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NAME & ADDRESS OF CONTRACTOR OR INSTITUTION: (State the division, department, or professional school, medical, graduate or other, with which this project should be identified.)

Department of Bacteriology and Immunology School of Medicine University of North Carolina Chapel Hill, N. C., 27514

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TITLE OF PROJECT:

Mechanisms of Innate Immunity: VIII. Further Studies on Antimicrobial Constituents in Specific and Azurophil Granules from PMN Leukocytes of Man and Animals

NAMES, DEPARTMENT, AND OFFICIAL TITLES OF PRINCIPAL INVESTIGATORS AND OTHER PROFESSIONAL SCIENTIFIC PERSONNEL: (not including graduate students) engaged on the project, and fraction of man-year devoted to the project by each person.

John K. Spitznagel, M.D., Professor and Principal Investigator, 15% 303-20-4594 Ardis D. Hoven, M.D., Research Associate, 50% James D. Folds, Ph.D., Assistant Professor, 10% Edith K. MacRae, Ph.D., Professor, 5% Richard F. Rest, Ph.D., Res. Assoc. postdoctoral, 50% Marjorie Cooney, Research Analyst, 37.5% Larry Martin, Laboratory Technician, 50% Ralph Penniall, Ph.D., Professor, 2%

NO. OF GRADUATE STUDENTS ON PROJECT: 1 NO. OF GRADUATE STUDENT MAN-YEARS: 1

SUMMARY OF PROPOSED WORK: (200-300 words, omit Confidential Data). Summaries are exchanged with government and private agencies supporting research, are supplied to investigators upon request, and may be published in AEC documents. Make summaries substantive, giving initially and for each annual revision the following: OBJECTIVE; SCIENTIFIC BACKGROUND FOR STUDY; PROPOSED PROCEDURE; TEST OBJECTS AND AGENTS.

Objectives: 1) To characterize further antibacterial constituents of PMN granules, and their relation to other granule and cellular constituents. 2) To measure their capacity to degrade bacterial products. 3) To assess their contribution to antimicrobial defenses and their influence on phagocytosed antigens. 4) To measure how they may be altered by infection, ionizing radiation, drugs, neoplasia, aging, and environmental pollutants.

Intraleukocytic microbicidal mechanisms are of two kinds: oxygen dependent and oxygen independent. Our studies have focused on the oxygen independent systems. Specific and azurophil granules of purified and homogenized PMN suspensions are isolated by sucrose density sedimentation from post nuclear supernatants. The granules are then extracted with appropriate detergents, buffers, or salt solutions. These extracts provide materials that can be tested directly for antibacterial and various catalytic activities. They also provide material from which biologically active materials can be separated by electrophoresis, chromatographic techniques and various combinations of these. Antibodies to these purified substances are raised in heterologous species and used as reagents to compare, localize and measure the antibacterial and other substances of interest in the PMN granules.

RESULTS TO DATE:

We have successfully resolved the granules of human PMN and characterized them with respect to their content of lactoferrin, myeloperoxidase, lysozyme, and beta-glucuronidase. We have prepared antibodies against lactoferrin and MPO that are useful in immunochemical studies on these substances.

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Signature of John K. Spitznagel

Signature of Principal Investigator March 18, 1974

DATE:

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INVESTIGATOR - DO NOT USE THIS SPACE

MECHANISMS OF INNATE IMMUNITY

VIII. Further Studies on Antimicrobial Constituents in Specific and Azurophil  
Granules from Polymorphonuclear Leukocytes of Man and Animals

A RESEARCH PROPOSAL

(Renewal)

Contract No. AT-(40-1)-3628

by

John K. Spitznagel

Department of Bacteriology and Immunology

School of Medicine

University of North Carolina

at Chapel Hill

to

The Atomic Energy Commission

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(rich in alkaline phosphatase, lysozyme and lactoferrin in rabbit PMN) degranulated first. Stossel has reported on degranulation of human and guinea pig PMN in response to oil droplets ingested by cell suspensions (Stossel, T. P., et al., 1971, J. Clin. Invest. 50: 1745). Not more than 10% of any of the granule associated substances he studied entered the vacuoles. No substance known to be incorporated in the specific granules of human PMN was tested except possibly alkaline phosphatase. Since our work casts some doubt on the presence in these granules of alkaline phosphatase, it would perhaps in such studies be better to use a substance such as lactoferrin as an indicator for secondary or specific granules. Henson has approached the problem differently from Bainton or Stossel (J. Immunol., 1971, 107: 1535; Ibid, 1547) and found that PMN can be induced to degranulate up against millipore membranes coated with antigen-antibody complexes (frustrated phagocytosis). He regards this as a model for tissue damage due to PMN in complex disease. Granule contents are in most, but not all cases, released into the media. But, alkaline phosphatase is an exception and, although it evidently appears quite early at the cell membrane-immune-complex interface, it remains insoluble. Our experience with alkaline phosphatase (Spitznagel, J. K., in Phagocytic Mechanisms in Health and Disease, see above) again suggests that alkaline phosphatase may actually not be a granule enzyme. But, it appears early at sites of phagocytic action, perhaps owing to association with the cell membranes. The conclusion from these examples is that degranulation and other functions of PMN cytoplasm can possibly be better interpreted in the future if the granules of PMN of the species in question are more fully explored and as more experience is accumulated with standardized stimuli to degranulation.

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In summary: Insights into the realities of intraleukocytic killing of bacteria have been occurring with increasing rapidity. The mechanisms include ones responsible for killing bacteria and ones that degrade or detoxify bacterial products. Death of bacteria in phagocytes may be due to substances with primary antimicrobial action such as cationic proteins, lactic acid or hydrogen peroxide, or due to interaction with enzymic systems that generate free radicals which are, in turn, antimicrobial. Degradation of bacteria is undoubtedly due to enzymes, proteases, glycosidases, nucleases and lipases.

We propose to continue to analyze the phagocytin activity of the human PMN for substances with direct antimicrobial capacity. Through collaboration with colleagues, we propose to make distinctions between direct effects and effects attributable to interactions in phagocytic vacuoles and oxidative metabolism. We also propose to continue studies on the proteases of human PMN. Our ultimate objective is to discover which functions can most profitably be measured in studies of disease and to adapt or devise methods for such studies.

Scientific Scope. As this work proceeds, we will study:

1. Antimicrobial substances of polymorph granules from human blood.

This involves resolution of the granules with centrifugation through sucrose density gradients; biochemical characterization of the granules; ultra-structural and enzyme histochemical characterization of the granules; testing of granules, granule lysates and granule extracts for antimicrobial activity; comparing their activities with activities of the whole cell homogenates and post 126 X g granule-rich supernatants from which the granules are separated; isolation of the antimicrobially active constituents

from the granules by gel filtration, electrofocusing, and other standard techniques; characterization of the active components of the granules and characterization of their antimicrobial activities.

2. Formation of phagocytic vacuoles and degranulation of human PMN and PMN from other species, dose response effects, vacuole formation with maximal stimuli and interaction of granule components in the vacuoles.

3. Lysosomal defects in polymorphs with potential defects in antimicrobial efficiency obtained from individuals with impaired resistance to infection.

