. Kim, Chung and Steinert).

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41084-01 LSB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Structural features of keratin and related intermediate filaments

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Peter Steinert, Ph.D.	Supv. Research Biologist	LSB/NIAMS
	Song Qing Gan, M.D.	Visiting Fellow	LSB/NIAMS
	Bernhard Korge, M.D.	Special Volunteer	LSB/NIAMS
	James Mack, Ph.D.	Sr. Staff Fellow	LSB/NIAMS

John Compton - Jackson Laboratories; Kathleen Green, Northwestern
S. Haynes, LMG/NICHK; Marc Lewis, BEIB/NIH; David Parry, Massey Univ., NZ
D. Roop, Baylor College; A. Steven, LSBR/NIAMS; J.S. Wall, Brookhaven Natl. Labs

COOPERATING UNITS *(if any)*
as above

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SECTION

INSTITUTE AND LOCATION
NIAMS, Bethesda, MD 20892

TOTAL MAN-YEARS:	PROFESSIONAL	OTHER
1.7	1.7	

CHECK APPROPRIATE BOX(ES)
☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The structure, function and expression of the keratin intermediate filaments of human and mouse skin, and the related intermediate filament proteins of other cell types, are being investigated. These studies are designed to understand the structural features that determine how the rod domains of the chains pack to form the filament core. Current models are being tested using electron microscopic methods as well as by analysis of the products generated on limited proteolytic digestion of intact filaments of subfilamentous forms of them. The glycine-rich end domains of, especially the keratin 1/10 filaments of epidermal cells, are unique in biology. We believe these organize into a glycine-loop configuration. Current studies are designed to determine how these are packed and how they might interact with other macromolecules co-expressed in epidermal tissues. Using genomic clones to the human keratin chains 1 and 10, transgenic mice have been constructed to examine the expression characteristics of the genes as well as to probe in vivo the likely functions of the various portions of the chains, such as rod domain segments and glycine-rich end domains.

Project Description

Objectives:

1. To determine the packing of the α-helical rod domain segments of the vimentin chains of type III intermediate filaments.

2. To determine the likely secondary, tertiary and possible quaternary structures of a synthetic polypeptide corresponding to the 1A segment of vimentin intermediate filaments,

3. To determine the possible organization and molecular dynamics of the chains of keratin 1/10 intermediate filaments.

4. To determine the possible secondary and likely higher-order structural features of the glycine-rich sequences of the end domains of keratins 1/10 and other related intermediate filamen proteins.

5. To determine how the intermediate filament associated protein and desmosomal protein, desmoplakin, may interact with keratin intermediate filaments.

Methods:

1. Scanning transmission electron microscopy of vimentin filaments disassembled in low ionic strength buffer are being utilized.

2. A 38-mer peptide corresponding to the 1A portion of vimentin has been synthesized and is being studied by sedimentation equilibrium, differential scanning calorimetry and solution state NMR methods.

3. Solid state NMR techniques are being used to determine the molecular dynamics of lysine-labeled and phenylalanine-labeled mouse keratin filaments.

4. Limited proteolytic digestion methods are being used to study the organization of the protein chains of intact or subfilamentous forms of mouse keratin intermediate filaments. Such particles are being analyzed by use of sedimentation equilibrium, sequencing and biochemical techniques.

5. A survey of protein sequence banks was used to search for possible homologies to glycine-rich sequences that are characteristic of keratins.

6. Structral homology studies using existing MATCH algorithms are being used to analyze the possible secondary structures of desmoplakin (and related bullous phemphigoid) sequences.

7. Genomic clones encoding the human keratin 1 and 10 genes are being used to prepare and characterize transgenic mice. The expression of the transgenes of such mice will be studied by use of immunofluorescence, in situ hybridization, Western, Southern and Northern blots of appropriate tissues.

