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U.S. ATOMIC ENERGY COMMISSION

SIE NO. 602

AEC CONTRACT NO. (R)

SUPPORTING DIV. OR OFFICE: Laboratory and University Division

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

707797

TITLE OF PROJECT:
Mechanisms of Innate Immunity. VI. Biological Properties of Polymorphonuclear Leukocyte Cytoplasmic Granules 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.

Dr. John K. Spitznagel, Professor and principal investigator
Dr. J. D. Folds, Assistant Professor
Dr. H. I. Zeya, Assistant Professor
Dr. G. Orlando, Research Associate
Mrs. M. Cooney, Research Analyst I
Miss Jennie Foushee, Research Aide

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

SUMMARY OF PROPOSED WORK: (200-300 words, omit Confidential Data). Summaries are exchanged with government and private agencies supporting research, are supplied to investigaters 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.

Objective is to i) characterize cytoplasmic particles from human and animal polymorphs and to isolate and identify antibacterial substances and systems from them ii) measure the effects of these systems on bacterial cells and their products iii) assess their contribution to antimicrobial defences and iv) measure how they may be altered by infection, ionizing radiation, neoplasia, aging and other influences. The results should add substantially to knowledge of antibacterial mechanisms and resistance to infection in health and disease. Approach is to isolate peripheral blood PMN or exudate PMN, disrupt these and resolve their cytoplasmic particles by centrifuging them through sucrose density gradients and then to study the chemistry and biological activity of granule extracts.

RESULTS TO DATE:
To date we find that the 6 antibacterial cationic proteins of rabbit PMN are associated with the peroxidase rich, largest, most rapidly sedimenting granules and most of the antibacterial action of rabbit PMN seems vested in these granules. Human polymorph lysosomes - while they in general resemble those of rabbits - show significant differences. Lactoferrin and lysozyme are in a granule separate from the one associated with alkaline phosphatase. A situation different to that in rabbits where these substances are evidently all associated with a single granule population. Human granules unlike any animal granule thus far studied have only a neutral protease, and it is confined to a single granule population.

	PROGRAM CATEGORY NO.
BUDGET	
PRIMARY	
SECONDARY	

John K. Spitznagel
Signature of Principal Investigator

DATE: Nov. 16, 1971

INVESTIGATOR – DO NOT USE THIS SPACE

CONTRACTS

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COLLECTION DOCUMENTS 1944-1994

SCIENTIFIC BACKGROUND

Results of research into mechanisms of inflammation and resistance to infection influence our work most strongly when they deal with structural metabolic, and biochemical changes that occur in PMN in association with phagocytosis, inflammation, the action of 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 at least four events in PMN: i) Degranulation (Hirsch, J. G. and Z. A. Cohn, 1960, J. Exptl. Med. 112: 1005); ii) Increased O_2 uptake due to activation of hexose-monophosphate-shunt (HMP) activity (Karnovsky, M. L., 1962, Physiol. Rev. 42: 143); iii) Transfer of cationic granule proteins to phagocytized bacterial cells (Spitznagel, J. K. and H. Y. Chi, 1963, Am. J. Path. 43: 607), and iv) Destruction of the bacteria (Cohn, Z. A., 1963, J. Exptl. Med. 117: 27). Toxins such as streptolysin S, cause lysis of PMN granules, and cells die (Weissman, G., 1964, Fed.Proc. 23: 1038), while drugs like colchicine may prevent degranulation (Malawista, S. E. and P. T. Bodel, 1967, J. Clin. Invest. 46L 786). Ionizing radiation may alter the antibacterial capacities of PMN (Selvaraj, R. J. and A. J. Sbarra, 1967, J. Bacteriol. 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,

What antibacterial substances do PMN possess, and how are they distributed in their cells? On the one hand histones and protamines possessing primary antibacterial activity are extractable from PMN (reviewed in Skarnes, R. C.