4. Biochemistry, immunochemistry and pathobiological significance of the neutral protease of human PMN.

5. Localization of lysosomal cationic proteins in polymorph lysosomes and their delivery to phagocytic vacuoles in man, rabbits and chickens.

6. Oxidative metabolism of mammalian, rodent and avian PMN: comparative studies.

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## GENERAL PLAN OF WORK

Preparation of PMN and PMN Granules. In all of our experiments it is necessary to prepare lysosomes and fractions of lysosomes. To do this from rabbit peritoneal exudate cells as we have in the past is straightforward and described in several of the accompanying publications (Ref. 3 and 4 in Mechanisms of Innate Immunity; VI Biological properties of polymorphonuclear leukocyte cytoplasmic granules and their components, Progress Report).

The methods used with buffy coat PMN from human blood are more involved because buffy coat includes lymphocytes, monocytes, platelets, basophils, and eosinophils which contribute granules, enzymes and other substances irrelevant to PMN and capable of causing confusion.

The PMN are obtained from fresh human blood collected in heparin. After most of the erythrocytes are removed by sedimentation in dextran (Fallon, H. J., et al., 1962, J. Lab. Clin. Med. 59: 779), the PMN along with residual erythrocytes, lymphocytes, monocytes, platelets, basophils and eosinophils are centrifuged out of the dextran-plasma mixture. Washed in a balanced salt solution, these cells are further fractionated by the technique of Bøyum (Scand. J. Lab. Invest. 21, Suppl. 97, 77, 1968) with sedimentation through a one step gradient of Ficoll and Hypaque. PMN appear as a button in this step and with them are eosinophils and residual erythrocytes. The erythrocytes are eliminated by hyposmotic lysis, but the eosinophils are difficult to remove. We avoid eosinophils and select blood donors with the lowest possible eosinophil counts.

Resolution of PMN Granules. The PMN button homogenized in .34 M sucrose (a sample is reserved for quantitative analysis) and centrifuged 10 min at 126 X g yields a granule-rich supernatant (C) and a nucleus

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rich pellet (D). The pellet D is reserved for quantitative analysis along with a sample of the supernatant C. The bulk of the supernatant is then applied to the top of a linear sucrose density gradient, in a 60 ml centrifuge tube, usually 30-53% w/w. The loading fraction is either 11% with respect to sucrose or adjusted to 25%. The tubes are placed in the centrifuge rotor (Beckman 25.2 with meniscus adjusted so that  $R_{min} = 6.4$  cm,  $R_{max} = 15.3$  cm. The rotor is placed in an L2-65B Beckman-Spinco centrifuge and accelerated to 4,000 rpm and maintained at this speed for 15 min. Then the rotor is accelerated to 21,000 rpm. A typical velocity sedimentation run subjects the granules to an integrated angular velocity of  $\int_0^t \omega^2 dt = 2.3 \times 10^{10} \text{ rad}^2 \text{ sec}^{-1}$ .

Analyses of Granules for Markers and Substances with Putatively Antimicrobial Action. In the tubes which have been thus centrifuged are three widely spaced bands of optical density. The most rapidly sedimented one (Band III) appears at about 32 ml from the top of the gradient. Next above it is Band II at 19 ml from the top and then at about 5.4 ml from the top is Band I. With proper technique Band III can be split into two components IIIs and IIIf. IIIs sediments slower than IIIf. The gradients are collected in 2.7 ml or 1.0 ml fractions (Csf) by upward displacement with 60% sucrose.

A number of quantitative biochemical and immunochemical assays as well as morphological procedures are then applied to analyze the fractions (Csf) collected from the gradients and in volumetric dilutions of the H, D, and C samples reserved during preparation of homogenates and gradients (see above). Quantitative analytical procedures include: total protein (Lowry, O., 1951, Biol. Chem. 193: 265.), alkaline phosphatase with p-nitrophenyl

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in diethanolamine buffer at pH 10 (Baggiolini, M., 1969, J. Cell Biol. 40: 529.), beta-glycerolphosphatase at pH 4.8 (phosphorus release determined with method of Chen, N., Anal. Chem. 28: 1756, 1956.), beta-glucuronidase (Fishman, W. H., 1963, p. 869, in H. U. Bergmeyer, Methods of Enzymatic Analysis, acad. Press, New York.), lysozyme (Shugar, D., 1952, Biochim. Biophys. Acta 8: 302.), peroxidase (Luck, H., p. 895, in H. U. Bergmeyer, Acad. Press, N. Y., 1963.). Immunochemical assays include lactoferrin (Masson, J. F., et al., 1969, J. Exptl. Med. 130: 643.) and myeloperoxidase MPO (Mage, M. G., et al., 1971, J. Ret. End. Soc. 9: 201.). Detergents are used to abolish latency of the granule constituents and measurements made in the presence of detergent are defined as 100% of activity. All these procedures are carefully checked to insure that they give results that are linear with respect to time (where appropriate) and to dilution of the substance to be measured.

The recovery of each substance from the D and C fractions is reconciled with the amounts found in H, the whole cell homogenate. The recovery of each constituent from each gradient is calculated and reconciled with the amounts in the volume of C applied to the gradients. Recoveries in D and C are usually better than 90%. Recoveries from gradients range from 70% to 100%. Slight losses from gradients are caused by adherence of some granules to the walls of the tubes. When these are taken into account recoveries approach 100%.

Ultrastructural Analysis of Fractions. Whole cells and fractions are fixed in 1.5% glutaraldehyde according to published methods (see Bainton, J. Exptl. Med. 130: above), washed, and then incubated in substrates for enzyme histochemistry for peroxidase (Graham, R. C. and M. J. Karnovsky,

1966, J. Histochem. and Cytochem. 14: 291.), alkaline phosphatase (Wetzel, B. K., et al., 1967, J. Histochem. and Cytochem. 15: 311.), and acid phosphatase (Ibid). Specimens that have been stained for enzyme activity and specimens which have not been so stained are then washed and fixed in osmium, dehydrated in propylene oxide and embedded in Epon. The blocks, cut with a diamond knife, provide ultra-thin sections to be mounted on grids and viewed with or without staining with uranium and lead. Enzyme histochemistry is controlled with specimens incubated without substrate but otherwise treated like the test material;

The gradient fractions are also subjected to electrophoresis on cellulose acetate or on polyacrylamide gels. The quantities of protein in the different bands are quantitated by measuring the dye uptake densitometrically. The figures are converted to micrograms of protein by constructing empirical curves equating dye uptake in selected bands with protein nitrogen determination, by measurements of enzymic activity or by immunochemistry (single radial diffusion of eluted materials).