Major Findings:

1. In collaboration with Drs. Steven (LSBR/NIAMS) and Wall (Brookhaven) we have found that unstained subfilamentous forms of vimentin filaments when examined in the scanning transmission electron microscope exist as an array of particles of several distinct conformations that provide important clues on the ways in which the individual protein chains associate to form the filaments. Most particles formed in 5 mM salt solutions correspond to dimers and are either about 50 nm or about 70 nm long, and many are bent or folded about their centers to form chevron-shaped species. These data conform to our earlier predictions and models of assembly, but are different from other reports in the literature which have stated that the smallest possible subfilamentous particles of vimentin in solution are tetramers about 50 nm long. Future work will continue analyses of larger subfilamentous particles, recovered in slightly higher ionic buffers.

2. Drs Mack (LSB) and Lewis (BEIB) have shown by sedimentation equilibrium centrifugation that a 38-mer synthetic polypeptide corrsponding to the 1A portion of the rod domain of hamster vimentin has been shown by to consist almost exclusively of a stable coiled-coil α-helical dimer in solution of 1.0 - 1.25 X PBS. This will now be used for: (a) solution state two-dimensional NMR, and (b) crystallization, in attempts to solve its likely tertiary structures. This will be the first attempt to study a coiled-coil sequence by these methods.

3. We have now shown that α-helix-enriched particles generated from limited proteolytic digests of mouse keratin intermediate filaments can provide useful information on the organization of protein chains in the filaments. Tetramer particles from the 1B portions of the chains were shown to consists of type I-type II heterodimers, thereby establishing for the first time the chain composiiton of the coiled-coil molecule of keratin filaments. Digestion of filaments dissociated at either pH 2.6 or 9.8 reveal an array of particles that should provide further useful information of the organization of the neighboring coiled-coil molecules in the filaments. Such particles will provide data in support of or against the several models that have been proposed in the literature in recent years.

4. We [Drs. Steven (LSBR, NIAMS), Haynes (LMG, NICHD), Korge, Gan and Mack (of LSB)] have proposed that the glycine-rich sequences of the keratin1/10 filaments form a glycine loop conformation, based on the recurrent aromatic residues which are thought to interact strongly which each other to form a hydrophobic "core". If so, the glycine-rich sequences will form a loop configuration. We predict therefore that the glycine residues will be highly mobile and flexible, while the aromatics will be less flexible. Our earlier solid state NMR data have confirmed the high degree of flexibility of the glycines. Attempts are now in progress by Dr. Mack to label keratin filaments in cell culture with both ^{2}H- and ^{13}C-phenylalanine labeled keratin filaments to study their dynamics as well. Because these labeling experiments are difficult (insolubility, toxicity and relatively low abundacy), we will explore the possibility of expressing the full-length cDNA clones we have for mouse and/or human keratins 1 and 10 in appropriate expression vector systems, so as to label the chains more directly, for structural studies on intact reassembled keratin filaments. Interestingly, we have recently discovered similar glycine-loop like sequences in other proteins of the epidermis, notably loricrins, and this sequence motif is also present in another class of proteins, single-stranded RNA binding proteins. We will continue to explore the occurrence and possible

structural features of this sequence motif using the structural and molecular biology approaches (and see below).

5. Using a 13.5 kbp genomic clone encoding the human keratin 10 gene isolated by Mr. Idler, Dr. Korge has further characterized the glycine-rich sequences, and found them to be significantly different from those of our previously-characterized cDNA clone and a published genomic sequence. Using PCR techniques, Dr. Korge is characterizing these sequences from different DNA sources, to determine the basis of this apparent polymorphism, and to determine whether these variations can account for the known allelic size variants of the human keratin 10 gene system. Variations of such sequences on both the amino- and carboxyl-terminal ends of the chain may have an important impact on the structure and function of the chain in the epidermis.

6. Transgenic mice bearing the human keratin 1 transgene have been prepared. in collaboration with Dr. Roop (Baylor). In collaboration with Dr. Compton (Jackson Laboratories), we have now also produced transgenic mice bearing the 13.5 kbp genomic fragment for the human keratin 10 gene. The expression characteristics of the transgene are in progress and will continue for several months. Once we have established that the fragment is correctly expressed in the epidermis, thus indicating that all of the necessary regulatory sequences are contained within the fragment, we will construct mutants to the glycine rich regions in an attempt to explore in vivo the likely structure and function of these sequences. However, because we do not have an antibody which will specifically recognize the human keratin 10 protein in the mouse background, we will have to engineer onto the human gene an additional sequence at the carboxyl-terminal end that will serve as a specific marker for the human gene. Subsequently, we hope to use immunogold techniques to study the expression of the mutant protein constructs.

