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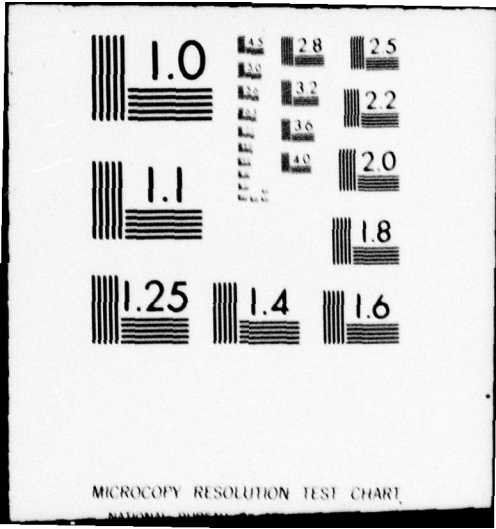
NEW ENGLAND MEDICAL CENTER HOSPITAL BOSTON MASS
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6 MECHANISM OF BACTERIAL KILLING BY THE PERITONEAL AND HEPATIC MACROPHAGE POPULATION: OXIDATIVE BACTERICIDAL MECHANISMS OF PHAGOCYtic CELLS

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20. Abstract (continued)

develop a system to examine the effect of oxygen tension on various aspects of neutrophil function, including phagocytosis, O_2^- and H_2O_2 production and bacterial killing.

Previous studies have shown that Kupffer cells, as well as hepatocytes and peritoneal macrophages, contain significant quantities of several glycosidase activities. The response of selected Kupffer cell glycosidases to *in vivo* inoculation of bacteria was examined using techniques previously developed. After administration of live *E. coli*, levels of N-acetyl- β -D-glucosaminidase and β -glucuronidase rose to twice control levels. Maximum activity was seen three days after a single inoculation of bacteria; levels had subsided to normal by five days. Bacteria killed by autoclaving had no effect on glycosidase levels under similar conditions.

At this point the direction of the research changed, and studies were begun on oxidative killing by neutrophils. Using *S. aureus*, an organism resistant to the oxygen-independent bactericidal mechanisms of granulocytes, we attempted to reproduce previous work showing survival of bacteria in the presence of neutrophils under nitrogen, except using techniques that would permit shorter periods of equilibration with the atmosphere before mixing the bacteria with the neutrophils. The techniques employed for these studies were unsuccessful, in that they appeared to damage the neutrophils.

We are presently developing more gentle techniques for the incubation of neutrophils under defined atmospheres, using nitrogen as the test gas and the abolition of O_2^- production by activated cells as the indicator of satisfactory equilibration.

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SUMMARY

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FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

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BODY OF REPORT

Background

1. Glycosidases of Kupffer Cells, Hepatocytes and Peritoneal Macrophages

Macrophages represent a heterogeneous population of cells which function as scavengers of particulate matter of diverse origins, including bacteria, products of cellular disintegration, and nonspecific foreign material (e.g., carbon particles) which may be taken up by the organisms. There is considerable evidence that in the course of disposing of ingested particulate materials, macrophages degrade them to soluble constituents. In particular, degradation by these cells of bacteria uniformly labelled with ^{14}C or ^{32}P has been shown to lead to the release of trichloroacetic acid soluble radioactivity from the microorganism⁽¹⁾.

Among the substances that must be handled by macrophages which have ingested bacteria is the bacterial cell wall⁽²⁾, a highly complex structure composed of a polysaccharide consisting of alternating residues of N-acetylglucosamine and N-acetylmuramic acid, cross-linked by short peptides to form a covalently linked two dimensional mesh. In addition to the cross-linked polysaccharide (the peptidoglycan), the cell walls of Gram negative organisms contain lipopolysaccharide (endotoxin). This substance is composed of lipid A, containing glucosamine oligosaccharides whose hydroxyl groups are completely substituted by fatty acids, to which

is attached a polysaccharide whose composition varies with the species of micro-organism⁽³⁾. Lipopolysaccharide is harmful to mammals⁽⁴⁾, but mammals are able to defend themselves against this agent, in part through the function of the macrophage, which has been shown to degrade endotoxin to biologically inert materials^(5,6).