Detection of Antimicrobial Action in Granules. We have usually done this with a modification of Muschel's photometric assay for antibacterial substances in serum. This involves dilution of extracts of granules or lysed granules in a buffer solution with a standard number of test bacteria; often these are Escherichia coli, but Staphylococcus aureus, Streptococcus faecalis, Proteus rettgeri, and other microorganisms have been used as well. After 60 min incubation in this mixture the 1 ml reaction mixture is diluted to 6 ml by addition of 5 ml of antibiotic assay broth. The tubes with bacteria treated with granule constituents, control tubes that contain buffer plus bacteria plus assay broth are mixed and incubated at 37°C until

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the turbidity in the control tubes reaches O.D. = .5. At that time the turbidities in all tubes is measured and recorded. Antibacterial action is recorded as the micrograms of protein in the test substance that reduces growth to 50% of the control (1 IEP50 unit) and the activity is defined as the number of IEP50 units per mg of protein. Different fractions can be compared for their activity. Dose response line slopes are frequently different for different fractions, however, and suggest that qualitative differences exist.

We are now working up another assay which will be similar to the Muschel technique insofar as exposure of microorganisms to the granule constituents is concerned. Bacterial killing, however, will be detected by spreading samples of the inhibition mixtures on agar plates and comparing growth from test and control suspensions. This method will permit more flexibility than the Muschel method. A greater variety of bacterial species can be tested including ones that grow as a pellicle in liquid media. Bactericidal and bacteriostatic action can be compared. The method may provide certain conveniences as well because the plates will be counted with an automatic colony counter. Thus, plates can be removed from the incubator and counted at once or stored and counted a few hours later.

Once we have measured antimicrobial activity in whole PMN homogenates or extracts and compared the activities from isolated granules with them, extracts and lysates of granules will be fractionated by standard techniques. Gel filtration, membrane filtration, electrophoresis in polyacrylamide gels, electrofocusing and other standard techniques will be used to resolve antibacterial substances. These antibacterial components will be checked for the enzymic activities listed above and for their reactivity with anti-human lactoferrin and anti-human MPO. Antibacterial activity recovered will be reconciled with activities in starting materials.

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If antibacterial substances appear likely to be antigenic, attempts will be made to raise antibodies to them so they can be detected and amounts measured by single radial diffusion or by radioimmunoassay (Leffell, M. S., 1972, *Infec. and Immun.*, in press). All this work is subject to limitations on availability of PMN. This has not been a problem because the blood bank has cooperated with us on bleeding donors. It could be a problem when large numbers of cells are needed to isolate a granule constituent. In the event that it is a problem, three sources of cells might be used. PMN can be obtained from normal persons by leukaphoresis or with an NCI-IBM Continuous Flow Blood Cell Separator (Celltrifuge, American Instrument Co.) or they can be obtained from patients with chronic myelogenous leukemia by either of the forgoing methods. The celltrifuge purchase is planned by the Clinical Research Unit for 1973.

Contribution of Cytoplasmic Granules to Phagocytic Vacuoles in Human PMN.

At present the process of degranulation and formation of phagolysosomes in PMN is poorly understood. This is especially true of human PMN partly because relatively little is known of granule composition in human PMN and partly because sensitivity, specificity, and ease with which constituents of interest can be assayed has been less than optimal. Moreover, phagocytic stimuli have been less than maximal if one judges by the proportion of supposedly granule bound materials which have been detected in the vacuoles (Stossel, T. P., et al., 1971, *J. Clin. Invest.* 50: 1745.). We believe our analysis of cytoplasmic granules from human PMN lysosomes and the methods we have adapted for this will have sufficiently extended the knowledge and capabilities required for such studies that it is worthwhile for us to study degranulation in human PMN. Miss Leffell has been doing

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this in our laboratory. She has adapted the method of Werb (Werb, Z. and Z. Cohn, 1972, J. Biol. Chem. 247: 2439) which is an adaptation of Wetzel and Korn (J. Cell Biol. 43: 90, 1969).

Polystyrene-latex spheres are coated with various substances and suspended with viable human PMN which are allowed to phagocytize for 30-60 min. Then the cells are homogenized in 30% (w/v) sucrose. The homogenate adjusted to 50% (w/v) in 5 ml sucrose is placed in the bottom of an 18 ml centrifuge tube and a stepwise gradient is prepared with 5 ml of 40% and 7 ml of 30% sucrose (w/v). The tubes are placed in an SW27.1 rotor and centrifuged for 60 min at 25,000 rpm in a Beckman-Spinco L2-65B centrifuge. After the tubes are removed from the centrifuge a layer of large membranous vesicles that enclose polystyrene latex beads is found on the meniscus at the top of the tube. Assays for 5' nucleotidase (Widnell, C. C. and J. C. Unkeless, 1968, Proc. Nat. Acad. Sci. USA 61: 1050), lactoferrin (see Masson above), lysozyme (see Shugar above), MPO (see Mage above) and Lowry protein (see above) reveal that about 3% of these specific and azurophil granule constituents are in the layer with the latex spheres. Alkaline phosphatase is also found there. Electron microscopy shows that the layer comprises spheres surrounded by membranes. Lactic dehydrogenase and the bulk of the granule enzymes are found in the bottom of the tube in the loading zone. Thus, the cytosol, the bulk of the granule constituents, and other cell constituents do not contaminate the vesicle fraction.

Miss Leffell now intends to ascertain the fraction of granule associated materials such as lactoferrin, MPO and neutral protease that can be induced to enter the vesicles with qualitatively and quantitatively different stimuli for degranulation.

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It is hoped that a maximal stimulus will be found which will force nearly 100% of the granule substances to enter the vacuoles. Difficulties may be anticipated in that maximally stimulated vesicles may be more fragile and vesicle recovery from homogenates may not be 100%. Exploration of alternative techniques for cell rupture, stabilization of membranes and conditions of centrifugation will be done if necessary to improve recoveries. In any event, the addition of this technique to the capabilities of our laboratory enables us to study the transport and the interaction of granule components, killing of microbes, degradation of microbial products and immunoglobulin complexed to antigen all in the vesicles themselves.

Lysosomes in Polymorphs with Potential Antimicrobial Defects from Individuals with Impaired Resistance to Infection. The experiment of nature, one of the proven models for studying the role of physiological mechanisms, is prepared by genetic accidents or disease that lead to failure of development or deletion of some substance or structure with resultant functional impairment of the organism. Such experiments of nature have already provided clues that O<sub>2</sub> uptake and HMP shunt activation are in some way linked to intracellular mechanisms that destroy ingested bacteria in PMN (Holmes, B., et al., 1967, J. Clin. Invest. 46: 1422), that peroxidase may be needed for intracellular destruction of Candida sp. (Lehrer, R. I. and M. J. Cline, 1969, J. Clin. Invest. 48: 1478), that G-6-PD mediates a mechanism that is involved in intracellular killing of bacteria (see Cooper, et al, above), and that deficiency of lysozyme, lactoferrin and the specific granules with which they are associated may reduce the antimicrobial efficiency of the human PMN (see Spitznagel, et al., above).