7. In collaboration with Dr. Green (Northwestern University) and Dr. Parry (Massey University, New Zealand), the near complete sequence of human desmoplakin has been determined. Interestingly, we have found several recurring peptides near the end of this chain which have high degrees of homology with the 1B portion of the rod domain of keratin filament chains. This suggests that such sequences may interact in vivo, and this therefore affords a possible mechanism of attachment of keratin filaments with desmosomes in epidermal (and other epithelial) cells. In order to explore this further, we have constructed a 57-mer synthetic polypeptide to this region. It will be used for microinjection experiments as well as production of specific antibodies.

Significance to biomedical research

A detailed understanding of the structure of keratin (and other related) intermediate filaments is necessary to understand their function in cells, their likely mode(s) of interactions with other cytoskeletal or cell peripheral components, and possible roles in keratinizing disorders of the skin. The use of transgenic systems may provide mouse model systems for the study of certain disorders.

Publications

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Stienert, P.M. (1990). The two-chain coiled-coil molecule of native epidermal keratin interemediate filaments is a type I - type II heterodimer. *J. Biol. Chem.* **265**:8766-8774.

Steinert, P.M. and Liem, R.J.H. (1990). Intermediate filament dynamics. *Cell* **60**:521-523.

Goldman, R.D., Zackroff, R.V. and Steinert, P.M. (1990). Intermediate filaments: an overview. In *Cellular and Molecular Biology of Intermediate Filaments*, ed. by Goldman, R.D. and Steinert, P.M., Plenum Press, New York, pp. 3-17.

Steinert, P.M. and Roop, D.R. (1990). The organization, structure and complexity of intermediate filament genes. In *Cellular and Molecular Biology of Intermediate Filaments*, ed. by Goldman, R.D. and Steinert, P.M., Plenum Press, New York, pp. 353-367.

Steinert, P.M., Mack, J.W., Korge, B.P., Gan, S.-Q., Haynes, S. & Steven, A.C. (1991). Glycine loops in proteins: their occurrence in certain intermediate filaments chains, loricrins and single-stranded RNA binding proteins. *Int. J. Biol. Macromol.* in the press.

Steinert, P.M. and Freedberg, I.M. (1991). Molecular and cellular biology of keratins. in *The Biochemistry and Physiology of the Skin*, ed by. Goldsmith, L.A., Oxford University Press, New York, in the press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41085-01 LSB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Expression, structure and function of filaggrin

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

PI	Peter Steinert, Ph.D.	Supv. Research Biologist	LSB/NIAMS
	Song Qing Gan, M.D.	Visiting Fellow	LSB/NIAMS
	Nedialka Markova, Ph.D.	Visiting Associate	LSB/NIAMS
	James Mack, Ph.D.	Sr. Staff Fellow	LSB/NIAMS
	William Idler, B.S.	Research Chemist	LSB/NIAMS
	Alasdair Steven, Ph.D.	Chief, LSBR, NIAMS	
	O. W. McBride, ' , DCBEC, NCI;	Sherri Bale, EEB, DCE, NCI	
	J. DiGiovanna, M.D. DB, DCBDC, NCI		

