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Role of the Phagocyte in Host-Parasite Interactions

XII. Hydrogen Peroxide-Myeloperoxidase Bactericidal System in the Phagocyte

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An antimicrobial system in polymorphonuclear neutrophils (PMN) consisting of myeloperoxidase and hydrogen peroxide has been proposed. This system appears to be activated during phagocytosis as a result of the stimulated metabolic activities. A lysed-granules (LG) fraction was prepared from guinea pig exudative PMN. LG alone possessed bactericidal activity which was related to the *p*H of the reaction; the lower the *p*H, the more marked the activity. When low concentrations of both H_2O_2 and LG were combined under conditions where neither factor alone exhibited significant killing power, there was a striking increase in bactericidal activity. This enhanced activity was much greater than an additive effect. The LG-peroxide antibacterial system was most active over a *p*H range of 4.0 to 6.0. The activity of the LG-peroxide system was essentially abolished by peroxidase inhibitors, NaN_3, KCN, and aminotriazole. The antibacterial activity of this system was nonspecific in nature, being equally effective against gram-negative and gram-positive organisms.

In the previous communication, evidence was presented indicating that phagocytizing cells produce increased concentrations of hydrogen peroxide and that this agent is involved in the intracellular killing of ingested bacteria (11). Recently, Klebanoff, Clem, and Luebke (9) described a bacteriostatic system in milk and found it to consist of lactoperoxidase, hydrogen peroxide, and thiocyanate. In addition, these investigators discovered that a peroxidase from leukocytes, myeloperoxidase, could substitute for lactoperoxidase. Since the polymorphonuclear neutrophil (PMN) produces hydrogen peroxide and also contains a high concentration of myeloperoxidase (1, 15), a study of the interaction of myeloperoxidase, H_2O_2, and bacteria was undertaken. It was felt that information to be gained from this study would aid in developing further the mechanism responsible for the killing of bacteria in the phagocyte.

MATERIALS AND METHODS

Bacteria. Test organisms were *Staphylococcus aureus*, *Staphylococcus albus*, *Streptococcus pyogenes*, *Streptococcus faecalis*, *Bacillus subtilis*, *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Shigella sonnei*. An overnight culture of a test organism grown in Trypticase Soy Broth (BBL) or Brain Heart Infusion (BBL) was standardized to a known concentration as described in the preceding paper (11). After standardization, bacteria were diluted in 0.1 M phosphate buffer of the appropriate *p*H to a concentration of approximately 2.5×10^5 organisms/ml. In some cases, bacteria in the logarithmic phase of growth were utilized.

Preparation of lysed granules (LG) from guinea pig PMN. Guinea pig exudate PMN were induced, isolated, and standardized as previously described (11). PMN were lysed initially by the sucrose lysis method of Cohn and Hirsch (2). Briefly, 3×10^8 PMN were mixed with 5 ml of 0.25 M sucrose and centrifuged at $400 \times g$ for 5 min. Then 4 ml of 0.25 M sucrose, containing 200 units of heparin to prevent the sticking of granules on cell fragments, was added to the pellet. The cell suspension was mixed for 1 min on a Vortex mixer, and then homogenized for 5 min in a motor-driven Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at $600 \times g$ for 5 min. The superantant fluid was poured off and the sediment was washed with sucrose. The supernatant fluid from this wash was combined with the initial supernatant fluid and centrifuged at $27,000 \times g$ for 15 min. The resulting supernatant fluid was discarded and the granular fraction was made up to a predetermined volume with 0.25 M sucrose. The granules were lysed by freezing and thawing six times in a dry ice-chloroform bath. The lysed granules (LG) were diluted in 0.1 M phosphate buffer of the appropriate *p*H and then used. PMN were maintained at 0 to 4 C throughout the entire procedure, and centrifugation was carried out in a Sorvall RC 2 centrifuge at 0 to 4 C.

Assay of bactericidal activity. Reaction mixtures were prepared in siliconized test tubes containing

0.1 ml of the stock suspension of the test organism and 1.0 ml of 0.1 M phosphate or citrate-phosphate buffer at the desired *p*H. The materials under study were added and the volume made up to 2.0 ml with the appropriate buffer. The tubes were incubated at 37 C with continuous shaking in a water bath. At predetermined time intervals (0, 15, 30, 60, and 90 min), 0.1 ml was removed, diluted, and plated on agar plates. Bactericidal activity at a particular time interval was assessed by comparing the bacterial concentrations in test reaction mixtures with the concentration found in control tubes containing only the test organisms and buffer.