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Because of my association with the Infectious Disease Division of the Department of Medicine, I have access to patients with impaired resistance to infection. We have clinical facilities for screening patients for defective intraleukocytic killing of bacteria and abnormalities of the metabolic concomitants of infection. We also have been privileged to have the cooperation of the Divisions of Hematology and Infectious Diseases specialists and immunologists at other medical schools in this and other states. We wish, therefore, to study the leukocytes with potential defects from individuals with resistance to infection depressed by various pathological processes, chronic infectious granulomatosis of childhood, Chediak-Higashi syndrome. Chronic and acute myelogenous leukemia, myeloproliferative diseases, uremia, drugs, toxins, infections and other problems can be mentioned. Patients' PMN will be assayed for ability to ingest and kill bacteria or Candida and to reduce nitro blue tetrazoleum. Then the PMN granule constituents will be assayed with the immunochemical, enzymatic and ultrastructural methods described under 1 above. If deviations from normal concentrations of granule constituents or granule morphology are found, further studies will be done. We anticipate about two such subjects will become available per month. It is planned that Dr. Ardis Hoven, a postdoctoral fellow, will carry on this work in our laboratory. We have a clinical research unit here which is available for the hospitalization of patients for clinical research projects.

#### COLLABORATIVE STUDIES

Outlined below are studies to be done in collaboration with other faculty members. Funds from this contract will be used from time to time

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to purchase animals and reagents and some technician time will be devoted to these projects when it is available.

Biochemistry, Immunochemistry and Biologic Significance of the Neutral Protease of Human PMN. (in collaboration with Dr. James Folds)

Dr. Folds has already found that as much as 90% of the non-specific proteolytic activity of human PMN is vested in granules that separate as a single population on sucrose density gradients. This appears to be a single enzyme separating on Sephadex columns and in electrofocusing experiments as a single peak.

Now he plans to further characterize this protein and examine its activity against a broad selection of substrates and its performance in the presence of protease inhibitors including iodoacetamide, cysteine, para-chloromercuribenzoate, mercaptoethanol, ethylenediamine tetracetic acid, diisopropyl fluorophosphate and others. He also plans to study its action on human immunoglobulins, tissue components such as basement membrane and elastin and microbial products such as diphtheria toxin and M proteins of group A beta-hemolytic streptococci. It is also planned to raise antibodies to this enzyme so that the molecule can be detected immunologically either in the cells or in tissues or body fluids. Such an antibody might conceivably be useful in studies on the activity of the enzyme in phagolysosomes.

Studies on the Origins, Packaging and Fate of Cationic Proteins (LCP) in Granules of Polymorphonuclear Leukocytes. (in collaboration with Professor Edith MacKae, Department of Anatomy) Ever since Spitznagel and Chi (Am. J. Path. 43: 697, 1963) described the presence of these very cationic, arginine-rich proteins in cytoplasmic granules of PMN, they have aroused the interest

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of morphologists (Horn, R. G. and S. S. Spicer, 1964, Lab. Invest. 13: 1; Bainton, D. F. and M. G. Farquhar, 1968, J. Cell Biol. 39: 286). Histochemical studies have unfortunately been restricted to techniques appropriate to light microscopy, staining with Fast Green (see Spitznagel, 1961, J. Exptl. Med. 114: 1063) and with Bieberich Scarlet (see Spicer above). Detailed studies of the ultrastructural relationships have not been possible and this has been an obstacle to precise definition of the biology of these proteins.

This stumbling block has been at least partly removed by Professor MacRae. She has developed a technique suitable for electron microscopic studies on cationic proteins rich in arginine and can distinguish between them and lysine-rich cationic proteins (MacRae, E., 1970, J. Cell Biol. 45: 235). With the use of this technique we propose to study the origin, distribution and fate of the cationic proteins of PMN granules. The study should extend considerably the work published by Spitznagel and Chi on the re-distribution of these proteins in phagolysosomes and clarify the origin of LCP as well as their packaging in relation to rough endoplasmic reticulum, Golgi apparatus, specific and azurophil granules of PMN. Ultrastructural studies of the comparative biology of cationic proteins in human, rabbit and chicken PMN will be possible. Chicken PMN will be especially useful because they lack MPO which is also a cationic protein. Bovine PMN which lack lysozyme may also be useful.

Oxidative Metabolism of Mammalian, Rodent and Avian PMN. (in collaboration with Professor Ralph Penniall, Department of Biochemistry) If one is to place the activity of any antibacterial mechanism of PMN in perspective it must be measured against what is known of the effects of oxidative

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metabolism. This is because much work (examples cited above) has inextricably linked it to intraleukocytic killing of bacteria. The studies of Brune (see above) demonstrate the presence of cationic proteins in the peroxidaseless PMN of chickens. These PMN are competent to kill microbes intracellularly. How do they do it? Klebanoff suggested that in human PMN increased  $H_2O_2$  production compensates for congenital lack of peroxidase. The question arises -- are chicken PMN compensating for their peroxidaselessness by producing more  $H_2O_2$ ? If they are not doing so, then one might suppose that the available mechanisms are sufficient -- cationic proteins, lactic acid, even the available  $H_2O_2$ .

The purpose of this study is to compare the oxidative metabolism of human, rabbit and chicken PMN resting and stimulated by phagocytosis to see if their  $O_2$  uptake burst, HMP shunt activity and  $H_2O_2$  production differ in any way. To do this, Professor Penniall has set up a variety of relevant methodologies adapted for use in this comparative study of phagocytosis. They are: manometric assay of respiration and concomittant  $^{14}CO_2$  production from glucose 1- and 6- $^{14}C$  glucose and  $^{14}C$  formate; polarographic and spectrophotometric assays of NADH and NADPH oxidative activities; spectrophotometric assays for glutathione reductase and peroxidase, lactic dehydrogenase ( $NAD^+$  and/or  $NADP^+$ ) glucose consumption, d-aminoacid oxidase, transhydrogenase, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. In addition, a modified Ficoll-Hypaque procedure has been devised for isolation of PMN from rabbit blood.

All of the above assays have been used to obtain basic values for PMN of rabbit's blood. Some of the parameters have also been measured in PMN of rabbit peritoneal exudates. At present difficulties are being worked

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out of the Ficoll Hypaque method in order to separate chicken PMN. The results of these studies will permit a comparison of oxidative metabolism of PMN from man, rodents and birds. These cells differ considerably in morphology both by light and electron microscopy. Not just the morphology, but also the chemistry of these granules are different. Human PMN possess the greatest peroxidase activity. Chicken PMN possess none. Rabbit PMN are in between. The cationic proteins appear to be distributed just opposite to this. If the  $H_2O_2$  production of the three cell types is essentially the same, then this would suggest there is no obligatory requirement that  $H_2O_2$  production be increased as a compensation for less peroxidase. If  $H_2O_2$  production is increased in chicken PMN compared with human, then this would support the Klebanoff hypothesis. If  $H_2O_2$  production by rabbit PMN is increased over human PMN, but less than in chicken PMN, that would provide Klebanoff further support.

Significance. These studies will extend current knowledge of mechanisms involved in killing and destroying microorganisms phagocytized by PMN of humans and other species. At present it is quite clear on the one hand that extracts of cytoplasm (phagocytin) from human and other PMN will kill bacteria and yeast. With rabbit PMN a spectrum of low molecular weight, very cationic proteins are largely responsible for this. The active constituents in human phagocytin have not been identified. On the other hand, it is quite clear that an intact oxidative metabolism is essential for normal antimicrobial activity in human PMN, but the essential connection between the metabolic pathways and the killing has to be defined. Our research should clarify the biology of the antibacterial substances in human phagocytin. If this is done, then convenient methods

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may be devised to assay these substances and the assays will be useful adjuncts in studying PMN of individuals subjected to various influences including infection, drugs, disease, and environmental pollutants.