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Filaggrin is a major differentiation product of terminally differentiating mammalian epidermal cells, that is thought to be involved in the aggregation and specific alignment of keratin intermediate filaments during the final stages of differentiation. Thus filaggrin is an important example of an intermediate filament-associated protein. We have isolated both cDNA and genomic clones which show that filaggrin is initially expressed as a large polyprotein precursor, filaggrin, that is subsequently proteolytically processed into individual functional filaggrin molecules. In the human system, the precursor consists of 3 allelic size variants, containing 10, 11 or 12 tandem repeats that segregated by normal Mendelian genetic mechanisms. In addition, these repeats show considerable sequence variation, so that any two repeats are only about 85% homologous to each other; so far, we find that of the 324 amino acid residues of the filaggrin repeats, about 40% of the positions are variable. We have constructed genomic fragments for the production of transgenic mice. We have begun a systematic analysis of regulatory sequences that control the expression of this gene system. We are studying the method of interaction of filaggrin with keratin intermediate filaments by use of solid state NMR techniques. Since there are a number of keratinizing disorders of the skin for which there is some evidence involving incorrect expression of the profilaggrin gene, we have begun a systematic search for the possible role of filaggrin in genetic diseases of keratinization.

Project Description

Objectives

1. To determine the structure of the profilaggrin gene in mouse and human epidermis.

2. To determine the likely mechanism of interaction between filaggrin and keratin filaments.

3. To explore the expression of the human profilaggrin gene and mutations of it in normal human and mouse skin, in transfected cells and in the transgenic mouse model system.

4. To characterize the regulatory sequences and factors involved in filaggrin expression in normal and abnormally keratinizing epidermis.

5. To explore the role of filaggrin in various genodermatoses.

Methods

1. Genomic as well as cDNA clones have been isolated from several libraries, subcloned and sequenced by standard procedures.

2. Solid state NMR techniques have been used with unlabeled mouse filaggrin and mouse keratin filaments labeled in vivo or in cell culture with ^{13}C-labeled leucine and glycine, and ^{2}H-labeled lysine.

3. Portions of genomic clones have been joined to form minigenes containing 0 or 3 filaggrin repeats as well as 7 kbp of upstream and 4 kbp of downstream sequences, which have been used for the production of transgenic mice.

4. Various portions of the putative upstream regulatory sequences of the human profilaggrin gene are being subcloned into CAT and other expression vector systems in attempts to identify potential positive and/or negative regulatory sequences.

5. Genetic linkage analyses as well as RFLPs of DNA samples obtained from kindred families of persons with normally keratinizing epidermis, as well as a variety of keratinizing disorders, are being done in order to explore the potential role of filaggrin in these disorders.

Major Findings

1. Dr. Gan has isolated and sequenced several cDNA clones containing one or more filaggrin repeats, as well as two genomic clones that contain sequences encoding the 5'- and 3'-ends of the human profilaggrin gene. We found considerable sequence variations, even between adjacent repeats on the same clone, and can be accounted for by simple single-base changes, yet about 40% of the amino acid positions can vary. Secondary structural studies predict

little overall change in the highly flexible conformation of the various filaggrin sequences. RFLP analysis of several randomly isolated DNA samples, as well as DNA from kindred CEPH families, reveals there are at least 3 allelic size variants in the human population, containing 10, 11 or 12 filaggrin repeats, which segregate by normal Mendelian genetic mechanisms.

2. Dr. Mack has shown that when filaggrin forms tight macrofibril bundles in vitro with keratin intermediate filaments, there is little overall change in the molecular dynamics of the glycine-rich end domains, which indicates that filaggrin does not interact with the filaments through their exposed end domains. However, when ^{2}H-lysine-labeled filaments were used, it appears that the molecular dynamics of the lysine residues is considerably constrained in macrofibrils versus filaments alone, which seems to provide direct evidence for the interaction of the filaggrin with the rod domain portions of the filaments. Thus filaggrin may be interacting with filaments by means of ionic salt bridges. This model seems consistent with the large body of sequence information described above.

3. Using minigene constructs containing 0 and 3 filaggrin repeats of the human profilaggrin gene, we have produced several founder transgenic mice. These are in the process of being bred to produce more F1 animals, as well as ultimately, homozygous mice for the transgenes. These mice will then be characterized in detail for the expression of the human filaggrin gene, by use of in situ hybridization, immunofluorescence using a human filaggrin specific monoclonal antibody, and Northern and Southern blots of various tissues to explore expression characteristics.

