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The Effect of Leukocyte Hydrolases on Bacteria II. The Synergistic Action of Lysozyme and Extracts of PMN, Macrophages, Lymphocytes, and Platelets in Bacteriolysis[1] (38261)

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(Introduced by T. N. Harris)

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Extracts of human and rabbit blood leukocytes, of rabbit granulocytes (PMN) and macrophages, which contained acid hydrolases and lysozyme, were found to be highly lytic to a variety of ^{14}C-labeled staphylococci and streptococci *in vitro* (1–3). On the other hand, extracts of lymphocytes were not lytic for these bacteria (2, 3). It was also found that bacteriolysis by the leukocyte extracts was strongly inhibited by cationic polyelectrolytes like protamine sulfate and histone, by leukocyte cationic proteins, and by anionic substances like heparin, chondroitin sulfate, DNA, alginic acid, and carrageenin (1, 3, 4). Since crystalline egg-white lysozyme failed to lyse these bacteria when tested alone, but collaborated effectively in bacteriolysis with crude trypsin, lysolecithin and phospholipase C (4), it was postulated that certain enzymes may function as preparatory agents to unmask the bacterial peptidoglycan for cleavage by lysozyme.

The present report deals with a hitherto undescribed phenomenon which shows that lysozyme, which is not lytic for the bacteria tested, acts synergistically with nonlytic extracts of lymphocytes, thymocytes, platelets, synovia and muscle to cause extensive lysis of certain streptococci and staphylococci. The lysis of the bacteria is accompanied by the release of the bulk of glucosamine from the bacterial cell-walls. The relationship of this phenomenon to the mechanism of bacteriolysis by PMN and macrophages, to the problem of macrophage activation and to the persistence of undegraded cell-wall components of bacteria in granulomatous inflammation will be discussed.

Materials and Methods. ^{14}C-labeled bacteria. Group A, type 4, streptococci, *Streptococcus faecalis* and *Staphylococcus albus* were obtained from the stock cultures of the Laboratory for Microbiology. A strain of *Streptococcus mutans* was kindly supplied by Dr. E. Kjems of the State Serum Institute, Copenhagen. All the bacterial strains were cultivated in brain–heart infusion broth (Difco) which contained 1 μCi/ml of D-glucose ^{14}C (The Radio Chemical Center, Amersham, England) (2). The bacterial cells were harvested from the stationary phase of growth, washed and resuspended in saline solution (0.9% NaCl) and adjusted to 1000 Klett units at 540 μm (Klett Summerson Colorimeter, Model 800-3). Such bacterial suspensions contained approximately 4-8 $\times$ 10^5 cpm/ml.

Leukocyte and tissue extracts. Human and rabbit peripheral blood was obtained in ACD, and the leukocytes and platelets were separated from the RBC by 3.5%

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Dextran in saline as described (2). Rabbit peritoneal PMN and macrophages were obtained 1 and 4 days respectively after the ip injection of 100 ml of a 5% solution of sodium caseinate (2). Rabbit lung macrophages were obtained by the method of Myrvik *et al.* (5). Rabbit popliteal and mesenteric lymphocytes and isolated by teasing the glandular tissue against stainless steel mesh. The purity of the various leukocyte preparations was regularly checked by staining with Giemsa. The peritoneal PMN usually contain less than 10% of other cell types while the macrophage preparations contained about 10% PMN. The lung macrophage preparations contained less than 10% of other cell types. The platelet suspensions isolated did not contain any other cell types. The purity of the platelet suspensions was also verified by checking their lysozyme content; preparations contaminated with PMN or macrophages always showed lysozyme activity. The lymphocyte and thymocyte preparations were about 90% pure but did not contain any detectable lysozyme activity.

One mg protein obtained from lysates of lymphocytes, thymocytes, platelets, blood leukocytes, peritoneal macrophages and peritoneal PMN, was equivalent approximately to 10^8, 10^8, 10^8, 10^7, 10^7, 10^7 cells, respectively. Rabbit synovia and muscle were obtained from normal animals and cut into small pieces. Extracts containing acid hydrolases were obtained from the various leukocytes by subjecting the cells to 6 cycles of freezing and thawing in liquid air (2). Extracts of synovia and muscle were obtained after homogenization with saline in a glass homogenizer fitted with a teflon pestle. All the cell and tissue extracts were cleared by centrifugation at 36,000*g* for 15 min in a refrigerated centrifuge, and the supernatant fluids were stored at $-20°$. The protein content of the extracts was determined by the method of Lowry *et al.* (6), using human serum albumin as a standard.