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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, these cationic proteins from rabbit PMN cytoplasmic granules (LCP) possessed primary antibacterial capacities. They had molecular weights between 4000 and 8000; 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, and see Progress Report this contract). On the other hand, metabolic factors may have a role in antibacterial actions of PMN: i) 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); ii) it has been suggested that an enzyme tentatively linked to the HMP shunt, NADH oxidase, is missing from PMN of children with X-linked chronic infectious granulomatosis, a disease characterized by chronic, recurrent staphylococcal and gram negative rod infections. The oxidase deficiency is thought to reduce H_2O_2 production and cause PMN bactericidal action to fail - a feature of this disease (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 at a slower rate than could

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normal PMN. The authors proposed that although peroxidase was lacking, still other effective antibacterial factors existed in the PMN. Other workers have found phagocytin (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: 688), but defects in phagocytin have not yet been implicated in disease production. 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). Clearly, the relative importance of the different bactericidal systems requires further assessment in man and animals, and heretofore undiscovered systems must be sought.

Patients with PMN defects may be infected by just one species of microbe, e.g., *Staphylococcus aureus*, *Candida albicans*, or gram negative rods, yet maintain resistance against other organisms (see Douglas and Fudenberg, 1969). 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 may, according to Mandell and Hook, 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. Possibly normal PMN possess several substances that kill microbes in a direct as well as a selective way. If one of these were deleted the cell might manifest a selective inability to kill bacteria. Evidence for this mechanism in humans has not yet been forthcoming but Davis et al.

(1968, see above) reported a patient whose PMN were only unable to kill S. aureus, and Lehrer et al. found their patient's PMN deficient in myeloperoxidase and only unable to kill C. albicans. This focuses attention on the possible existence of multiple antimicrobial factors with selective action. The LCP isolated from rabbit PMN by Zeya and Spitznagel have these qualities. Each of them has a restricted range of action but together they account for almost the entire range of antibacterial activity of the parent PMN. The degranulation of PMN during phagocytosis is another possible source for selectivity in bacterial killing. Since there are several different kinds of PMN cytoplasmic granules (Zeya, H.I. and J.K. Spitznagel, 1969, Science 163: 1069), selective degranulation might limit exposure of bacteria to only a fraction of the antimicrobial mechanisms in PMN.

Besides their antimicrobial activities PMN contribute to inflammation in various ways (reviewed by Cochrane, C.G., 1968, adv. in Immunol. 9: 97-162). They also influence, if only indirectly, the immune response. Some evidence suggests they destroy antigen (Walsh, T.E. and C.A. Smith, 1961, J. Immunol. 66: 303; Cohn, Z.A., J. Exptl. Med. 120: 869), but these investigators used semi quantitative techniques and gram negative rod "agglutinogens" and this suggests further investigation with different antigens and quantitative techniques that can discriminate between antibody classes might yield interesting results. Moreover, the fact that macrophages seem to be influential in responses to some antigens (Spitznagel, J.K. and A.C. Allison, 1970, J. Immunol. 104: 119-139) and the fact that basic proteins can increase uptake of protein antigens by cells (Ryser, H.J-P. 1968, Bull. Swiss Acad. Med. Sc. 24: 363) focus attention on the possibility that cationic proteins from PMN could influence macrophage behavior in the handling of an antigen.

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How these antibacterial and other factors are delivered to scenes of bacterial invasion in a usable form was answered by Cohn and Hirsch (Cohn, Z. A., and J. G. Hirsch, 1960, J. Exptl. Med. 112; 983). They showed that the cytoplasmic granules of PMN are lysosomes and contain acid hydrolases and antibacterial (phagocytin) activity. Their phagocytin was probably 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, 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 (1969) have confirmed this and found that the antibacterial cationic proteins of rabbit PMN are confined to a unique heavy granule fraction. This work shows interesting correlations with morphological 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). Further complexity is introduced into the problems surrounding degranulation mechanisms by the fact that granule constituents can combine with and reversibly inactivate each other under certain circumstances (Zeya, H. I., 1969, Fed. Proc. 28). All of these factors will have to be considered in developing a useful hypothesis of degranulation.