Peroxidase activity. The myeloperoxidase activity of the LG preparation was assayed with guaiacol (10). Two reaction mixtures, each containing 1 ml of 20 mM guaiacol (Merck & Co., Inc., Rahway, N. J.), 2 ml of 10 mM phosphate buffer at *p*H 7.0, and 0.1 ml of an appropriate dilution of the LG preparation, were prepared. To one reaction mixture was added 20 ml of 10 mM H_2O_2; the other reaction mixture served as a blank. The increase in absorbance at 470 mμ was measured with a Beckman DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). One unit of myeloperoxidase was arbitrarily considered as that causing an increase in absorbance of 0.001 unit per min.

Inhibitors. All inhibitors were of reagent grade. 3-Amino-triazole was purchased from the Nutritional Biochemicals Corp., Cleveland, Ohio. Stock solutions were initially prepared in distilled water, then further diluted with 0.1 M phosphate buffer at *p*H 6.0 and used.

RESULTS

Bactericidal activity of LG. Since PMN granules are presently considered to contain antibacterial factors along with various hydrolytic enzymes, initial experiments were concerned with establishing the bactericidal activity of LG in our experimental system. The summarized results of several experiments carried out at *p*H 6.0 are presented in Table 1. These data indicate that LG exhibit an antibacterial effect which, with the exception of the highest concentration, was related to the concentration of granules. The highest concentration used demonstrated less of an effect than some of the lower concentrations. In fact, when an even greater number of granules than that shown was employed, the test organisms grew. It is interesting that in only one case was bactericidal activity observed at the 15-min period. The optimal concentrations of LG appeared to be those equivalent to granules from 2×10^8 to 4×10^8 PMN. With these concentrations, more than 99.9% of the *E. coli* cells were killed by 60 min. Lower concentrations of LG had not achieved this extent of bactericidal activity by 90 min.

Other investigators have reported that the in vitro activities of various antibacterial factors in PMN are enhanced when the *p*H is lowered (7). Thus, experiments along this line were carried out to determine whether similar results could be obtained with our LG preparation. A concentration of LG that exhibited only slight bactericidal activity at *p*H 6.0 was selected and then tested against *E. coli* over a *p*H range from 4.0 to 7.0. The results of these experiments are shown in Table 2. It is quite apparent that the antibacterial effect of the concentration of LG used was markedly enhanced at the lower hydrogen-ion concentration. More than 99.9% of the test organisms were killed by 15 min at *p*H 4.0 and 4.5, whereas less than 30% were killed during the same period at *p*H 6.0 or above. Killing at *p*H 5.0 and 5.5 was somewhat lower than that exhibited at the lower hydrogen-ion concentrations, but the same extent of killing was eventually obtained. At *p*H 6.0 and above, not only was the rate of killing reduced but also the extent of killing. It should be noted that no significant bactericidal effect on *E. coli* was found with any of the different hydrogen-ion concentrations employed.

TABLE 1. *Bacterial activity of lysed granules from guinea pig polymorphonuclear neutrophils (PMN) on Escherichia coli*

PMN equivalent[a]	Per cent bacteria killed[b]			
	15 min	30 min	60 min	90 min
$\times 10^7$				
60.0	0	25.6	70.0	91.2
40.0	0	91.7	>99.9	—
20.0	19.3	78.4	>99.9	—
4.0	0	37.4	85.2	97.8
2.0	0	19.6	75.3	93.7
0.8	0	0	24.6	52.6
0.4	0	0	0	0

[a] Lysed-granule preparation equivalent to granules isolated from the specified number of PMN.
[b] Initial concentration of *E. coli* in the reaction mixtures was 10^4/ml and experiments were carried out at *p*H 6.0.

Effect of different H_2O_2 concentrations on the LG-peroxide system. Experiments designed to determine the optimal H_2O_2 concentration for the LG-peroxide system against a bacterial population of approximately 10^4 to 3×10^4 organisms were carried out. The results (Table 3) indicate that an H_2O_2 concentration of 5×10^{-4} M gave the greatest extent of killing when used with LG. However, this concentration alone killed a rather high percentage of the test organisms. A lower concentration of H_2O_2, 5×10^{-5} M, also showed enhanced killing when combined with LG, but this concentration was less toxic to the *E. coli*. Lower concentrations, while less toxic to the test organisms, did not demon-

TABLE 2. *Effect of pH on bactericidal activity of lysed granules on Escherichia coli*[a]

pH	Per cent bacteria killed			
	15 min	30 min	60 min	90 min
4.0	99.3	>99.9	—	—
4.5	98.3	>99.9	—	—
5.0	87.8	97.9	>99.9	—
5.5	39.4	93.3	98.5	>99.9
6.0	0	29.2	86.6	92.7
6.5	0	12.6	75.4	88.4
7.0	0	7.8	53.6	88.2

[a] In a reaction mixture the initial concentration of organisms was 9.0×10^3/ml and the lysed-granule preparation was equivalent to granules from 10^7 polymorphonuclear neutrophils.