Assays of enzyme activities. The lysosomal enzymes acid phosphatase, β-glucoronidase, mannosidase, *N*-acetylglucosaminidase, aryl sulfatase, cathepsin D, and lysozyme were determined in all the extracts as described (2). Lysozyme was removed from the different extracts by absorption with bentonite (7).

Degradation of bacteria by cell and tissue extracts. ^{14}C-labeled bacteria (5×10^4 cpm/ml of reaction mixture) were incubated for 16 hr at 37° in a shaking water bath with leukocyte or tissue extracts (0.1–1.0 mg protein/ml of reaction mixture); 0.1 *M* acetate buffer pH 5.0 was used in all the experiments. The soluble radioactivity which remained in the supernatant fluid after centrifugation of the reaction mixtures as 2500*g* for 20 min was determined in a scintillation counter using Toluene-Triton X-100 as scintillation fluid as described (2). The lytic effect of egg-white lysozyme (Sigma Chemical Co., St. Louis, MO) on the labeled bacteria was tested in the absence and presence of leukocyte and tissue extracts. The combined effect of lysozyme with stromata of human RBC and with human albumin, globulin and fibrinogen was also tested on the labeled bacteria.

Inhibition of bacteriolysis. The lysis of the bacteria by the leukocyte and tissue extracts was prevented by heparin and chondroitin sulfate (Sigma Chemical Co.) as described in detail (1, 3).

Determination of glucosamine. The supernatant fluids derived from the various lytic systems were analyzed for the presence of glucosamine by the method of Gatt and Berman (8).

Results. Lysis of bacteria by cell and tissue extracts. Figure 1 shows that while *S. albus* is very susceptible to lysis by extracts of human and rabbit blood leukocytes, this microorganism is less affected by extracts of macrophages (3, 4). On the other hand, no appreciable lysis of the bacteria occurred with extracts of lymphocytes, thymocytes, platelets, synovia or muscle. Similar results were obtained with *Strep. faecalis* and *Strep. mutans.* Under the same conditions group A streptococci were completely resistant to lysis by leukocyte lysates (1–4). Since lysozyme did not lyse any of the bacteria employed, but did so effectively in the presence of a nonlytic mixture containing crude trypsin, lysolecithin and phospholipase C (4), it was postulated that certain

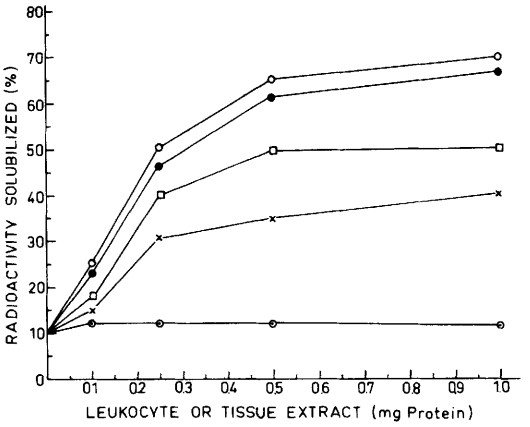

FIG. 1. Lysis of *Staphylococcus albus* by cell and tissue extracts. Human blood leukocytes ○——○; Rabbit blood leukocytes ●——●; Rabbit lung macrophages □——□; Rabbit peritoneal macrophages ×——×; Extracts of rabbit lymphocytes, thymocytes, platelets, muscle and synovia ⊙——⊙.

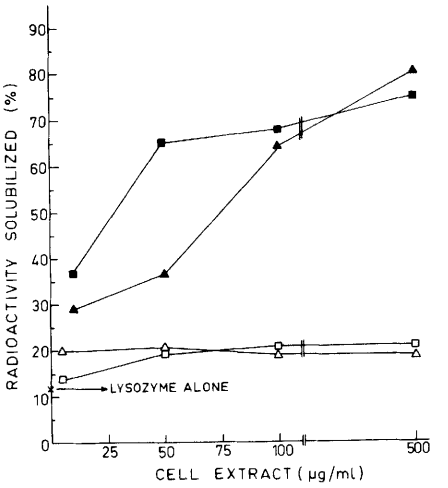

FIG. 2. Lysis of *Streptococcus mutans* by the combined action of lysozyme and extracts of lymphocytes and platelets. Lysozyme (50 µg/ml) ×——×; lymphocyte extract □——□; platelet extract △——△; lysozyme (50 µg/ml) + lymphocyte extracts ■——■; lysozyme (50 µg/ml) + platelet extract ▲——▲.