Traditionally studies of PMN have been morphological and enumerative with rather limited use of histochemistry. Studies on marrow have been employed but are also morphological, tedious and not suitable for studies involving large numbers of subjects and observations. Since it is known that the granules of PMN are integral to their phagocytic function and since granule development proceeds sequentially, different classes of granules being formed as these cells differentiate, it is clear that detection and quantitative measurement of constituents specific for each granule type will provide a measure of sufficiency of PMN composition and of maturity heretofore unavailable. The use of immunochemistry for these assays has, because of its high specificity, distinct advantages. A good example is the assay of MPO. We have found that human eosinophil peroxidase is ten times more active than MPO. Thus, if PMN are contaminated by 4% eosinophils the spectrophotometric assay may show as much peroxidatic activity as 40% PMN would produce. MPO measured immunochemically is specific; hence, eosinophil peroxidase does not interfere with the assay. This now makes it possible to perform meaningful assays for MPO on relatively small amounts of blood from either normal or diseased individuals. An immunochemical assay for PMN protease would be of similar utility. Pulmonary secretions from individuals chronically subjected to environmental pollutants such as oxides of nitrogen and sulfur might easily be monitored for these enzymes, both of which are potentially destructive of tissue if they are released from the PMN. The same secretions might be monitored for lactoferrin and the ratios of these enzymes might be expected to reveal the status of the PMN entering the tracheo-bronchial tree.

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produce PMN that are unlike normal PMN and can phagocytize but cannot kill the phagocytized bacteria -- PMN that fail to show normal increases in  $O_2$  uptake and HMP shunt activity during phagocytosis (J. Clin. Invest. 46: 1422, 1967.). The presence in PMN of certain enzymes also suggests this hypothesis. Myeloperoxidase, located in the larger PMN granules (Baggiolini, M., et al., 1969, J. Cell Biol. 40: 509. and see Progress Report), plus  $H_2O_2$  plus halides or thiocyanate are bactericidal in vitro (Klebanoff, S. J., 1968, J. Bact. 95: 2131.). Moreover, it has been shown that an enzyme tentatively linked to the HMP shunt, NADH oxidase, is missing from PMN of some children with X-linked chronic infectious granulomatosis. The oxidase deficiency putatively lowers  $H_2O_2$  production and causes PMN bactericidal action to fail (Baehner, R. L., and M. L. Karnovsky, 1968, Science 162: 1277.). Lehrer, et al. (Lehrer, R. I., J. Hanafin and M. J. Cline, 1969, Nature 223: 78.) reported that the PMN of a patient with candidiasis lacked myeloperoxidase. It is noteworthy that while this patient's PMN could not kill Candida albicans, they were able to kill bacteria, albeit at a reduced rate compared with normal PMN. The authors proposed that although peroxidase was lacking, still other effective antibacterial factors existed in the PMN.

Subsequently, Klebanoff (Klebanoff, S. J., 1970, Science 169: 1095) has expressed similar views after examining PMN from Lehrer's patient and has suggested (in Phagocytic Mechanisms in Health and Disease, ed., Williams and Fudenberg, p. 3 ff., Intercontinental Book Corp., New York, 1972) that peroxidase deficient cells show compensatorily increased  $H_2O_2$  production.

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Iodination of bacteria, related by Klebanoff to MPO activity and bactericidal action in PMN, is not indispensable to bacterial killing (McCall, C. E., et al., 1971, Clin. Res. 19: 47). However, a patient with recurrent infections and total absence of glucose-6-phosphate dehydrogenase activity from her PMN (Cooper, et al., 1971, J. Clin. Invest. 49: 21a) has been reported to show impaired intraleukocytic bactericidal action, suggestive evidence that HMP shunt activity and/or  $H_2O_2$  are essential. Spitznagel, et al., have reported a patient with recurrent infections and with selective absence of lysozyme and lactoferrin-rich granules from his PMN. These PMN showed reduced intraleukocytic killing of certain bacteria, but were essentially normal with respect to the usual metabolic concomitants of phagocytosis seen in PMN (Spitznagel, J. K., et al., 1972, J. Clin. Invest. 64: 930a). The possibility that lactoferrin is instrumental in intraleukocytic killing of bacteria has been suggested (Masson, et al., 1969, J. Exptl. Med. 130: 643.). Leffell and Spitznagel have shown lactoferrin is present and latent in peroxidase negative granules and, therefore, specific granules of human PMN. The results with this patient suggest that lysozyme, lactoferrin, or substance X associated with the missing granules influence killing of certain bacteria in human PMN and that HMP shunt activity with its resultant  $H_2O_2$  production is not necessarily sufficient for expression of their full spectrum of antimicrobial action. In this same relationship it is noteworthy that colchicine and vincristine can inhibit  $O_2$  uptake and HMP shunt activation and not reduce killing of bacteria in PMN (Malawista, S. E., 1971, Blood 37: 519). It should be useful in deciding on the antimicrobial role of oxidative metabolism and  $H_2O_2$  production to compare them in chicken, rabbit and human PMN since these species of PMN vary greatly with respect to cationic protein and MPO.

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What other evidence suggests that antimicrobial mechanisms may exist, independent of peroxidase and  $H_2O_2$  in human PMN? Phagocytin, an extract of PMN cytoplasm with direct antimicrobial action, has been found (Hirsch, J. G., 1956, J. Exptl. Med. 103: 613) in human PMN (Quie, P. G., J. G. White, B. Holmes and R. A. Good, 1967, J. Clin. Invest. 46: 668). But, defects in phagocytin have not yet been implicated in human disease nor has the origin of this activity in human PMN been elucidated. Davis, et al., (Davis, W. C., S. D. Douglas and H. H. Fudenberg, 1968, Ann. Int. Med. 69: 1237) have reported a patient with a failure of PMN bactericidal action selective for phagocytized S. aureus. Douglas has suggested that PMN dysfunction is probably pleomorphic (Douglas, S. D. and H. H. Fudenberg, 1959, Hosp. Prac. 4: 29). Patients with PMN defects may only be infected by Staphylococcus aureus, Candida albicans, or gram negative rods while maintaining resistance against other organisms (see Douglas and Fudenberg, 1969; Spitznagel, et al., 1972, J. Clin. Invest. 64: 930a). Mandell and Hook (Mandell, G. L. and E. W. Hook, 1969, Am. J. Med. 47: 473), working with presumably  $H_2O_2$  deficient PMN from a patient, attributed the selectivity of his defects in bacterial killing to an inability of certain bacteria to produce catalase. Unable to produce catalase, but able to produce  $H_2O_2$ , such bacteria presumably kill themselves in the presence of PMN myeloperoxidase. Other factors may have been involved, however, for the patient survived 17 years and acquired infection with only a few of the many catalase producing bacteria. This further suggested that normal PMN possess several substances that kill microbes with direct as well as a selective mechanism. If one of these were deleted the cell might manifest a selective inability to kill bacteria. Rigorous evidence for such a result secondary to a selective

defect in human or animal PMN has not yet been forthcoming, but Davis, et al. (1968, see above) reported a patient whose PMN were only unable to kill S. aureus, Lehrer, et al. found their patient's PMN deficient in myeloperoxidase and only unable to kill C. albicans and Spitznagel, et al. found their patient's cells, which were deficient in lysozyme and lactoferrin, killed Staphylococcus aureus and Candida albicans, but failed to kill enterococci, Proteus species and Escherichia coli.