The degradation of bacterial cell walls by macrophages implies the existence in these cells of hydrolases capable of depolymerizing macromolecular cell wall constituents. In particular, glycosidases would be necessary to degrade the carbohydrate portion of endotoxin as well as to cleave the glycosidic linkages of peptidoglycan. The existence in hepatic lysosomes of a large number of glycosidases potentially capable of participating in the degradation of these substances has been known for some time⁽⁷⁾. This laboratory has recently analyzed the distribution of several of these glycosidases among hepatic cell types. These studies showed that hepatocytes, Kupffer cells and peritoneal macrophages all contained approximately equal quantities of the glycosidase activities examined. Further studies with Kupffer cells showed that the glycosidases were located in the lysosomes, as expected for enzymes that participate in the degradation of foreign materials by phagocytic cells⁽⁸⁾.

The enzyme content of macrophages has been shown to vary with the physiological state of the cell. Both in vitro and in vivo studies have shown that the exposure of macrophages to suitable agents causes their content of hydrolytic enzymes to increase significantly while they undergo morphological changes consistent with an

increased capacity to deal with ingested material⁽⁹⁾. Whether Kupffer cell glycosidases undergo similar changes is not yet known.

2. Oxidative Killing Mechanisms of Neutrophils

Upon exposure to opsonized bacterial, normal granulocytes undergo changes in their oxidative metabolism (the "respiratory burst") which result in a marked increase in oxygen uptake, a rise in H_2O_2 and superoxide ($O_2^{\cdot -}$) production and a tenfold increase in the amount of glucose metabolized via the hexosemonophosphate shunt⁽¹⁰⁻¹⁷⁾. These changes in metabolism are thought to be related to the activation of oxygen-dependent bactericidal mechanisms, since patients whose granulocytes cannot undergo these metabolic alterations (e.g., patients with chronic granulomatous disease or severe glucose-6-phosphate dehydrogenase deficiency) are unusually susceptible to bacterial infections^(18,19). Hydrogen peroxide in particular has been implicated in a bactericidal process, since Klebanoff has shown that bacteria are readily killed when incubated with hydrogen peroxide and halide ion in the presence of myeloperoxidase, an enzyme present in large amounts in the primary granules of polymorphonuclear leukocytes⁽²⁰⁾.

Though there is much evidence indicating that the peroxide-halide-myeloperoxidase system is involved in bacterial killing by granulocytes, it is clearly not the only oxygen-dependent killing mechanism employed by these cells. This is shown by the fact that patients whose granulocytes are devoid of myeloperoxidase on a hereditary basis have little trouble with bacterial infection, and appear to have nor-

mal life expectancies⁽²⁰⁾. This is contrary to what would be expected if the peroxide-halide-myeloperoxidase mechanism were the only oxygen dependent bactericidal mechanism employed by granulocytes, since under such circumstances, patients with myeloperoxidase deficiency would be expected to be as susceptible to bacterial infections as patients with chronic granulomatous disease.

With regard to the nature of the non-myeloperoxidase requiring oxidative bactericidal mechanisms of granulocytes, it is possible to envision hydrogen peroxide itself as one of the agents involved, since in sufficiently high concentrations this compound is bactericidal in the absence of myeloperoxidase and halide⁽²⁰⁾. However, recent observations showing that activated granulocytes generate large amounts of O_2^- ⁽¹⁰⁻¹⁴⁾ have led to suggestions that this compound, or substances such as $\cdot OH$ and singlet oxygen which can be formed from this compound in secondary reactions⁽²¹⁻²³⁾, may also play a role in the myeloperoxidase-independent bactericidal activity of granulocytes. Work has been published indicating that in artificial systems, these substances are able to kill certain strains of bacteria⁽²⁴⁻²⁶⁾, although other strains of bacteria appear not to be susceptible to the effect of these agents under otherwise similar conditions. A recent paper by Johnston et al. has provided strong evidence for the participation of $\cdot OH$ in the action of human granulocytes on several pathogenic bacteria⁽¹³⁾.