SCIENTIFIC SCOPE

As work on innate resistance to infection continues, we will study:

i) Specificity and mode of antibacterial action of cationic and other PMN lysosomal proteins.

ii) Role of PMN in immune responses.

iii) role of different lysosomes in phagocytosis and destruction by PMN of bacteria, yeasts and fungi.

iv) Production of lysosomal constituents of PMN; the influence of infection, drugs, X-rays, genetic mechanisms and aging.

The general plan of work is outlined below.

General Plan of Work

Specificity and mode of action of cationic and other lysosomal proteins from rabbit polymorphonuclear leukocytes (PMN)

In all of our experiments it is necessary to prepare lysosomes and fractions of lysosomes. The general method for this is described first.

Exudates stimulated by intraperitoneal injection of glycogen will provide the PMN from which cytoplasmic granules will be prepared. Homogenized in .34 M sucrose and centrifuged at 400 X G 10 minutes to remove nuclei, unbroken cells, and large debris, the exudate cells provide a suspension from which a series of 3 granule fractions can be prepared by centrifugation through a sucrose density gradient (20% to 50%) in a zonal rotor (Beckman II-14) at 23,000 X G for 20 min. Lysosomal cationic proteins can then be separated from the heaviest fraction, band III (ref. 11 in bibliography of Progress Report). Either of two methods for separation are applicable, sucrose density gradient electrophoresis (Zeya, H.I. and J.K. Spitznagel, 1968, J. Exptl. Med. 127: 927) or gel filtration over sephadex G 75 (ref. 10 in bibliography of Progress Report). Cellulose acetate electrophoresis of the pooled fractions will show whether the fractions are homogeneous and verify identity of the proteins in the fraction. The amount of protein in the fraction will be measured by the Lowry technique.

The several other granule and protein fractions which will result from these procedures will provide material for analysis of other PMN cytoplasmic components.

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Specificity

These studies extend earlier ones on the range of bacterial killing by lysosomal cationic proteins (LCP), give clues to their modes of action, and may help to analyze the basis for differences in host resistance to various microbes. Using the modified antibacterial assay of Muschel (Zeya, H.Il and J.K.Spitznagel, 1968, J. Exptl. Med. 127: 927), we will test the antibacterial potency of the different LCP fractions against previously untested strains of Staphylococcus aureus and Streptococci of different groups. In addition, the effects of the fractions will be tested against strains of Pseudomonas aeruginosa, Aerobacter, Klebsiella, Serratia marcescens, Salmonella typhimurium, Mycobacterium tuberculosis, atypical mycobacteria and fungi including Candida albicans, Cryptococcus neoformans, Blastomyces dermatiditis. These experiments will provide results with which to compare the antibacterial effects of material similarly prepared from other species and strains of animals and from humans (from peripheral blood), and with which to contrast the relative importance of LCP and other antibacterial substances of PMN.

Mechanism of Action

In early attempts at analyzing the action of LCP, crude extracts of rabbit PMN lysosomes were the source of antibacterial factors (Zeya, H.I. and J.K. Spitznagel, 1966, J. Bact. 91: 755). Now the availability of more homogeneous fractions make further studies on their action worth while. Because there are five or more proteins involved, each with its own physicochemical features and each toxic for a characteristic spectrum of microorganisms, it is likely their mechanisms of action differ.

i) Effects of LCP on growth and viability of bacteria. Procedures devised by Muschel (see above Specificity of Action) and by Hirsch (Zeya, H.I. and J.K.