These differences in susceptibility to infection along with related defects in PMN also focus attention on the possible existence of multiple antimicrobial factors with selective action. The LCP isolated from rabbit PMN by Zeya and Spitznagel had these qualities. Each of them had a restricted range of action, but together they accounted for almost the entire range of antibacterial activity of the parent PMN. The presence of LCP in human PMN is just now being explored in order to see if they may be involved in the pathogenesis of defects. The degranulation of PMN during phagocytosis is another possible source for selectivity in bacterial killing (Holmes, B. in Phagocytic Mechanisms in Health and Disease, ed., Williams and Fudenberg, Intercontinental Book Corp., N. Y., 1972). Since there are several different kinds of PMN cytoplasmic granules (Zeya, H. I. and J. K. Spitznagel, 1971, Lab. Invest. 24: 229; Welsh, I.R.H. and J. K. Spitznagel, 1971, Infec. and Immun. 4: 97; Spitznagel, J. K., in Phagocytic Mechanisms in Health and Disease, ed., Williams and Fudenberg, Intercontinental Book Corp., N. Y., 1972), selective degranulation might limit exposure of bacteria to only a fraction of the antimicrobial mechanisms in PMN. Clearly, the relative importance of the different bactericidal systems requires

further assessment in man and animals, and heretofore undiscovered systems must be sought.

Aside from their bactericidal or fungicidal actions, PMN exercise a digestive function which, with success that depends partly on the species of microorganism, dispose of microbial products. Among important bacterial products are proteins which may be exotoxins. Moreover, phagocytized bacteria are probably coated with host proteins, immunoglobulin G, for example, and complement components as well, before being phagocytized. These proteins are then digested in the PMN along with the bacteria. If for any reason digestion turns out to be incomplete, the remnant peptides might well be altered but not inactivated both in respect to toxicity and antigenicity (Spitznagel, J. K., in Proceedings of the Fourth International Congress on Pharmacology, p. 216 ff., Schwabe and Co., Basel, 1971). They might, in fact, be rendered more toxic or their antigenic specificity changed.

In this connection we are interested in the neutral protease of human PMN. Janoff recently reviewed studies on PMN proteases (Janoff, A., 1972, *Am. J. Pathol.* 68: 579). He had localized the neutral protease to a total granule fraction from homogenized PMN. We have shown that these enzymatic activities are, in fact, confined to the azurophil granules of human PMN (Folds, J. D., I.R.H. Welsh and J. K. Spitznagel, 1972, *Proc. Soc. Exptl. Biol. and Med.* 139: 461). In this same work we have found one other protease, an aminopeptidase which is associated with a granule distinctly separable from the neutral protease. Unpublished work from our laboratory done with gel filtration and electrofocusing now suggests that the neutral protease and the elastase may, in fact, be associated with the same molecule; at least they cannot be separated from each other.

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Thus far there appears to be a genuine restriction on the number of proteases in human PMN. Whenever we find an acid protease it seems associated with the lymphocytes, monocytes, or platelets contaminating some cell suspensions. Since the neutral protease is optimally active at about pH 7, it suggests that if it functions in the vacuole the vacuole has not always a high hydrogen ion concentration, as has been generally supposed. These proteases deserve further investigation in order to extend our knowledge of how bacteria are digested, how tissues are damaged, and with respect to autoimmune disease, how immunogenic fragments of tissue and serum proteins can be produced.

How these antimicrobial and other factors are delivered to scenes of bacterial invasion in a usable form was indicated by Cohn and Hirsch (Cohn, Z. A. and J. G. Hirsch, 1969, J. Exptl. Med. 112: 983). They showed that the cytoplasmic granules of rabbit PMN are lysosomes and contain acid hydrolases and antibacterial (phagocytin) activity. The rabbit phagocytin probably included the combined antibacterial lysosomal cationic proteins of PMN (see Zeya and Spitznagel, J. Exptl. Med. 1965). Although it is known that cytoplasmic granules of PMN fuse their membranes with phagocytic vacuoles containing bacteria (Zucker-Franklin, D. and J. G. Hirsch, 1964, J. Exptl. Med. 120: 569), the events that produce this effect are unknown. Furthermore, the matter is now more complicated because Baggiolini, et al. (Baggiolini, M., J. G. Hirsch and C. DeDuve, 1969, J. Cell Biol. 40: 509-541) have found that PMN cytoplasmic granules are heterogeneous. Zeya and Spitznagel. (Zeya, H. I. and J. K. Spitznagel, 1971, Lab. Invest. 24: 229 and 24: 237) have confirmed this and found that the antibacterial cationic proteins of rabbit PMN are confined to the azurophil granule fraction.

This work shows interesting correlations with enzyme histochemical studies by Spicer, et al. (Horn, P. G. and S. S. Spicer, 1964, Lab. Invest. 13: 1) and Bainton and Farquhar (Bainton, D. F. and M. G. Farquhar, 1968, J. Cell Biol. 39: 286 and 299).

The work of others (Bainton, D. F., et al., 1971) might suggest that the cytoplasmic granules in adult PMN from human blood are too heterogeneous in size to resolve into homogeneous populations with velocity sedimentation (Bainton, D. F., et al., 1971, J. Exptl. Med. 134: 907). We have found that they can, in fact, be resolved with due attention to purification of cell suspensions and details of homogenization and centrifugation (Spitznagel, J. K., et al., in preparation). Welsh and Spitznagel (see above) have found that the general scheme of granule classes worked out by Bainton and Farquhar in rabbit PMN applies to human PMN, i.e., there are MPO positive azurophil or primary granules and MPO negative (secondary) or specific granules. Nevertheless, there are differences in composition that may be significant. For example, the alkaline phosphatase is associated with membranous vesicles as isolated on gradients and not with the secondary granules. There is probably further biochemical and morphological heterogeneity among the azurophil granules (Spitznagel, in Phagocytic Mechanisms) as well as differences in fragility among the various granules.