enzymes may expose the resistant peptidoglycan of these bacteria to cleavage by lysozyme. It was therefore of interest to determine whether the nonlytic extracts of lymphocytes and platelets found to contain lysosomal enzymes (9, 10) (see also Materials and Methods) not lysozyme, would also collaborate with lysozyme in bacteriolysis. Figure 2 indeed shows that lysozyme acts synergistically with the nonlytic extracts of lymphocytes and platelets to cause extensive lysis of the bacteria. A similar collaboration was found between lysozyme and nonlytic extracts of thymocytes, synovia and muscle. On the other hand, no bacteriolysis occurred when lysozyme was tested in collaboration with RBC stromata or with albumin, globulin and fibrinogen. As in the case of the PMN and macrophage extracts (2) the mixtures of lysozyme with the tissue extracts solubilized the bulk of the glucosamine of the cell walls. Preliminary studies on the electron microscopy of the bacterial cells revealed that extensive lysis of the cell wall was accompanied by the disintegration of the cell interior. These results are essentially similar to those described for cells treated with PMN and macrophage enzymes (1–4). Figure 3 shows that as little as 5 µg/ml of lysozyme was sufficient to "activate" lymphocyte and platelet extracts in bacteriolysis. At this point it was of interest to examine the possibility that lysozyme would also enhance the lysis of bacteria by extracts of PMN and macrophages which contained lysozyme. Figure 4 shows that 50 µg/ml of lysozyme markedly potentiated the lytic effects of extracts of blood leukocytes, lung macrophages and peritoneal PMN on *Strep. mutans* (one mg protein extracts of blood leukocytes, lung macrophages or peritoneal PMN, contained approximately 50 µg/ml of lysozyme). Similar enhancement of bacteriolysis was obtained with *Staph. albus* and *Strep. faecalis*. Group A streptococci again proved to be extremely resistant to lysis by such mixtures. The results indicate that even "professional" phagocytes may be deficient in lysozyme, and that an extra supply of this enzyme may be needed for the full expression of their bacteriolytic capacities.

The mechanisms of bacteriolysis. The results presented in Figs. 2–4 suggest that the lytic systems present in both PMN and macrophages probably comprise 2 groups of enzymes: a preparatory group, also present in lymphocytes, thymocytes, platelets,

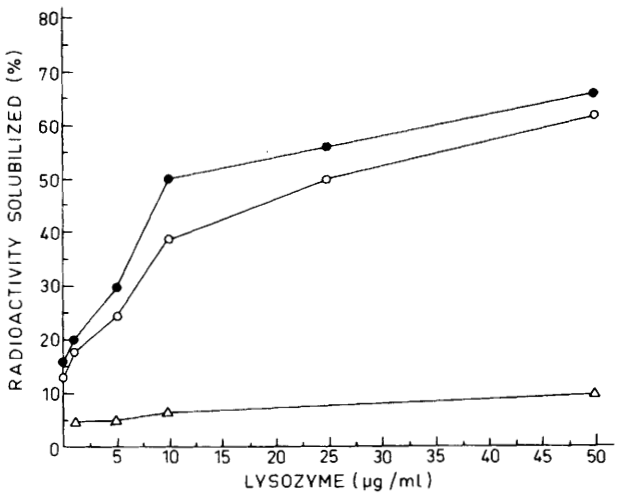

FIG. 3. The effect of increasing concentration of lysozyme on the lysis of *Streptococcus mutans* by cell extracts. Lysozyme △——△; lymphocyte extract (0.5 mg/ml) ●——●; platelet extract (0.5 mg/ml) ○——○.