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Spitznagel, 1966, J. Bact. 91: 755) provide tests for viability of bacteria exposed to LCP fractions dissolved in buffer solutions. Muschel's test probably measures bactericidal action but we will verify this by subculturing bacteria treated with LCP onto agar plates. Whether or not growth of the microorganism is required for the lethal action of the LCP will also be investigated. The ways in which pH, salt concentrations and anaerobic conditions influence the antibacterial action of LCP will be the subject of some experiments and we will investigate antagonism of LCP action by other tissue components; proteins (acidic and basic), lipids, nucleic acids, acid mucopolysaccharides, glycogen, iron and divalent cations.

ii) Effects on bacterial permeability The most cationic of the lysosomal proteins has, in addition to its positive charge, a number of hydrophobic amino acids and conceivably its action resembles that of cetyl-trimethyl ammonium bromide or the polymyxins which increase bacterial permeability. Leakiness, if the LCP induce it, will be recognized when small molecular components, mucleotides, amino acids or inorganic ions (phosphate or sulfate) leak out into the media (Zeya, H.I. and J.K.Spitznagel, 1966, J. Bact. 91: 755; Spitznagel, J.K. and L.A.Wilson, 1966, J. Bact. 91: 393). Conversely, hydrolysis of substrate molecules to which the cell is normally impermeable can be used to show that permeability is increasing.

iii) Site of binding to bacterial cell Fluorescein conjugated to LCP will reveal sites in, or on, bacteria to which the LCP bind when the bacteria are studied under a U.V. microscope. LCP tagged with ^{125}I may be traceable to points of binding in bacterial cells which have been treated and then fixed, embedded, sectioned, mounted on grids with emulsion, and examined by electron microscopy. These techniques will be available.

iv) Effects on synthesis of macromolecules Depending on the outcome of

these studies, we will incubate a thymineless E. coli with LCP and ^{3}H-thymidine to see if DNA synthesis occurs with incorporation of the thymidine. Incorporation of ^{14}C-alanine might be studied in the presence of LCP to see if protein synthesis is blocked.

Role of PMN in immune response

i) Effects on adjuvants. The method to be described depends on the fact that the adjuvant action of endotoxin for immunization of CBA mice with bovine serum albumin (BSA) is dose dependent (Spitznagel, J.K. and A.C. Allison, 1970, J. Immunol. 104: 119-139). The purpose is to study effects of PMN--especially their lysosomal components--on the capacity of adjuvants to enhance immune responses, of antigens to stimulate antibody production, and of cells--macrophages and lymphocytes--to perform immunological functions. The experimental animals will be CBA mice. Syngeneic PMN from peritoneal exudates will take up, in tissue culture, endotoxin that has been iodinated with ^{131}I. When the cells have been washed and ^{131}I counts on them measured in a crystal scintillation spectrometer, endotoxin bound to them will be calculated. Intraperitoneal injection of these cells, their concentration adjusted to contain the desired dose of endotoxin, will serve as adjuvant in experimental mice. In other experiments adjuvant action of just endotoxin treated with lysosomal components of PMN will be compared with that of cell associated or untreated endotoxin. An equal amount of free endotoxin will be injected as a control into other mice. Other controls will include mice injected with similar numbers of PMN in balanced salt solution, mice injected with no cells and no endotoxin, and when appropriate, mice injected with endotoxin mixed with lysosome fractions just before injection. The primary immunizing injection is crystalline BSA, given peritoneally. One hundred μg of BSA given 20 days later will serve as the secondary injection for each mouse. Bleedings taken just before and 10 days after the

secondary antigen injection will yield serum for determinations of primary and secondary antibody responses. Antibody responses will be measured by a modification of the Farr technique for antigen binding capacity (ABC) (Brownstone, A., N.A.Mitchison and R. Pitt-Rivers, 1966, Immunology 10: 481). Crystalline ^{131}I BSA is the antigen. ABC's measured in individual mouse sera provide statistics from which are calculated geometric means and standard errors for each experimental group. The effects of PMN associated endotoxin on antibody production against BSA can then be compared with the effects of endotoxin or cells given alone. Antibody responses in all groups can be compared with responses in mice that only received antigen.