Studies of the sequence and effect of degranulation in rabbit PMN have been published by Bainton and Farquhar (J. Cell Biol. 47: 11a, 1970) who found alkaline phosphatase was the first enzyme detectable by enzyme histochemistry in the membrane of phagocytic vacuoles formed as bacteria were phagocytized. Because alkaline phosphatase was associated with specific granules of PMN from rabbits, they concluded the specific granules

MECHANISMS OF INNATE IMMUNITY

VII. Antimicrobial Constituents in Specific and  
Azurophil Granules from Polymorphonuclear Leukocytes  
of Man and Animals

A RESEARCH PROPOSAL  
(Renewal)

Contract No. AT-(40-1)-3628

by

John K. Spitznagel  
Department of Bacteriology and Immunology  
School of Medicine  
University of North Carolina

to

The Atomic Energy Commission

F 1939

*John K. Spitznagel*  
\_\_\_\_\_  
John K. Spitznagel, M.D., Principal Investigator      APR 5 1974      11/15/72  
Date

*A. H. Shepard, Jr.*  
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A. H. Shepard, Jr., Assistant Vice President and Treasurer      11/21/72  
University of North Carolina, Chapel Hill      Date

*George R. Holcomb*  
\_\_\_\_\_  
George R. Holcomb, Dean of Research Administration      11/22-72  
University of North Carolina at Chapel Hill      Date

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Scientific Background. Results of research into resistance to infection and mechanisms of inflammation influence our work most strongly when they deal with structural, metabolic, and biochemical changes that occur in PMN with phagocytosis, entry into inflamed tissues, exposure to drugs, or ionizing radiation. The cytoplasmic granules of PMN play a significant part in these mechanisms and are a center of research interest in our laboratory.

This discussion considers some of the published research that affects our work on these granules, phagocytosis, and other factors in PMN function. Phagocytosis of bacteria leads to several events in PMN: 1) formation of a phagocytic vacuole and degranulation (Hirsch, J. G. and Z. A. Cohn, 1960, J. Exptl. Med. 112: 1005.; Zucker-Franklin, D. and J. G. Hirsch, 1964, J. Exptl. Med. 120: 569.); 2) increased O<sub>2</sub> uptake and activation of hexose-monophosphate-shunt (HMP) activity (Karnovsky, M. L., 1962, Physiol. Rev. 42: 143.); 3) transfer of cationic granule proteins to phagocytized bacterial cells (Spitznagel, J. K. and H. Y. Chi, 1963, Am. J. Path. 43: 607.); 4) iodination of materials in phagocytic vacuoles in human PMN (Klebanoff, S. J., 1967, J. Exptl. Med. 126: 1063.) and 5) destruction of the bacteria (Cohn, Z. A., 1963, J. Exptl. Med. 117: 27.).

Stimuli other than phagocytosis influence PMN. Toxins, such as streptolysin S, cause lysis of PMN granules and cells die (Weissman, G., 1964, Fed. Proc. 23: 1038.) and drugs like colchicine may prevent degranulation while tissue products, the prostaglandins (E<sub>1</sub>) inhibit the extrusion of granule enzymes from PMN (Malawista, S. E. and P. T. Bodel, 1967, J. Clin. Invest. 46: 786.; Weissman, G., et al., 1970, Nature New Biology 231: 131.). Ionizing radiation may alter the antibacterial capacities of PMN (Selvaraj,

R. J. and A. J. Sbarra, 1967, J. Bact. 94: 149), but whether this effect is produced on mature cells, on progenitor cells, or is due to the immaturity of PMN from bone marrow regenerating after X-irradiation is not clear.

PMN may kill bacteria directly with constituents possessing primary antimicrobial capacity or with mechanisms mediated through metabolic activities that generate short lived toxic entities such as free iodine. These possibilities are considered in sequence.

What antibacterial substances with primary antimicrobial capacity do PMN possess and how are they distributed in these cells? On the one hand, histones and protamines possessing primary antibacterial activity are extractable from PMN (reviewed in Skarnes, R. C. and D. W. Watson, 1957, Bact. Rev. 21: 273.). Spitznagel (1961, J. Exptl. Med. 114: 1063.) adapted methods for histochemically detecting cationic proteins and found such proteins coated bacteria in abscesses as well as bacteria phagocytized by PMN in tissue culture. Histochemical evidence showed these proteins came from PMN cytoplasmic granules and thus were not nuclear histones, an inference borne out by biochemical studies done later (Zeya, H. I. and J. K. Spitznagel, 1963, Science 142: 1085; Ibid, 1966, J. Bact. 91: 750; Ibid, 1966, J. Bact. 91: 744.). Isolated, LCP from rabbit PMN cytoplasmic granules possessed primary antibacterial capacities. They had molecular weights between 4,000 and 8,000; some of them contained over 30% arginine and 14% cystine and were poor in aromatic amino acids (Zeya, H. I. and J. K. Spitznagel, 1968, J. Exptl. Med. 127: 927; Ibid, 1971, Lab. Invest. 24: 229 and 24: 237.).

More recently Brune (Brune, K., M. S. Leffell and J. K. Spitznagel, 1972, Infec. and Immun. 5: 283.) has shown that chicken PMN lacking

peroxidase were quite able to kill ingested bacteria and yeast. These chicken PMN possessed at least two granule types. The very large ones contained lysozyme and cationic proteins similar electrophoretically to those of rabbit PMN azurophil granules (Brune, K., and J. K. Spitznagel, 1973, J. Infec. Dis., in press.). Extracts of these PMN granules possessed antimicrobial capacities.

It has been difficult to demonstrate LCP in total granule fractions of human PMN. The sources of the difficulties probably include: the buffy coat PMN which are often incompletely separated from formed elements other than PMN, elements that contain histones and other basic proteins that may be confused with LCP, or anionic substances that can complex with LCP and interfere with their resolution; the PMN themselves may contain strongly anionic substances that can complex with the LCP; and finally, it is possible, and the histochemical behavior of human buffy coat PMN suggest this, that compared to rabbit exudate PMN there is less LCP in human buffy coat PMN. Welsh has shown, however, that antibacterial extracts of human buffy coat PMN granules contain proteins more cationic than lysozyme or peroxidase. These proteins, with cellulose acetate electrophoresis, behave like the LCP of rabbit PMN and are more cationic than lysozyme. But, on polyacrylamide gels they behave as molecules or aggregates larger than lysozyme. Their properties are currently under study in our laboratory.

Why is oxidative metabolism thought to play a part in antibacterial actions of PMN? It is largely because Holmes, et al. applied the findings of Karnovsky (see above) and showed that children with chronic infectious granulomatosis, a disease characterized by inability to cope with infections,

## RESEARCH PROPOSAL

Polymorphonuclear leukocytes (PMN) in innate resistance to infection and the antimicrobial constituents that can be detected, localized and characterized in their cytoplasmic fractions are the subjects of these studies. In the past we have shown that azurophil granules of rabbit PMN possess antimicrobial capacity mediated by several cationic proteins (LCP) which display selective activity. More recently we have shown the cytoplasmic granules of chicken PMN possess similar cationic proteins. In addition, the granules of PMN from both species possess lysozyme, the chicken PMN in fact possesses two different ones. The specific granules of the rabbit possess lactoferrin as well and it is possible that chicken PMN possess an analogous protein, conalbumin.