TABLE 3. *Effect of different concentrations of H_2O_2 on bactericidal activity of lysed-granule (LG) peroxide system against Escherichia coli*

H_2O_2 concn	Per cent killed at 15 min[a]	
	H_2O_2	LG[b] + H_2O_2
M		
5×10^{-4}	34.5	99.9
5×10^{-5}	15.4	99.5
1×10^{-5}	12.7	9.4
5×10^{-6}	2.3	4.7
1×10^{-6}	0	0

[a] Initial concentration of *E. coli* was 9.6×10^3/ml and experiments were carried out at *p*H 6.0.

[b] Lysed-granule preparation from 7.5×10^6 polymorphonuclear neutrophils and contained approximately 300 peroxidase units.

strate any increased bactericidal activity when reacted with LG. Thus, 5×10^{-5} M H_2O_2 was used in most experiments. When significantly larger numbers of test organisms were employed, higher H_2O_2 and LG concentrations were required. It should be mentioned that the concentration of LG used had no bactericidal activity on the test organism.

Effect of pH on the LG-peroxide antibacterial system. Since phagocytosis is known to produce a decrease in intracellular *p*H (13) it was of interest to determine the effect of *p*H on the bactericidal activity of the LG-peroxide system. The combined results of several experiments are presented in Table 4. A definite relationship between *p*H and bactericidal activity was demonstrated. The most striking killing occurred at a *p*H range from 4.0 to 6.0. Although some bactericidal activity was exhibited at *p*H 7.4, it was not comparable to that observed at the lower *p*H. In most cases more than 90% of the total bactericidal effect was demonstrated by the first 15 min of incubation.

TABLE 4. *Effect of pH on bactericidal activity of lysed granule-peroxide system against Escherichia coli*[a]

pH	Per cent killed	
	15 min	30 min
4.0	>99.9	—
4.5	>99.9	—
5.0	99.5	>99.9
5.5	99.0	99.1
6.0	98.3	98.8
6.5	19.6	19.6
7.0	12.3	12.3
7.4	30.4	39.8

[a] In a reaction mixture the initial concentration of *E. coli* was 1.4×10^4/ml; the lysed-granule preparation was equivalent to granules from 7.5×10^6 polymorphonuclear neutrophils and contained approximately 300 peroxidase units. The H_2O_2 concentration was 5×10^{-5} M.

Effect of peroxidase inhibitors. To ascertain clearly that myeloperoxidase was the active factor responsible for the synergistic effect of LG, the effect of various peroxidase inhibitors on the LG-peroxide antibacterial system was determined. The results of these experiments are presented in Table 5 and indicate that peroxidase is involved in the bactericidal activity of the LG-peroxide system. All the inhibitors essentially abolished the bactericidal activity of the LG-peroxide system at 15 min. Some activity was noted at 30 min in the case of KCN. The concentrations of inhibitors utilized in this study showed little or no effect when tested with the bacteria alone.

Effect of the LG-peroxide system on various test organisms. Table 6 demonstrates the marked synergistic effect of LG on the bactericidal activity of H_2O_2 against a variety of test bacteria. Under the experimental conditions utilized, LG had little or no antibacterial effect against most of the test organisms. It is interesting that many of the gram-positive test organisms were more susceptible to the bactericidal effect of the H_2O_2 than were the gram-negative organisms. In particular, the two species of *Staphylococcus* were quite susceptible to the hydrogen peroxide and this bactericidal activity was not significantly enhanced in the presence of LG. The *S. pyogenes* was also susceptible to H_2O_2 activity, but showed increased killing when LG was added. *B. subtilis* was quite unusual. This organism was slightly susceptible not only to the H_2O_2 but also to the LG preparation. The bactericidal activity of the LG-peroxide system on

TABLE 5. *Effect of various peroxidase inhibitors on killing of Escherichia coli by lysed granule-peroxide antibacterial system*[a]

Inhibitor	Per cent killed	
	15 min	30 min
None	98.8	99.5
AT[b] (10 mM)	0	0
NaN_3 (1 mM)	0	8.5
KCN (1 mM)	8.5	38.0

[a] In the antibacterial system the lysed-granule preparation was equivalent to granules from 7.5×10^6 polymorphonuclear neutrophils containing approximately 300 peroxidase units, and the concentration of H_2O_2 was 5×10^{-5} M. The initial concentration of *E. coli* was 10^4 organisms/ml. The experiments were carried out at *p*H 6.0.