ii) Effects on antigen Mice produce antibody in proportion to the antigen injected into them (Spitznagel, J.K. and A.C.Allison, 1970). Experiments of others have shown that PMN simply destroy antigenicity (Cohn, Z.A.,1964, J. Exptl. Med. 120: 869-83). They used semi-quantitative methods for antibody measurements, complex antigens and antigen input that could not be accurately measured. They used rabbit PMN to treat antigen and mice to test the antigen--an *E. coli* agglutinogen--that probably primarily stimulated IgM production. The experimental model described above improves upon that design because, for example, it features quantitative measurements of antibodies and use of syngeneic animals. Details of experiments will not be described. The general plan would be to incubate PMN with ^{131}I iodinated BSA and then inject them into mice. The antibody responses would be determined and compared with those given by mice that were injected with BSA alone.

iii) Effects on macrophages and lymphocytes in immune responses It is well known that lymph node cells and spleen cells can reconstitute mice rendered immunologically incompetent by X-irradiation. It has been shown more recently

that macrophages allowed to take up soluble protein antigens such as BSA enhance their immunizing capacity. By treating lymphocytes or macrophages with PMN lysosome components *in vitro* and then injecting the treated cells into recipient animals it may be possible to show that PMN have a potential influence on these cells *in vivo*. For instance, BSA taken up by macrophages is a potent antigen when the living macrophages are injected into normal syngeneic mice; treatment of the macrophages with PMN lysosome components might greatly depress or even stimulate the ensuing immune response in the recipient mice. The outcome would have a bearing on a possible role of PMN in indirectly controlling immune responses to antigens.

Role of different PMN cytoplasmic granules in phagocytosis

It is now clear that the lysosomal or cytoplasmic granules of both rabbit and human PMN are heterogeneous. What is not clear is precisely which enzymes cluster together in the same particles and, do these particles fuse randomly with PMN phagocytic vacuoles or do they fuse in an ordered fashion. Of course if the granule fusion takes place in an ordered fashion one would then like to know if the order is determined by host factors or parasite factors.

In the past two methods have been used--histochemical staining and measurements of enzymes by *in vitro* enzyme assays. In phagocytosis the same techniques have been used except that the apparent loss of latency by the enzymes and their concomitant release into supernatant fluids or cell sap have been taken as indices of participation of granules. There are several disadvantages to these approaches but one of the most important is that several isozymes that all attack the same substrate may be involved in the various granules and one could hardly know with the traditional techniques which isozyme was being measured,

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The additional and independent specificity of immunological methods is needed. We propose, therefore, to continue immunochemical studies of rabbit PMN lysosomes and initiate similar studies on human PMN lysosomes. Antibodies to lysosomal components can be used to measure the enzyme and other molecules from the lysosomes by radial immunodiffusion or they can be used to trace them with immunofluorescence, ferritin or radioactive tags.

Immunofluorescence may then be a useful supplement to the tools of histochemistry and cytology traditionally used in studies of this problem. We have prepared (Spitznagel, J.K., P.M. Absher and H.I. Zeya, 1970, in preparation) potent, specific antisera against the granules of rabbit PMN cytoplasm. We can now do the same with human PMN granules. These antisera are specific for components that are unique to each of the three subpopulations of PMN granules that can be separated in the centrifuge (Zeya, H.I. and J.K. Spitznagel, 1969, Science 163: 1069).

Antibodies (IgG), specific for different granule fractions and conjugated with fluorescein or rhodamine, will serve as reagents to stain cytoplasmic granules in PMN mounted, by means of centrifugation at low speeds, on glass slides. The stained cells will be examined by methods of histochemistry and fluorescence microscopy. Controls for the specificity of fluorescence staining will be cells treated with non-immune fluorescence labeled IgG, specifically absorbed fluorescence labeled immune IgG, non-immune IgG without a label, and

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immune IgG that has no fluorescent label. The results should reveal relationships between PMN cell anatomy and the three granule fractions obtained by centrifuging PMN granules through a sucrose density gradient. This should further clarify, for example, which of the three granule populations are the so-called specific ones. If we can organize electron microscope studies of this problem in collaboration with others we will do this. The department is adding a new electron microscope and technician so a collaborative effort is a real possibility. These techniques may be extended to studies on effects of phagocytosis, drugs, and other events on PMN granules.