synovia and muscle, which is not directly lytic to the bacteria tested, and lysozyme, which is exclusively present in PMN and macrophages, but not in the other cells, and which is capable of cleaving the mucopeptide after it has been modified by the preparatory enzymes. A further support for this suggestion was obtained from experiments which showed that extracts of blood leukocytes could be rendered nonbacteriolytic by treatment with bentonite, and that such treated extracts completely regained their bacteriolytic properties upon the addition of lysozyme (Fig. 5). Preliminary experiments have shown that absorption of leukocyte extracts with bentonite also resulted in the removal of the bulk of their β-glucuronidase content, but had little effect on all the other lysosomal enzymes tested. In other experiments we tested the combined effect of lymphocyte extracts with lysozyme which had been either heated to 100°, autoclaved at 120° or boiled for 10 min with HCl or NaOH at 0.01 *M*. In each case the lysozyme activity was checked with *M. lysodeikticus* (2). It was found that only after boiling at 100° in the presence of NaOH did lysozyme completely lose its enzymatic activity and its capacity to act synergistically with the nonlytic extracts of lymphocyes or platelets. These results further support the indication that active lysozyme is the key enzyme essential for this synergistic lytic system.

Further studies on the mechanism of bacteriolysis were performed. Labeled *S. albus* and *Strep. mutans* were incubated for 2 hrs at 37° with 1-mg protein aliquots of lymphocyte or platelet extracts. The cells were then washed with 0.1 *M* acetate buffer pH 5.0 and resuspended in the buffer. Lysozyme was added to the cells at 20 µg/ml. In other experiments, the labeled cells were first incubated with lysozyme and washed, and extracts of lymphocytes or platelets were then added. The reaction mixtures were further incubated for 16 hr at 37°. It was found that marked bacteriolysis occurred when the cells were preincubated either with lysozyme or with the cell extracts. These results indicate that relatively short exposure is sufficient for the modification of the bacterial cell-walls by either lysozyme or the cell extracts, which render them susceptible to lysis upon the addition of the corresponding nonlytic agents. The possibility was further examined that the short exposure of the bacteria to lymphocyte extracts or to lysozyme might have resulted in the adsorption of these agents upon the bacterial surface, and that a simple wash with buffer was not sufficient to

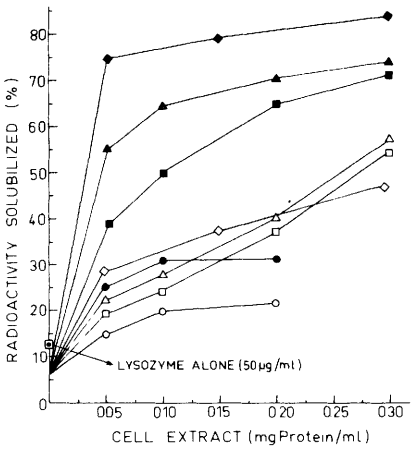

FIG. 4. The effect of lysozyme on the lysis of *Streptococcus mutans* by leukocyte and macrophage extracts. Lysozyme ⊡——⊡ (50 µg/ml); Extracts of rabbit peritoneal macrophages ○——○; peritoneal macrophages + lysozyme (50 µg/ml) ●——●; lung macrophages □——□; lung macrophages + lysozyme (50 µg/ml) ■——■; human blood leukocyte extract △——△; human blood leukocyte extract + lysozyme (50 µg/ml) ▲——▲; peritoneal PMN ◇——◇; peritoneal PMN + lysozyme (50 µg/ml) ◆——◆.

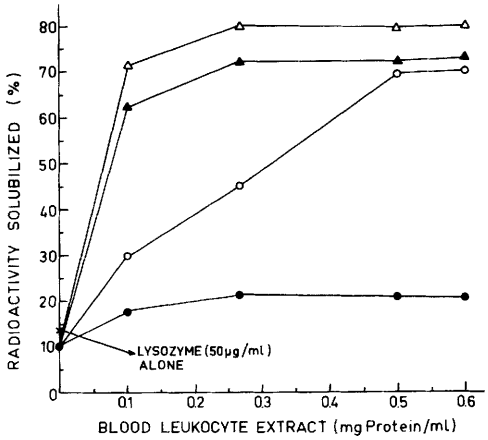

FIG. 5. The effect of bentonite on the lysis of *Streptococcus mutans* by human blood leukocyte lysates. Lysozyme (50 µg/ml) ×——×; blood leukocyte lysates ○——○; blood leukocyte lysates absorbed with bentonite ●——●; leukocyte lysates + lysozyme (50 µg/ml) △——△; blood leukocyte lysates absorbed with bentonite + lysozyme (50 µg/ml) ▲——▲.