Results

1. Studies on the Glycosidases of Kupffer Cell from Rat Liver

With the completion of studies designed to characterize the glycosidases of Kupffer cells in uninfected animals⁽⁸⁾, investigations were begun in animals infected with bacteria. E. coli 0111:B4:H was the organism selected for use in these studies, because the structure of its cell wall lipopolysaccharide is known.

For the initial experiments, rats were inoculated by tail vein with ca. 10^8 organisms per animal, and selected Kupffer cell glycosidases were measured at various intervals of time after infection. Some animals received live bacteria, while others received organisms that had been killed by autoclaving. Control rats received normal saline or Hanks' buffered saline solution. At 48 hours, β -D-glucosaminosidase levels in rats that had received live bacteria were twice normal, while levels in animals inoculated with killed bacteria were not different than control. By contrast, β -glucuronidase and β -D-galactosidase levels were the same in all three groups of animals. Similar results were seen after five days.

Subsequent studies were carried out under more precisely defined conditions to establish more accurately the time course of the changes in glycosidase activity. In these experiments, rats were inoculated by tail vein with 5×10^7 live E. coli per 100 g. body weight. At various times after inoculation, Kupffer cells were isolated

and their content of β -D-glucosaminosidase and β -glucuronidase was measured. The results are shown in Figure 1. Unlike the previous experiments, the levels of both glycosidases rose after inoculation. Peak activities were observed three days after administration of the bacteria, and by five days the levels of enzymes had begun to decline again. The magnitudes of the increases were similar to those undergone by β -D-glucosaminosidase in the previous experiment (Figure 1).

2. Studies on Oxygen-dependent Bactericidal Mechanisms of Phagocytic Cells

In response to a favorable reply to a request for permission to undertake a major change in the direction of the research supported by USAMRDC Contract No. DAMD 17-74-C-4055, my laboratory has begun studies on the oxygen-dependent bactericidal mechanisms of neutrophils. Certain studies on this topic had already been accomplished in this laboratory. Although support for these studies came from a source other than an Army Contract, the results are pertinent to the work to be accomplished under the present Contract and are therefore included in this report.

a. Studies carried out in this⁽¹⁰⁻¹²⁾ and other⁽¹³⁻¹⁴⁾

laboratories over the past three years have shown that O_2^- is produced by granulocytes during the respiratory burst that accompanies phagocytosis. In seeking a possible direct role for O_2^- in the bactericidal activity of granulocytes, experiments were performed to ascertain whether bacteria are killed by a system consisting of xanthine oxidase plus purine, a known source of O_2^- and H_2O_2 , and, if so, whether O_2^- participates directly in this process. (For the purposes of this experiment, "direct participation" referred to participation in any manner other than as a source of H_2O_2). These experiments showed that both E. coli and S. epidermidis were killed by this system, provided that xanthine oxidase was present at sufficiently high concentration. With E. coli, killing was prevented by catalase but not by superoxide dismutase, indicating that the bactericidal agent was H_2O_2 . With this organism, O_2^- did not participate directly in the killing, but merely served as a source of H_2O_2 , which arose by the spontaneous dismutation of O_2^- to oxygen and H_2O_2 . With S. epidermidis, on the other hand, killing was prevented by both catalase and superoxide dismutase. This finding showed that the bactericidal agent was neither O_2^- nor H_2O_2 , but a compound formed by the reaction of O_2^- and H_2O_2 was proposed as the agent responsible for the killing of S. epidermidis⁽²⁴⁾.