4. Selected portions of the profilaggrin gene corresponding to the 5'-upstream, intron, coding and 3'-downstream sequences are being subcloned into several expression vector systems by Drs. Gan and Markova. We are currently using commercially-available CAT vectors, and vectors kindly given us by Dr. Yuspa (LCCTP, DCE, NCI) and Dr Schaffner (Switzerland). Once clones including both + and - orientations are prepared, these will be used for transfection analyses to test possible promoter enhancer activities.

5. Dr. DiGiovanna (DB, DCBDC, NCI) is currently identifying individual patients as well as families of patients with a variety of keratinizing disorders, including Darier's disease, various ichthyoses, epidermolytic hyperkeratoses, and related disorders, for both clinical diagnosis, as well as to collect blood samples. Dr. Bale (DCE, NCI) is coordinating these samples and their transformation with EBV so as to maintain a permanent source of DNA of these patients. In collaboration with Drs. Bale and McBride (LB, DCBDC, NCI) these DNA samples are being used for RFLP and genetic linkage analysis studies to explore the possible role of the profilaggrin gene system in the molecular basis of these genetic disorders. This work is being done in coordination with a consortium of other dermatology investigators interested in genodermatoses.

Significance to biomedical research

Elucidation of the structure and expression of the profilaggrin gene system in both normal and abnormally keratinizing epidermis is crucial to an understanding the function of the gene. Using our clones and antibodies, we

are now in a unique position for the first time to initiate studies on the likely involvement of this gene system in a number of genodermatoses.

Publications

Rothnagel, J.A. and Steinert, P.M. (1990). The structure of the gene for mouse filaggrrin and the composition of the repeating units. *J. Biol. Chem.* **265**:1862-1865.

Gan, S.-Q., Idler, W.W., Markova, N., McBride, O.W. and Steinert, P.M. (1990). The structure and polymorphisms of the human profilaggrin gene. *Biochemistry*, in the press.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41086-01 LSB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Expression, structure and function of loricrin, a major cell envelope protein

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Peter Steinert, Ph.D.	Supv. Research Biologist	LSB/NIAMS
	William Idler, B.S.	Research Chemist	LSB/NIAMS
	In-Gyu Kim, M.D., Ph.D.	Visiting Fellow	LSB/NIAMS
	Kozo Yoneda, M.D., Ph.D.	Visiting Fellow	LSB/NIAMS
	U. Lichti, Ph.D.	Sr. Staff Fellow	LCCTP, DCE, NCI
	D. Roop, Ph.D.		Baylor College of Med.
	A. Steven, Ph.D.	Chief, LSBR	NIAMS

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☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

The cell envelope constitutes a thick layer of highly insoluble protein on the inside of the plasma membrane of cornified epidermal cells and of other stratified squamous epithelial cells. Of several putative protein components, none has hitherto proven to be a major component because of major differences in amino acid composition, levels of mRNA or of expressed protein. We have recently identified a new protein, termed loricrin, that fulfills all of the requirements of a major constituent of the cell envelope. Loricrin clones were first identified in a mouse epidermis, and now in more detail, from human epidermis. Loricrins are glycine-rich proteins that contain the highly flexible glycine loop motif. They are crosslinked in cell envelopes by isodipeptide $N^{epsilon}$-(gamma-glutamyl)lysine bonds. The possible role of loricrins in keratinizing disorders of epidermis is being investigated by transgenics, PCR, RFLP and genodermatoses analyses.

Project Description

Objectives

1. To characterize the crosslinking of loricrin to the cell envelope.

2. To characterize the biosynthesis of loricrin in the epidermis.

3. To study the structural organization of the possible glycine loop motif of loricrin and its possible interaction with other cytoplasmic constituents.

4. To isolate and characterize the human loricrin gene system.

5. To determine the expression characteristics of loricrin in transgenic mice.

6. To determine the possible role of loricrin in keratinizing disorders of the epidermis.

Methods

1. Protein chemical methods are being used to isolate, purify, and sequence peptides containing isodipeptide crosslinks.

2. Specific antibodies to mouse and human filaggrins and loricrins are being used for immunogold analysis and indirect immunoflourescence.