remove these agents. We therefore employed a 5% solution of NaCl instead of acetate buffer to try to elute the agents from the cells. Treatment with salt was found by us to elute protamine sulfate from the surface of *M. lysodeikticus* (2). It was found that bacteria treated with lymphocyte extracts followed by a wash with 5% NaCl were not lysed upon addition of lysozyme. On the other hand, cells first treated with lysozyme and then washed with salt underwent lysis upon the addition of lymphocyte extracts. The results indicate that while the lymphocyte enzymes were probably eluted from the cells, lysozyme remained adsorbed upon them. It is also possible, however, that lysozyme had exerted some changes in the bacterial cell walls, within the short incubation period, which did not lead to lysis but exposed the cell walls to a final lysis by the lymphocyte enzymes. Further studies on the mechanism of bacteriolysis by lysozyme and cell extracts are under way.

The effect of inhibitors on bacteriolysis. Since heparin and chondroitin sulfate were previously found to inhibit bacteriolysis by extracts of blood leukocytes and by enzymes derived from PMN and macrophages (1–3), it was of interest to examine the effect of these inhibitors on the bacteriolysis induced by a mixture of lysozyme and lymphocyte or platelet extracts. It was found that 50 µg/ml of either heparin or chondroitin sulfate caused a 95% inhibition of lysis of *Strep. mutans* and *S. albus* by such lytic mixtures. Since neither heparin nor chondroitin sulfate inhibited lysozyme activity, it was assumed that the anionic polyelectrolytes probably inhibited the preparatory enzymes present in the lymphocytes and platelets. It was also found that bacteria pretreated with lymphocyte extracts and heparin, then washed with buffer, did not undergo lysis upon the addition of lysozyme. On the other hand, bacteria pretreated with heparin and lysozyme, followed by a wash with buffer, underwent lysis upon the addition of lymphocyte extracts. These results further indicate that the anionic substances probably inactivated the preparatory enzymes.

pH optimum for bacteriolysis. A further comparison between the preparatory lytic system present in lymphocytes and in blood

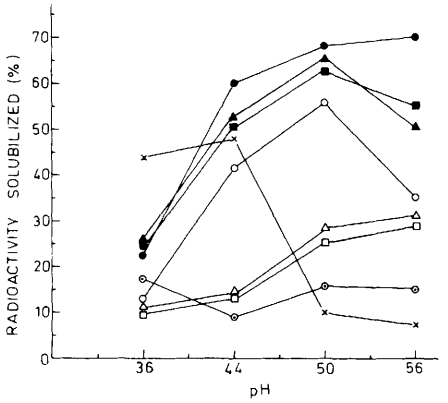

FIG. 6. The effect of pH on the lysis of *Streptococcus mutans* by human blood leukocyte, lymphocyte and platelet extracts and lysozyme. Bacterial cells in acetate buffer ⊙——⊙; Lysozyme (25 μg/ml) ×——×; Platelet extract (1 mg protein/ml) □——□; Platelet extract + lysozyme (25 μg/ml) ■——■; Lymphocyte extract (1 mg protein/ml) △——△; Lymphocyte extract + Lysozyme (25 μg/ml) ▲——▲; Blood leukocyte extract (1 mg protein/ml) ○——○; Blood leukocyte extract + Lysozyme (25 μg/ml) ●——●.

leukocytes was made using acetate buffers of different pH values. Figure 6 shows that the pH optimum for the lysis of bacteria by rabbit lymphocyte extract and lysozyme or by blood leukocyte extracts is 5.0. It was also found that both systems are strongly inhibited by citrate and oxalate and by EDTA. The enhanced lysis of streptococci incubated in acetate buffer of low pH is in accord with the findings that lysozyme acts synergistically with acids in bacteriolysis.