b. Attempts were made to study the killing of S. aureus by granulocytes as a function of oxygen tension. S. aureus was selected because of a study by Mandell showing that this organism is resistant to the oxygen-independent bactericidal mechanisms of granulocytes⁽²⁷⁾. In preliminary studies of phagocytosis under anerobic conditions, granulocytes were found to take up emulsified opsonized paraffin oil droplets at a normal rate, but to only 75 percent of the extent seen with the same cells incubated in air. Extensive studies were then conducted using a strain of S. aureus purchased from the American Type Culture Collection to determine the optimum conditions for investigating killing by granulocytes under hypoxic conditions. In these studies, the quantity of granulocytes, the ratio of granulocytes to bacteria and the times of incubation were varied. 10^7 granulocytes per ml. was found to be a suitable concentration of cells. Killing was maximum at a bacteria/cell ratio of 1:1, was easily detectable at a ratio of 5:1, but did not appear to take place at a ratio of 10:1. The elaboration of leukocidal proteins by the microorganisms was not studied.

Attempts to measure anerobic killing were then made. These were unsuccessful, probably for technical reasons. The experiments were carried out in 15 ml. Warburg flasks. Granulocytes in 1 ml. of medium were placed in the vessel itself, while 0.5 ml. of bacterial suspension was placed in the sidearm. The atmosphere in the flask was then changed by several cycles of evacuation followed by flushing with N_2 . The evacuation was accompanied by considerable foaming in the granulocyte suspension. After exchange of the atmosphere the bacteria were tipped from the sidearm into the reaction vessel. Incubations were conducted on a rocker at 37° , and were

terminated with 10 ml. of distilled water injected through the serum port with which the flask was stoppered. Determinations of the numbers of surviving bacteria revealed no killing under N_2 , nor were bacteria killed in control incubations carried out in a similar manner except that room air was used instead of N_2 . Killing was observed, however, in other control incubations which had not been carried through the cycles of evacuation and gassing. We have concluded that the granulocytes were damaged by the evacuation-gassing cycles, and in the future we intend to gas without evacuation.

c. Experiments are planned that will measure the dependence of O_2^- production on oxygen tension. For these experiments, as for measurements of killing under anaerobic conditions (see above), it will be necessary to devise a method for equilibrating the reaction mixture with a defined atmosphere without evacuating the system. Attempts to abolish O_2^- production by neutrophil suspensions which have been bubbled with N_2 through a serum port in a sealed tube have failed, indicating that oxygen has not been removed from the reaction mixtures by the techniques so far employed. Presently we are attempting to deoxygenate the reaction mixtures by incubating them under an atmosphere of N_2 in a sealed flask, using incubation mixtures of relatively small volume to maximize the surface area through which equilibration of the mixture with the overlying atmosphere is attained. Abolition of O_2^- production in the incubation mixture will be used as an indicator of satisfactory equilibration.

Conclusions

1. Glycosidases of Kupffer Cells

These experiments have shown that the inoculation of bacteria into rats leads to a rise in the level of Kupffer cell glycosidases. The concentration of enzyme reaches a maximum at three days, then begins to decline. Thus, the behavior of Kupffer cell glycosidases resembles the response of other macrophage lysosomal hydrolases to stimulation. The duration of the increase in previous studies has been longer than observed here, a difference that is likely to be related to the differences in the nature of the stimuli used in the various experiments.

2. Oxygen-dependent Bactericidal Mechanisms

When suitably activated, neutrophils generate a large amount of O_2^- that is used for bacterial killing. Most is used after conversion to hydrogen peroxide, but we, and others, have found some instances in which O_2^- appears to participate as a precursor of hydroxyl radical. With regard to oxygen-independent bactericidal mechanisms, experiments so far have been inconclusive for technical reasons. We are presently developing techniques by which these mechanisms can be investigated.

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