[b] 3-Aminotriazole.

TABLE 6. *Bactericidal activity of lysed granule (LG)-peroxide system on various bacteria*

Test organism	Per cent killed at 15 min[a]		
	LG[b]	H_2O_2[c]	LG + H_2O_2
Staphylococcus aureus	6.7	47.4	56.4
S. albus	0	86.2	97.1
Streptococcus pyogenes	0	37.7	98.8
S. faecalis	0	4.6	97.5
Bacillus subtilis	23.0	21.0	99.6
Listeria monocytogenes	0	6.5	99.0
Escherichia coli	0	13.4	99.4
Pseudomonas aeruginosa	0	2.1	99.6
Shigella sonnei	0	16.0	99.9

[a] Initial concentration of organisms was 10^4 to 3×10^4/ml.

[b] Lysed-granule preparation equivalent to granules from 7.5×10^6 polymorphonuclear neutrophils and contained approximately 300 peroxidase units.

[c] Final concentration of H_2O_2 was 5×10^5 M, and all experiments were carried out at *p*H 6.0.

this organism, however, demonstrated that the LG and H_2O_2 effects were not merely additive. *L. monocytogenes* and the three gram-negative test organisms were all extremely susceptible to the LG-peroxide system. Bacteria in the logarithmic phase of growth appeared to be slightly more susceptible to the LG-peroxide system than were stationary-phase cells. It should be mentioned that, when intact granules were substituted for lysed granules in the LG-peroxide system, little or no enhanced killing was observed.

DISCUSSION

In 1955 de Duve and his associates (3) postulated the existence of a new cytoplasmic organelle, the lysosome. Although lysosomes were initially identified in rat liver cells, these subcellular structures have now been found in a wide variety of cells and are especially plentiful in cells capable of phagocytic activity (19). It has been fairly well established that lysosomes are involved in the destruction and degradation of phagocytized particulate material. According to current evidence these cytoplasmic granules discharge their contents into vacuoles containing the ingested material (19). The lysosomes are known to contain a vast array of hydrolytic and proteolytic enzymes with acid *p*H optima. The liberation or activation of the lysosomal enzymes involves the rupture or lysis of the lysosomal granule and thus a reduction in the concentration of these granules would be expected during phagocytosis. That this degranulation does occur has been well documented (8, 14). However, in addition to the hydrolases, several antibacterial factors have also been found in the lysosomes: lysozyme (2, 4), phagocytin (2), and cationic proteins (17, 18). While there is good evidence linking the eventual dissolution of phagocytized material with the various lytic enzymes, definitive data implicating the various antibacterial factors of lysosomes in the intracellular killing of ingested bacteria are lacking. It has been postulated that the liberated lysosomal enzymes may be responsible for the intracellular killing of bacteria. However, there is now evidence indicating that enzymatic activity is indeed separable from antibacterial activity (16, 17).

In view of the fact that PMN lysosomes do contain various bactericidal factors, our initial experiments were concerned with establishing whether a bactericidal effect could be demonstrated with the LG preparations. These experiments, while confirming the antibacterial nature of the LG preparation, also revealed that the bactericidal activity of the factors involved was related to the *p*H; the bactericidal activity was much more active at acid *p*H than around neutrality. Thus, killing is rapid in the *p*H range of 4.0 to 5.0, but above this range the bactericidal effect is rather slow-acting. This finding is analogous to results reported by Hirsch in his studies with phagocytin (6). Unfortunately, definitive studies on the intracellular *p*H of phagocytizing leukocytes have not been done. While it can be stated with a considerable degree of confidence

that the *p*H in the phagocyte is lowered, probably as a result of increased lactic acid formation during phagocytosis, the exact extent of the decreased intracellular hydrogen-ion concentration is not known. It has been determined that many bacteria are readily killed once they have been ingested by phagocytic cells. For example, Hirsch (7) has indicated that the average intracellular survival time for many microbes is 10 to 15 min. Rowley (12), using mouse peritoneal macrophages, has shown that most of his test organisms were killed at essentially the same rate, with the half-life being 6 to 9 min. Only *S. aureus* demonstrated a longer survival time. Thus, it seems not unreasonable to assume that the bactericidal factor or factors responsible for intracellular killing in phagocytes should be fast-acting. It is possible that the factors in the LG preparation may be involved in the intracellular killing of bacteria, assuming that the hydrogen-ion concentration within the phagocyte is sufficiently depressed. However, if the intracellular *p*H is only slightly decreased, then these factors probably would not be involved in the bactericidal activity of the phagocyte. Therefore, it would seem likely that other antibacterial factors, more active either over a broader *p*H range or at slightly acid *p*H values, may be operating in the phagocyte.