The effect of heat on the lytic systems. Figure 7 shows that blood leukocyte lysate which conained approximately 25 μg/ml of lysozyme lost most of its lytic activity after heating at 75° and 100° for 15 min. The heating also inactivated the lysozyme activity of the extracts. It is also shown that the readdition of lysozyme completely restored the lytic activity of the heat-treated leukocyte extracts, indicating that the preparatory agents present in leukocytes are heat-stable. The figure also shows that the preparatory agents in lymphocytes and platelets are less heat resistant than the leukocyte extracts. In other experiments it was found that lymphocyte extracts or blood leukocyte lysates treated with bentonite to remove lysozyme failed to restore the lytic capacity of the heat-inactivated leukocyte extracts. The finding that lysozyme is susceptible to heat in the original leukocyte extracts, but is very stable when heated in acetate buffer (see above), is also of interest. Other experi-

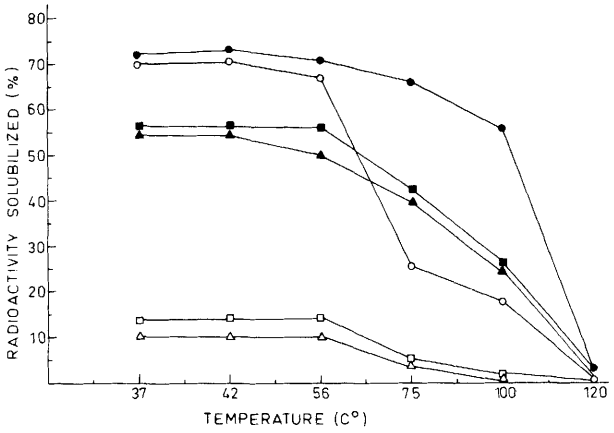

FIG. 7. The effect of heat on the lysis of *Streptococcus mutans* by leukocyte extracts. The extracts were heated for 30 min at 37°, 42° and 56° for 30 min and for 10 min at 75° and 100°. Blood leukocyte extract containing approximately 25 μg/ml of lysozyme ○——○; Blood leukocyte extracts + egg white lysozyme (25 μg/ml) ●——●; Platelet extract (1 mg protein) △——△; Platelet extract + lysozyme (25 μg/ml) ▲——▲; Lymphocyte extract (1 mg protein) □——□; Lymphocyte extract + lysozyme (25 μg/ml) ■——■.

ments have shown that lysozyme is also rapidly inactivated when heated in the presence of human albumin.

Preliminary experiments have also shown that both human and rabbit serum and synovial fluids of patients with arthritis possess a lysozyme-dependent lytic system for certain Gram positive bacteria. Unlike the lytic system present in blood leukocytes the lytic system of serum and synovial fluid is inactivated at 75°. Further studies on these lytic systems are under way.

Discussion. The data presented indicate that the bacteriolytic system present in blood leukocytes consists of two groups of substances. The first is a group of preparatory, nonlytic agents, probably of lysosomal origin, which are active at pH 5.0, are heat-stable, and are rapidly inactivated by anionic polyelectrolytes. This group of agents, probably enzymes, is present also in nonphagocytic cells such as lymphocytes, thymocytes, and platelets, as well as in extracts of synovia and muscle. The second type of agent is lysozyme, which is exclusively present in phagocytic cells. This is not lytic to most Gram-positive bacteria but can act synergistically with the nonlytic agents of many cells to cause extensive lysis of the bacteria. The conclusion that bacteriolysis can be a two-stage mechanism is also based on previous findings (4) that lysozyme collaborates effectively with a nonlytic mixture containing crude trypsin, lysolecithin and phospholipase C in the bacteriolysis of Gram-positive and Gram-negative organisms. Further evidence for the presence in phagocytic cells of a group of lytic enzymes comes from blood leukocytes by bentonite or by heat (Fig. 7) abolishes its bacteriolytic properties and that such activity is fully regained upon the addition of egg-white lysozyme.

The mechanism by which the nonlytic preparatory enzymes or agents act in concert with lysozyme in bacteriolysis is still not fully understood. It may, however, be suggested that certain acid hydrolases act by unmasking the peptidoglycan for lysis by lysozyme. This suggestion is based on the observations that streptococci, which are extremely resistant to lysis by lysozyme, disintegrate following the removal of surface polysaccharide and further treatment with lysozyme (12, 13). Other studies have shown that streptococci undergo lysis by lysozyme only after treatment with detergents and with trypsin (14, 15). Similarly, the removal of the lipopolysaccharide of Gram negative bacteria (16, 17), or the formation of "holes" in the LPS layer by antibodies and complement (18), renders these bacteria susceptible to lysis by lysozyme.

