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The inhibition by basic and acidic polyelectrolytes of the degradation of bacteria by leukocyte enzymes:

Relation to the persistence of microbial constituents in inflammatory sites**

Although much is known about the role played by bacteria in the pathogenesis of human disease and on the bactericidal systems of phagocytic cells, very little work has been done on the mechanisms by which leukocytes degrade bacterial cell constituents. It is assumed that the breakdown of the bacterial envelopes and cell-wall constituents is caused by the lysosomal enzymes of both granulocytes (PMN) and macrophages, but the nature of the enzymatic systems which lead to bacterial breakdown is not fully known.

Most of the evidence for bacterial degradation *in vivo* comes from indirect serological studies showing the appearance of numerous antibodies to different microbial constituents in the sera of animals undergoing infections. Other evidence for bacterial breakdown comes from electron microscopical studies (Ginsburg *et al.*, 1974) showing the ultrastructural alterations in bacteria following phagocytosis by both PMN and macrophages. While certain bacterial species such as staphylococci and some Gram-negative bacteria are readily broken down by phagocytic cells, other organisms such as streptococci, acid-fast bacilli, and fungi are very resistant to degradation. The structures of bacteria which are particularly resistant to degradation are the peptidoglycan-polysaccharide and lipopolysaccharide complexes of the cell walls. The biological importance of the persistence of undegraded cell walls in tissues is that it may lead to granulomatous reactions (Ohanian and Schwab, 1967; Ginsburg *et al.*, 1974) and to the dissemination of insoluble bacterial material to other inflammatory tissue sites (Ginsburg *et al.*, 1974). The literature on these subjects has been recently reviewed (Ginsburg *et al.*, 1974; Van Furth, 1970; Ginsburg, 1972).

Several studies have described the partial degradation of micro-organisms following phagocytosis by macrophages from different animal species (Stahelin *et al.*, 1956; Cohn, 1963*a*; Ayoub and Wanamaker, 1967; Spector *et al.*, 1970; Glick *et al.*, 1972). There are, however, few data on the nature of the enzymes or the

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bacterial structures solubilized. The techniques for collecting polymorphs and monocytes and for the isolation of leukocyte lysosomes and the solubilization of their enzyme contents (Cohn and Hirsch, 1960) makes it possible to investigate lysis of bacteria *in vitro*.

Previous studies from our laboratory have shown that ^{14}C-labelled *Staph. albus* and *Strep. faecalis* were degraded to a large extent by lysates of human, rabbit and mouse leukocytes (Ginsburg *et al.*, 1973, 1974; Lahav *et al.*, 1974). On the other hand, group A streptococci and several Gram-negative bacteria were very resistant to degradation. The solubilization of radioactivity from the susceptible bacteria was accompanied by the release of the bulk of glucosamine and other cell wall constituents and by massive cellular breakdown as revealed by electron microscopy (Ginsburg *et al.*, 1973; Lahav *et al.*, 1974). It was also found that the lysis of bacteria by leukocyte enzymes was inhibited to a large extent by several basic and acidic polyelectrolytes such as histone, protamine sulfate, heparin, chondroitin sulfate, alginic acid and by carrageenan (Ginsburg *et al.*, 1973, 1974).

The present report describes the inhibition of bacteriolysis by macromolecular substances, including certain dyes and lipids, by normal human immunoglobulins and by synovial fluids from joints from patients with rheumatoid arthritis. The possible relation of the inhibition of bacteriolysis to the persistence of microbial cell-wall components in chronic inflammatory exudates and the role played by both PMN and macrophages in the processing of bacterial antigens will be discussed.

MATERIALS AND METHODS

The techniques of labelling bacteria with ^{14}C-glucose, the preparation of lysates from different leukocyte populations and the determination of bacterial lysis was described in detail in previous publications (Ginsburg *et al.*, 1973, 1974; Lahav *et al.*, 1974). Briefly, suspensions of ^{14}C-labelled bacteria in 0.1 M acetate buffer, pH 5.0, containing approximately 60,000 cpm/ml, were incubated for 18 hours at 37 °C with leukocyte lysates and the percentage of solubilized ^{14}C was determined in the supernatant fluids. The inhibition of bacteriolysis by a variety of cationic and anionic polyelectrolytes, by lipids and dyes as well as by normal human immunoglobulins and synovial fluids was determined on incubation mixtures containing 1 mg of enzyme protein, labelled bacteria and inhibitors. Results were expressed as the percentage of inhibition of radioactivity release after 18 hr of incubation as compared with incubation mixtures in the absence of inhibitor.