3. Secondary structural predictive and NMR techniques are being used.

4. Standard molecular biology techniques are being used to isolate and characterize genomic fragments carrying the human loricrin gene, which will subsequently be used for the production of transgenic mice.

5. Both skin and blood samples are being collected from patients with keratinizing disorders of the epideremis to use RFLP, PCR and genetic linkage analyses.

Major Findings

1. In collaboration with Dr. Lichti, we have pulse-labeled newborn human (foreskin) epidermis with ^{3}H-lysine and have isolated labeled peptides containing isodipeptide crosslinks. Limited manual amino acid sequencing of several of these peptides has shown the presence of recognizable loricrin peptide sequences. This established that loricrin is (a) directly crosslinked to the cell envelope by such bonds and (b) supports our notion that loricrin is a major component of the cell envelope. Further studies are in progress to isolated additional crosslinks to establish kinetic information about the deposition and subsequent crosslinking of loricrin and other putative protein components of the cell envelope.

2. Using specific antibodies to mouse filaggrin and mouse loricrin, we (Drs Steven, LSBR/NIAMS and Roop, Baylor) have used immunogold techniques to study the deposition of these two proteins during terminal stages of epidermal differentiation. We have found that filaggrin labels large irregularly shaped keratohyalin granules, termed F-granules. Loricrin antibodies label small round granules that we termed L-granules. The proteins of the two granules have different fates, however: loricrin subsequently becomes deposited on the intracellular side of the cell periphery, whereas filaggrin is located between the keratin filaments of the cells. Further work is in progress to study the co-expression of these two proteins in normal as well as abnormally kratinizing human epidermis.

3. Loricrins contain large stretches of glycine-rich sequences , analogous to the keratins, and we are in the progress to doing NMR structural studies to probe the molecular dynamics of the glycines and recurrent aromatic residues. Using established predictive algorithms, the glycines are likely to be highly flexible, while the aromatics are likely to be less flexible. Loricrins represent another new class of proteins containing the glycine-loop structural motif.

4. Dr. Hohl and Mr. Idler have isolated genomic clones encoding human loricrin. The gene contains an intron only in the 5'-untranslated regions. We have found no sequence variations between several cDNA clones and genomic clones. Yet mouse and human loricrins display major variations in sequence. Dr. Yoneda is currently using PCR methods to further characterize loricrin sequences in different human and other species.

5. Dr. Yoneda is in the process of constructing this gene fragment in a form suitable for use in making transgenic mice, which will subsequently be used to make deletion mutants to explore the function in vivo of the glycine loop sequences.

6. In collaboration with Dr. McBride, the human loricrin gene has been mapped to the 1q21 position of the genome. Since this is also the same locus of the profilaggrin and involucrin genes, futher genetic linkage analyses are in progress to determine the exact linkage of these 3 genes. Since certain epidermal bullous disorders have also been mapped to this locus, we have initiated a collaboration with Drs. DiGiovanna and Bale to gather patient blood material in order to begin genodermatosis research.

Significance to biomedical research

Detemining the structure and expression of the human loricrin gene system is of vital importance in understanding the role of loricrin in normal epidermal differentiatation and in assessing its potential role in keratinizing disorders of the epidermis.

Publications

Mehrel, T., Hohl, D., Rothnagel, J.A., Longley, B., Lichti, U., Bisher, M.E., Steven, A. C., Steinert, P.M., Yuspa, S.H. and Roop, D.R. (1990). Characterization of a novel cell envelope precursor, loricrin. *Cell* **62**:1103-1114.

Bisher, M.E., Roop, D.R., Steinert, P.M. and Steven, A.C. (1990). Immunocytochemical mapping of the biosynthetic pathways of two major proteins expressed in terminally differentiating epidermal keratinocytes. *Proc. XIIth Congr. E.M.*, San Francisco Press Inc., San Francisco, pp. 940-941.