Our data also suggest that even "professional" phagocytes like PMN and macrophages are relatively deficient in lysozyme (Fig. 4), and that this agent is the limiting factor in bacteriolysis by leukocytes. In order to fully express their lytic capacities macrophages may need to acquire extracellular lysozyme either from mucous secretions or from disintegrated PMN and macrophages present in inflammatory exudates. Lysozyme may perhaps also be available from living macrophages capable of secreting large amounts of this enzyme (19). The acquisition of extracellular lysozyme may also be achieved by pinocytosis, as has been described (20). Since bacteriolysis can occur both intracellularly (21), following phagocytosis, and extracellularly, following the release of the bacteriolytic systems from disintegrated leukocytes, it is obvious that an ample supply of preparatory enzymes may be available. In this respect, the findings that streptococcal cell-wall components can release certain lysosomal enzymes from intact macrophages (22) is also of importance.

The collaboration between the preparatory enzymes of lymphocytes and thymocytes with lysozyme may also be relevant to the problem of macrophage activation (23). It may be postulated that some of the lymphokines (24) liberated from sensitized lymphoid cells by the specfic antigens may have the properties of the preparatory enzymatic system described in this study. Such lymphocyte factors may play an important role in cellular immunity to bacteria (25). It must however be demonstrated that such preparatory enzyme systems can also be released from intact lymphocytes.

Since the preparatory nonlytic agents are also present in platelets, it would be important to establish the relationship of these thermostable factors to the β-lysins present in these cells, which are engaged in the killing of Gram positive bacteria (26). The lytic system present in the leukocytes should also be related to the myeloperoxidase-hydrogen peroxide-halide system described by Klebanoff (27). This system possesses microbicidal properties towards many microorganisms. Since we have found (unpublished observations) that the lytic system described in this study is not inhibited either by azide, KCN, or by mercurials, it is assumed that the energy-dependent myeloperoxidase system may not be involved in the lysis of bacteria. Thus one should differentiate between the energy-dependent bactericidal reactions and the lytic system which is probably nonenergy-dependent, but which is mediated by preformed lysosomal enzymes.

Finally, the role played by inhibitors of bacteriolysis (1, 4) in the persistence of undegraded microbial cell-walls in inflamed tissues must also be considered. Since inflammatory exudates may contain a variety of anionic polyelectrolytes, like chondroitin sulfate, DNA and heparin sulfate, and cationic polyelectrolytes like histone and leukocyte cationic proteins, derived from damaged leukocytes and tissue cells (4), and since macrophages have been shown to be able to pinocytose large quantities of some of these materials (20), which, we have shown, can inhibit bacteriolysis by leukocyte enzymes (1, 3, 4), it is possible that the degradation of microbial cell wall components in inflammatory areas would be hampered or retarded. The persistence of microbial cell-wall entities in tissues have been shown to cause severe and prolonged granulomatous lesions (3, 28, 29). Undegraded cell-wall components may also be translocated within macrophages to other sites of inflammation and thus cause the spread of granulomatosis (30). Further work on the bacteriolytic systems of leukocytes, serum and synovial fluids is in progress.

Summary. Extracts containing acid hydrolases and lysozyme derived from human and rabbit blood leukocytes, from rabbit peritoneal and lung macrophages and from peritoneal PMN are highly lytic for ^{14}C-labeled *Staphylococcus albus, Streptococcus faecalis,* and *Streptococcus mutans.* On the other hand, extracts of human and rabbit platelets and of rabbit lymphocytes, thymocytes, synovia and muscle are not lytic for these bacteria. A phenomenon is described in which lysozyme which is not lytic to these bacteria collaborates with nonlytic extracts of lymphocytes platelets, thymocytes, synovia and muscle in the lysis of Gram positive bacteria. Lysozyme also enhances the lysis of bacteria by extracts of PMN and macrophages. It is postulated that the bacteriolytic system present in PMN and macrophages comprises a group of preparatory nonlytic enzymes, also present in lymphocytes, thymocytes, platelets, and other tissues that prepare the peptidoglycan to cleavage by lysozyme. The preparatory enzyme systems of leukocytes and tissues have a pH optimum of 5.0 and are strongly inhibited by heparin and chondroitin sulfate. The relationship of the synergism between lysozyme and tissue enzymes in the degradation of microbial cells is discussed in relation to the pathogenesis of granulomatous inflammation.

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