Effects on PMN lysosomal components of infection, drugs, radiation and aging

What influence have these variables on the numbers, kinds, or composition of PMN lysosomes? It is known that they influence the number of PMN that are being made at any given time. Do PMN emerge from bone marrow as standardized cells or will their composition be influenced, for example, by infection? If their composition varies, this might be due to immaturity of cells that are being rushed out of the bone marrow, it could be due to qualitative or quantitative changes in biosynthetic processes in the PMN precorsors, or it could be due to both causes.

In a typical experiment, we will induce experimental Staphylococcus aureus skin infections in rabbits and take bone marrow and exudate PMN from them at 18, 42, 72 and 96 hours. After counting the cells and taking samples on which to determine total cell protein by the Lowry method, we will homogenize the cells, centrifuge them at 400 X G x 10 minutes and then centrifuge the supernatant material through sucrose density gradients. The gradients will provide three fractions of cytoplasmic granules and the contribution of each of these

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fractions to total cell protein will be determined by the Lowry method and appropriate calculations. The granule fractions, especially the two heavier ones, will provide material that will be examined by cellulose acetate electrophoresis, enzyme assays, and Muschel's test for antibacterial activity. In the Muschel's test, antibacterial action against two species of bacteria will be tested: S. aureus and group D Streptococcus. These two species are killed by different rabbit LCP and their response should show whether the staphylococcal infection has a preferential effect on PMN antistaphylococcal LCP compared with PMN antistreptococcal LCP. Normal rabbits will yield marrow and exudate cells to be analyzed as controls. Similar experiments will be designed to compare effects of infection on PMN lysosomal enzymes, myeloperoxidase and other enzymes. By selecting suitable animals or devising appropriate drug or X-ray treatments it should be possible to use these procedures to study the effects of drugs, X-ray, genetic mechanisms and age on cytoplasmic granules of PMN. Studies on PMN (peripheral blood) of patients with different diseases or under various treatments also would be useful.

SIGNIFICANCE OF THIS RESEARCH

The significance of the research rests on its aim, which is a better understanding of antibacterial actions of polymorphs and innate resistance to infection. As the specificities and modes of action of cationic and other lysosomal proteins of PMN are better understood, it will be easier to analyze differences which exist within and between species in resistance to various infections (Spitznagel, J. K., and H. I. Zeya, 1964, Trans. Asso. Amer. Phys. 77: 126). This is probably also true for what can be discovered about the different kinds of polymorph cytoplasmic granules and their actions during phagocytosis. These differences in resistance between species have long hampered studies of

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experimental infection. A thorough understanding of similarities and differences between antibacterial actions in PMN of various laboratory animals and man might show how to manipulate the animals to improve experimental design and interpretation.

Experiments on the effects of genetic changes, drugs, X-ray and aging are useful because they may have predictive value and may provide information useful in diagnosis, prevention and treatment of defects in PMN function caused by these agents. Such experiments benefit from the results of investigations on mechanisms of antibacterial action in polymorphs. Finally, since it is frequently unclear in what degree and way innate mechanisms are operating to modify specific, acquired, immune responses to infections, experiments on the effects of PMN on immune responses may yield useful information on these questions.