RESULTS

The lysis of bacteria by leukocyte lysates

Figure 1 shows the lysis of ^{14}C-labelled bacteria by extracts of human peripheral blood leukocytes. As can be seen, *Staph. albus* and *Strep. faecalis* were readily lysed, but group A streptococci, *Shigella flexneri* and *Listeria monocytogenes* were lysed only to a small extent. The results also show that the absence of a terminal N-acetylglucosamine from the polysaccharide of the A-variant streptococci does not render this mutant to be more susceptible to lysis.

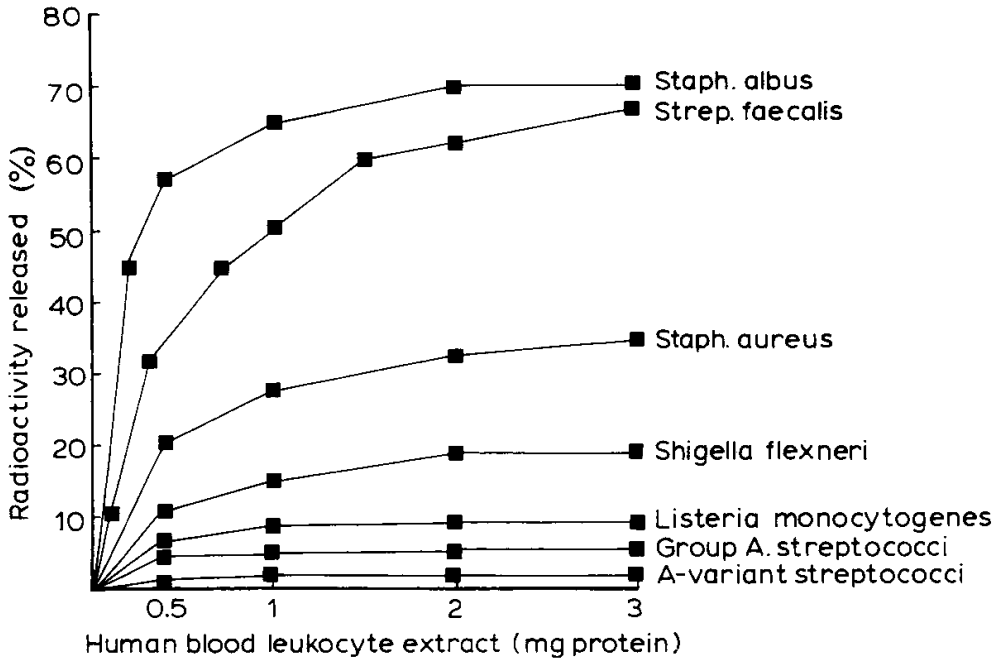

FIG. 1. The lysis of ^{14}C-labelled bacteria by extracts of human peripheral blood leukocytes.

Figure 2 shows the lysis of *Staph. albus* by lysates derived from different leukocyte populations. (Similar results were obtained with *Strep. faecalis*.) PMN of humans and rabbits and lysates of blood leukocytes were superior to macrophage lysates in lysing bacteria. Under similar conditions platelet enzymes and lysates from mouse lymph node cells (not shown) were inactive. The nature of the enzymes