DEPARTMENT OF HEALTH AND HUMAN SERVICES - PUBLIC HEALTH SERVICE

NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER: Z01 AR 41087-01 LSB

PERIOD COVERED
October 1, 1989 to September 30, 1990

TITLE OF PROJECT *(80 characters or less. Title must fit on one line between the borders.)*
Epidermal transglutaminases

PRINCIPAL INVESTIGATOR *(List other professional personnel below the Principal Investigator.) (Name, title, laboratory, and institute affiliation)*

P.I.	Peter Steinert, Ph.D.	Supv. Research Biologist	LSB/NIAMS
	William Idler, B.S.	Research Chemist	LSB/NIAMS
	In-Gyu Kim, M.D., Ph.D.	Visiting Fellow	LSB/NIAMS
	Soo II Chung, Ph.D.	Sr. Investigator	LB/NIDR
	Jung Ho Han, Ph.D.	Visiting Fellow	LB/NIDR
	Jeff Gorman, Ph.D.	Sr. Scientist	CSIRO, Geelong,Australia
	Hee Chul Kim, Ph.D.	Visiting Associate	LB/NIDR

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☐ (a) Human subjects ☒ (b) Human tissues ☐ (c) Neither
☐ (a1) Minors
☐ (a2) Interviews

SUMMARY OF WORK *(Use standard unreduced type. Do not exceed the space provided.)*

Transglutaminases form isodipeptide crosslinks between acceptor amide groups of glutaminyl residues and donor epsilon-NH_2 groups of lysines. In the epidermis, and other stratified squamous epithelial tissues, these enzymes are thought to be involved in the crosslinking of putative protein components to form the insoluble cell envelope. Using molecular biology approaches, we have found that there are 3 different transglutaminase activities in normal human and mouse epidermis. These are known as the K, C and E enzymes. Work is in progress to isolate and characterize clones encoding each of these and to determine the likely functions of these different activities in normal and abnormally keratinizing epidermis.

Project Description

Objectives

1. To isolate and characterize cDNA clones encoding the 3 different transglutaminases and to determine their likely protein structures.

2, To localize these activities in epidermal and related epithelial tissues and to examine their expression characteristics.

3. To isolate and detemine the genomic structures of these activities.

4. To determine the likely substrate preferences of these activities.

Methods

1. Synthetic oligonucleotides and PCR methods will be used to isolate both cDNA and genomic clones from appropriate cell culture, foreskin and genomic libraries, using standard molecular biology techniques.

2. Indirect immunofluoresecence, in situ hybridization, immunogold, Northern, Southern and Western blotting techniques are used to localize the expression of the transglutaminase activities.

3. In vitro assays of synthetic peptides derived from loricrins and involvucrin are being used to assess the Km values of the enzymes.

Major Findings

1. Full-length cDNA clones have been isolated by Dr. Hee Chul Kim and Mr. Idler for the transglutaminase K enzyme activity expressed in cultured epidermal keratinocytes as well as intact epidermal tissues. The protein is 813 amino acids long, has a molecular weight of 89.3 kDa, possesses a typical active site consensus sequence of transglutaminases, has sequences characteristic of a membrane-bound protein, and has properties expected for the partially purified enzyme.

2. Using PCR methods from peptide sequences obtained by Dr. Gorman, Dr. In Gyu Kim and Dr. Han have isolated clones for the transglutaminase E activity. Work is in progress to obtain complete sequences for the mouse and human enzymes, because protein chemical analyses of Dr. Hee Chul Kim and Dr. Chung shows that they are very different in properties. Sequences that are unique to the E and K enzymes will be utilized in the production of specific antibodies for more detailed expression work. Similarly, specific clones will be used to isolate genes for them.

Significance to biomedical research

Characterization of the structures and function(s) of these three different transglutaminase activities in human epidermis is important in understanding the processes or normal epidermal differentiation, as well as assessing their potential roles in keratinizing disorders of the epidermis.

Publications

Kim. H.C., Idler, W.W., Kim, I.G., Han, J.H., Chung, S.I. and Steinert, P.M. (1990). The complete amino acid sequence of the human transglutaminase K enzyme deduced from the nucleic acid sequences of cDNA clones. *J. Biol. Chem.* in press.