In the meantime mounting evidence has linked the oxidative metabolism of mammalian PMN, including those of humans, to intraleukocytic antimicrobial action. Important to this system are the hexosemonophosphate shunt, availability of oxygen and chloride as well as myeloperoxidase. Recently new concepts have been suggested within the context of the oxygen dependent systems. Super-oxide anion  $\bar{O}^{\cdot}$  and singlet oxygen have been proposed by different investigators as mediators of intraleukocytic killing. The role of these putative mediators is in the process of being determined by other workers. While there is no doubt that the oxidative mechanisms form powerful antimicrobial systems, it is clear that there are back-up systems which function reasonably well. Back-up systems must be important in humans with genetically determined myeloperoxidase deficiency. Such persons do not have increased susceptibility to infection. Their leukocytes kill microbial cells more slowly than do normal PMN but they do kill. Chicken PMN have no peroxidase yet they kill microbes efficiently and

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chickens resist infection adequately. Moreover, PMN placed in anaerobic systems kill several kinds of bacteria, an observation which correlates with the fact that PMN seem to continue their function in abscesses which may provide an anaerobic microenvironment. It seems clear therefore that there are both oxygen dependent and oxygen independent antimicrobial systems in PMN. So far the cationic proteins, lactoferrin and lysozyme seem most likely to provide the oxygen independent system.

It is the purpose of this research to investigate primarily the oxygen independent killing capacities of human and animal PMN. We also propose to try where possible to place these mechanisms in proper context as to their importance and mode of action with respect to the oxygen dependent mechanisms. Our approach to this has been to try to isolate important organelles of PMN and the constituents of these organelles so that they can be studied in isolation and then studied together in controlled conditions. We hope in this way to develop methods for the precise and unambiguous detection and measurement of antimicrobial substances in resting and in phagocytizing PMN. We propose to continue this approach with the plan outlined in our previous proposal, which immediately follows this section. In the Progress Report we have described the accomplishments of this laboratory for the past year in which we have characterized the normal human PMN granules and shown that it is feasible as well as desirable to be able to use immunochemical techniques to follow certain events that involve both oxygen independent and oxygen dependent systems. The use of immunochemical methods offers several advantages. They are specific for molecular structure, they provide a method for measuring enzyme molecules in the presence of inhibitors that would block catalytic measurement, and they provide detection and measurement of molecules for which no catalytic measurement is available.

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We will prepare isolated granules for study of their antimicrobial capacity and then fractionate their components by the techniques in common use for resolution of protein mixtures. The constituents will be tested for their antimicrobial activity against a variety of microbes. We will attempt to elucidate the way in which the granules are assembled and transported in resting cells since this is crucial to the understanding of the way they are activated during phagocytosis. The formation of phagolysosomes will be studied further and the contribution of the different granule constituents to this will be investigated with the techniques we have developed. We will also attempt to understand the changes that take place in the cytoplasmic membrane in response to contact with and binding to particles coated with opsonins. This will be facilitated by our ability to isolate the cell membranes. We will continue to look for patients with defective granules in their polymorphonuclear leukocytes, hoping to learn with them more about the role of PMN constituents and functions in intraleukocytic killing. In the same way we will carry on comparative studies employing the peroxidaseless PMN of chicken in order to study oxygen antimicrobial systems in the absence of MPO. Finally, we will continue to study the role of the proteases of the PMN in the degradation and killing of phagocytized bacteria and other microbial forms. The effects of various environmental stresses such as intercurrent illness, immunologically mediated diseases, high energy radiation, drugs, and environmental pollutants will be studied where possible.

Detailed methods of procedure are described in the previous proposal, a copy of which herewith follows.

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## BUDGET

From July 1, 1974 through June 30, 1975

1. Salaries and Wages

J. K. Spitznagel, M.D., Prof. and Principal Investigator - 15% time, 12 months (Social Security no. [REDACTED])	\$ 2,200
Richard F. Rest, Post-Doctorate Assoc., 50%	3,000
(Also Ardis D. Hoven, M.D., Res. Assoc., 50%; James D. Folds, Ph.D., Asst. Prof., 10%; Edith K. MacRae, Ph.D. Professor, 5%; and Ralph Penniall, Ph.D., Professor, 2%)	0
Marjorie Cooney, Research Analyst, 15 hrs. wk.	2,000
Larry Martin, Lab Technician, 20 hrs. wk.	3,000
Sarah Freedman, Secretary	300

Hospitalization and Disability Insurance (\$16/mo. of employment for each fulltime employee)	250
Fringe Benefits: Social Security 5.85% salaries and wages	
Retirement 8.95% salaries and wages	1,554

2. <u>Travel</u> : Domestic (travel to meetings, two persons)	500
Foreign	500

3. <u>Supplies and Animal Care</u>	5,742
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4. <u>Publications, reprints, medical illustration</u>	400
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5. <u>Miscellaneous items</u> : Xerox, subscriptions, telephone tolls.	500
Maintenance and equipment repair	500

Indirect Costs - 48.13% salaries and wages, provisional and unaudited	5,054
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TOTAL	\$25,500
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John K. Spitznagel, M.D., Principal Investigator

## JUSTIFICATION

Salary of Principal Investigator: The amount budgeted for Dr. Spitznagel's salary amounts to 6 2/3% of his total salary. However, Dr. Spitznagel gives at least 15% of his time to the research supported by this grant. The difference in salary between the amount or percent budgeted and the amount of actual time spent is made up from other sources.

Foreign Travel: The Principal Investigator has been invited to participate in the International Congress of Immunology to be held in Brighton, England, July 22-26, 1974. This invitation is the result of earlier activities suggested by this grant. Moreover, the meeting should be a valuable source of current information concerning research activities in PMN leukocyte functions and other aspects of inflammation and immune responses. It is requested therefore that the AEC allow \$500 toward this trip, which is now estimated to cost \$800. The balance of the cost will be obtained from other sources.

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Contract No.: AT-(40-1)-3628

FINANCIAL STATEMENT

(1) Total actual project cost to date for the current period	\$ 21,005
(2) Estimated total cost for remainder of period	12,995
(3) Total actual and estimated cost chargeable to AEC for current period based on percentage of cost agreed upon as contained in A-III of Appendix "A" to Contract	34,000
(4) Accumulated costs chargeable to AEC (include costs reported in certified statement for preceding period(s) and the costs stated in Item (3) above)	113,993.80
(5) Accumulated AEC Support Ceiling as stated in Article III of Contract	114,643 (reduced to \$114,300)
(6) Total estimated AEC funds remaining under Contract (subtract Item (4) from (5)) which may be used to reduce amount of new funds required from AEC for proposed renewal period	649.20 (\$354.20)

Other financial support

National Institutes of Health grant 5 R01-AI02430 "Metabolic Aspects of Bacteria"

Project period: 12/01/73 to 11/30/74. Amount of award: \$37,575.00

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FACILITIES AVAILABLE

Laboratory - 1050 square feet

Animal quarters under full-time supervision of Dr. James Pick  
(D.V.M.) and staff.

General laboratory equipment, glassware, fume hoods, analytical  
balance, etc.

Special Items

Beckman L2-65B preparative ultracentrifuge

Beckman TI-14 and other rotors

Sorvall RC-2 refrigerated centrifuge

Packard Tricarb liquid scintillation spectrometer

Packard Autogamma crystal scintillation spectrometer

Acta V recording spectrophotometer

Paper electrophoresis

Polyacrylamide gel electrophoresis

Density gradient electrophoresis

Isoelectric Focusing Apparatus

Immuno-electrophoresis

Chromatographic equipment with fraction collector

Fluorescence microscope

pH meter - Beckman Century SS-1

Coulter Counter - A Model

In Department

Amino acid analyzer

Electron microscopes

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