Leukocytes, and more specifically PMN, are known to be rich in myeloperoxidase activity (1). The myeloperoxidase has been found to be localized in lysosome-like granules (15), and it behaves in much the same manner as better-known lysosomal enzymes: relatively inactive in intact granules, but quite active when these granules are lysed or ruptured (W. H. Evans and M. Rechicigal, Jr., *in press*). Since, as previously mentioned, an antimicrobial system whose chief constituents are thiocyanate, peroxide, and peroxidase has recently been described (9), the possibility of a similar system in phagocytes was considered. The results obtained in this study indicate that a peroxidase-peroxide system may indeed be operable in the PMN. It should be pointed out that added thiocyanate was not required in the LG-peroxide system, and, in fact, its presence somewhat inhibited the antibacterial activity of the system (McRipley and Sbarra, *unpublished data*). The finding that the LG-peroxide system was more active at lowered *p*H is consistent with both the *p*H optimum for myeloperoxidase activity and the acid *p*H that occurs within the PMN during phagocytosis. The low concentrations of both myeloperoxidase and hydrogen peroxide that are required makes the in vivo functioning of this system more plausible. It should be especially noted that the myeloperoxidase-containing LG preparation used in this study is prepared from a relatively small number of PMN. Other studies have employed a much higher initial PMN concentration to isolate bactericidal factors (2, 5). Finally, the finding that the initial concentration of many test organisms was reduced by more than 95% during the first 15 min of an experiment, and the nonspecific nature of the bactericidal activity, lend credence to the possibility of the operation of the myeloperoxidase-peroxide system in the PMN.

It might be argued that the LG preparation is actually a rather crude myeloperoxidase preparation and is contaminated with the entire array of hydrolytic enzymes and antibacterial factors. However, in an attempt to circumvent the possible effect of any of the bactericidal factors, a concentration of LG which alone demonstrated little or no bactericidal activity over a short incubation period at a slightly acid *p*H was employed. Furthermore, the fact that peroxidase inhibitors were so efficient in inhibiting the LG-peroxide system not only implicates myeloperoxidase as the active factor in LG, but also indicates that the myeloperoxidase was free and available. Most important, by using relatively low concentrations of PMN and *p*H values that are not drastically acidic, a much desired and closer approximation to the physiological environment is achieved. It is felt that a functional bactericidal system in the phagocyte must be operable under these conditions.

In the previous paper, evidence for hydrogen peroxide formation and its relationship to the phagocytic and associated metabolic activities in PMN was presented (11). The synergistic effect of myeloperoxidase on the bactericidal activity of hydrogen peroxide indicates that the bactericidal system in the PMN is composed of at least two different agents. Further, it is indicated that this system is activated as a result of phagocytosis. This activation is thought to occur as follows. The increased glycolytic activity observed with phagocytizing cells seems to supply the necessary energy for engulfment; also, it lowers the intracellular *p*H. The increased flow of glucose through the hexose monophosphate shunt pathway (HMP) results in an increased turnover of reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) and a concomitant significant elevation of H_2O_2. This is brought about as a result of a release or activation of $NADPH_2$ oxidase and myeloperoxidase from lysosomes or lysosome-like particles. The activation is thought to occur as a result of the lowered intracellular *p*H. The activated oxidase would oxidize $NADPH_2$, producing H_2O_2, and would also make available additional NADP, a limiting coenzyme of the

HMP. The activated myeloperoxidase would now react with the hydrogen peroxide and through its peroxidative activity would probably kill the engulfed bacteria. The final digestion and degradation of the bacteria would be accomplished by means of various hydrolases.

Whereas the in vitro activity of the myeloperoxidase-peroxide antibacterial system appears to be fairly conclusive, the in vivo functioning of this system is admittedly speculative, even though both constituents of the system are present in the PMN. Experiments are now underway to substantiate the operation of the myeloperoxidase-peroxide system in the PMN.

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