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MECHANISMS OF INNATE IMMUNITY

VI. Biological Properties of Polymorphonuclear Leukocyte Cytoplasmic Granules 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 U.S. Atomic Energy Commission

John K. Spitznagel, M.D., Principal Investigator — Nov. 16 1971 Date

A. H. Shepard, Jr., Assistant Vice President and Treasurer — Nov 23, 1971 Date
University of North Carolina, Chapel Hill

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We propose to continue and extend the program of investigation we outlined in our comprehensive proposal submitted in November, 1969. The body of that document is therefore included in this one. Several changes, proposed or accomplished, are noteworthy:

Changes in techniques:

We would like to automate some of our enzyme analysis. At present we do 8 different analysis on 50 samples each time we examine 1 person's polymorphs. This is 400 procedures and keeps our technicians busy doing these to the exclusion of almost all else. Thus we can do about one person's polymorphs a week. We could greatly accelerate our program if we had an autoanalyzer II from Technicon. Technician time so freed could be utilized for work on electron microscope specimens which we are now preparing more often.

Changes in personnel:

Dr. I. R. H. Welsh has received his Ph.D. degree and has returned to the Republic of South Africa where he has been awarded a position as senior scientist with the South African Atomic Energy Commission. Dr. Kay Brune's fellowship has terminated, and he has returned to the Pharmakologische Anstalt at the University of Basel. Dr. Gerry Orlando has joined our group on a half time basis. We have a new technician, Mrs. Jennie Foushee, a graduate of this institution's training program for Research Aides

In addition to these changes in personnel within our own group, we are entering into closer collaboration with other workers: UNC-Dr. Ralph Penniall, Professor Biochemistry; Mr. Mark Goodwillie, graduate student in Department of Biochemistry; Bowman-Gray School of Medicine - Dr's Charles McCall and Robert Cooper, Dept. of Medicine; Dr. Lawrence DeChatelet, Dept. of Biochemistry.

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The scope of our studies for the period March 1972-February 1973 will encompass the following areas: i) Specificity and mode of action of lysosomal components. In man we will study the antimicrobial action of extracts from the different particles from polymorph cytoplasm and use a spectrum of gram positive, gram negative and fungal organisms in the studies. We will specifically study actions of purified components when they, and if they, are available, e.g. lactoferrin and cationic protein including lysozyme, peroxidase and other cationic proteins not known to be enzymes.

From rabbit PMN we will continue to isolate cationic lysosonial proteins and study their mode of action on bacteria. In addition the studies on chicken PMN (See progress report) will be extended to identify their modes of antimicrobial action and compare them with mammalian PMN. Chicken PMN,because they lack myeloperoxidase, are advantageous for comparative studies of antimicrobial action of polymorphs. We will study their cytoplasmic particles,and Dr. Penniall and Mr. Goodwillie will study their oxygen metabolism.

ii) Role of PMN in immune response. In collaboration with Dr. Folds, we will continue to study the proteases of human PMN and their action on various antigens including human immunoglobulins.

iii) Role of different lysosomes in phagocytosis

This is being studied by Miss M. S. Leffell, an advanced graduate student. She has adapted a method so that she can isolate phagocytic vacuoles from PMN and at the same time isolate their cytoplasmic granules in a sucrose density gradient. She will study their degranulation biochemically with the aid of gradient centrifugation and morphologically with the electron microscope.

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iv) Production of lysosomal constituents

I shall continue my studies to characterize the cytoplasmic particles of human circulating PMN and observe the effects of age, exercise, fever, drugs, ionizing irradiation and other environmental influences as material becomes available. In addition I shall investigate PMN lysosomes from human marrow, first separating the cells with discontinuous gradients according to their maturity and then analyzing the constituent lysosomes of each developmental stage.

Dr. Zeya will continue to study in rabbit bone marrow the changes that occur due to bacteremia, drugs and other environmental influences.