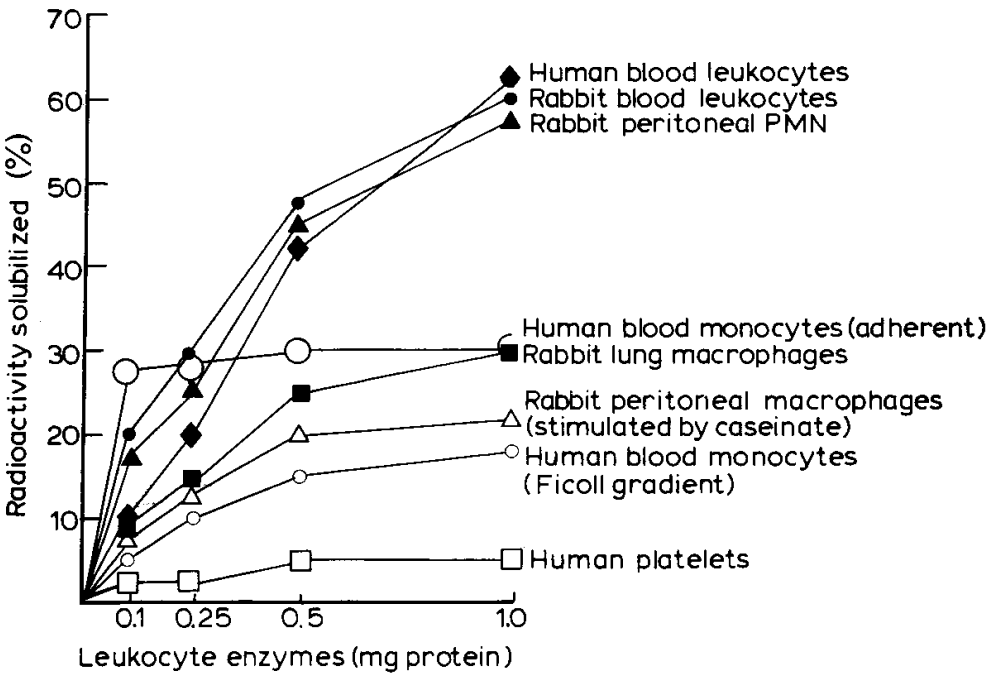

FIG. 2. The lysis of *Staph. albus* by lysates derived from different leukocyte populations.

which lyse the bacterial cells is still not known. However, although all the leukocyte lysates are rich in lysozyme, this enzyme, at the concentrations present in the lysates, failed to solubilize any appreciable amounts of radioactivity from any of the bacterial cells. Previous studies (Lahav *et al.*, 1974) have shown that the degree of lysis of staphylococci and streptococci by leukocyte lysates could not be correlated with their content of lysozyme, N-acetylglycosaminidase, mannosidase, glucuronidase and cathepsin D. More recent experiments (to be reported in detail) have shown that an artificial mixture containing crude trypsin, lysozyme, phospholipase C and lysolecithin could effectively replace leukocyte lysates in the lysis of bacteria. Moreover this artificial 'cocktail' lysed *E. coli*, *Shigella flexneri* and *Listeria monocytogenes* previously shown not to be markedly affected by leukocyte lysates. This enzyme mixture also enhanced the lysis of bacteria by leukocyte enzymes. The nature of the enzymes present in the crude trypsin preparations used is still not known but crystalline trypsin could not replace crude trypsin in this system and more studies employing other enzyme mixtures may prove valuable for the determination of the nature of the lytic system present in leukocytes.

The inhibition of bacteriolysis by macromolecules

In view of the high lytic effects of leukocyte lysates on certain bacteria, a search was made for naturally occurring substances, likely to be present in inflammatory exudate, which would modify the lysis of bacteria by leukocyte lysates. If indeed such inhibitory substances occur in body fluids, their potential for inhibiting the lysis of bacterial cell wall components by lysosomal enzymes is apparent. Figure 3 and Table 1 show that a variety of cationic and anionic polyelectrolytes, certain