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Contract No: AT-(40-1)-3627

BUDGET FOR SIXTH YEAR

From March 1, 1972 thorugh February 28, 1973

1.	Salaries[1]	
	Dr. John K. Spitznagel, Professor and Principal Investigator - 15% time, 12 months	$3,500
	Dr. H. I. Zeya, Assistant Professor - 50% itme[2]	0
	Dr. James D. Folds, Assistant Professor - 20% time[3]	0
	Dr. G. Orlando, Research Associate[4]	0
	M. Cooney, Research Analyst	2,593
	J. Foushee, Laboratory Assistant	5,060
2.	Supplies and Animal Care	6,000
3.	Equipment	
	Lease of Autoanalyzer II	3,200
4.	Travel	900
5.	Telephone, etc.	260
6.	Publication Costs	252
		$ 21,765
	Fringe Benefits	1,587
	Overhead	3,698
		$ 27,050

1) Fringe benefits and overhead not included. Fringe benefits - 14.225% of salaries and wages. Overhead - 33.16% of salaries and wages (provisional and unaudited).

2) Dr. Zeya's salary comes from an award from The Leukemic Society of America.

3) Dr. Folds' salary comes entirely from State Funds.

4) Dr. Orlando's salary is derived entirely from outside sources.

John K. Spitznagel, M.D.
Principal Investigator

1034324

JUSTIFICATION OF BUDGET

The amount of $3,200 is requested under equipment in this budget for the purpose of renting a Technicon autoanalyzer II (less print-out module). This includes:

Sampler IV
Proportioning pump
Colorimeter RSB with voltage stabilizer
Recorder, Single pen
Outlet strip
Kit of accessories

Purchase price: $7,090.00

The rental is on a 12-month basis, but if rental continues for 36 months, Technicon considers the unit purchased. I feel this unit will greatly expedite now routine analysis of polymorph granules of human and animal origin. This will free scarce technician time for other activities. In addition, it will enhance accuracy. When a technician pipettes 40-50 assays mistakes are apt to occur. Machines don't make this kind of mistake.

Thus, in the interests of greater efficiency, we propose to obtain the instrument. We propose to rent it because the funds are not sufficient to purchase it.

1034325

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

FINANCIAL STATEMENT

For the period: March 1, 1971 - February 29, 1972

	Budget	Expenditures as of Nov. 1 1971	Extimated Additional Expenditures	Total Expenditures
Personnel	$ 13,346	$ 8,882	$ 4,464	$ 13,346
Fringe Benefits	1,888	1,057	631	1,688
Overhead	4,426	2,945	1,481	4,426
Supplies	5,200	5,000	400	5,400
Travel	900	900	0	900
Publications	800	550	250	800
Equipment	290	0	290	290
Miscellaneous	200	100	100	200
TOTALS	$27,050	$19,434	$ 7,616	$ 27,050

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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 L-2-658 preparative ultracentrifuge

Beckman TI-14 and other Rotors

Sorvall RC-2 refrigerated centrifuge

Packard Tricarb liquid scintillation spectrometer

Packard Autogamma crystall scintillation spectrometer

Beckman DB recording spectrometer

Paper electrophoresis

Polyacrylamide gel electrophoresis

Density gradient electrophoresis

Isoelectric Focusing Apparatus

Immunoelectrophoresis

Chromatographic equipment with fraction collector

Fluorescence microscope

In Department

Amino acid analyzer

Electron microscopes

1034327

Curriculum Vitae

Geraldine S. Orlando Ph. D.

Born [redacted]

Present Address [redacted]

Marital Status; Married, 2 children

Education and Degrees

After being educated in the public school system of New York City, I entered [redacted] in September 1960. I majored in [redacted]. During this period of time I engaged in several genetic research projects with Dr. Marien professor of biology. I graduated with a [redacted] in 1964. I then attended [redacted] I studied under Dr. A. Saz, chairman of the Department of [redacted], inorder to obtain a [redacted]. After completing the required course work, I took a Master's by-pass examination and continued in the graduate school as a Ph. D. candidate. I studied immunology under Dr. J.A. Bellanti and also Ann L. Jackson. The title of my thesis was "The IgM Immune Response to Salmonella Typhimurium"

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