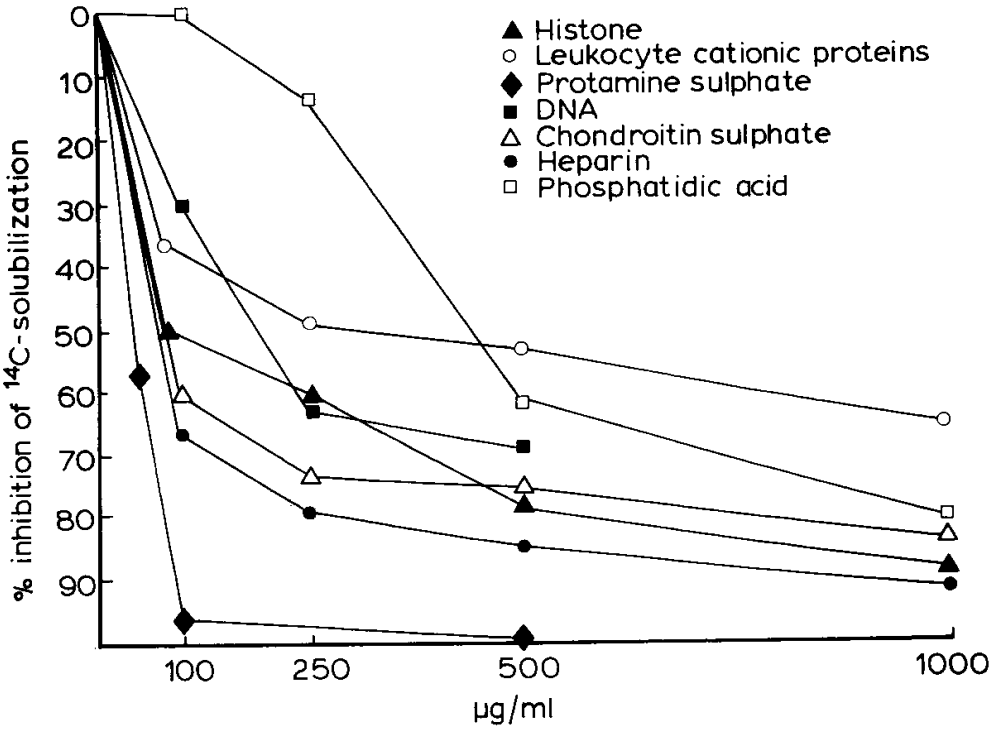

FIG. 3. The effect of macromolecules on lysis of bacteria by human leukocyte lysates.

TABLE 1. *The effect of macromolecules on lysis of bacteria by human leukocyte lysates*

Leukocyte lysates (1 mg protein) + inhibitors (1 mg/ml)	*% Inhibition of ^{14}C released from*	
	Staph. albus	*Strep. faecalis*
Alginic acid	95	93
Carrageenan	90	90
Dextran sulfate	70	15
Hyaluronic acid	5	25
Lipopolysaccharide *(E. coli)*	5	0
Glycogen	0	0
Trypan blue	95	90
Congo red	100	100

dyes, and phosphatidic acid, are potent inhibitors of lysis of *Staph. albus* induced by human blood leukocyte lysates. (Similar results were obtained with *Staph. aureus* and *Strep. faecalis*.) These inhibitors were also very effective against monocyte and macrophage enzymes. Moreover, smaller amounts of inhibitors were sufficient to block lysis by these extracts. Since polyanions (Mora and Young, 1959; Skarness and Watson, 1955; Clark and Higginbotham, 1970) and trypan blue (Lloyd *et al.*, 1968) are potent inhibitors of certain lysosomal enzymes it is thought that some of the key enzymes in the leukocyte lysates responsible for breaking cell-wall structures of bacteria have been inhibited.

Of special interest is the inhibition of bacteriolysis by chondroitin sulfate, heparin, leukocyte cationic proteins, histone and DNA as these may occur in inflammatory exudates.

Preliminary results have also shown that a 'cocktail' of inhibitors containing 50 μg/ml each of chondroitin sulfate, DNA, and heparin and 1% serum caused approximately 70% inhibition of lysis of *Staph. albus* by human blood leukocyte lysates. When, however, 50 μg/ml of protamine sulfate was added to the above mixture the inhibition of lysis was somewhat depressed. Since this mixture turned very turbid it was assumed that protamine sulfate precipitated out some of the negatively charged polyelectrolytes. These results suggest that a delicate balance between cationic and anionic polyelectrolytes present in tissue exudates may determine the degree of inhibition of lysis of bacteria by leukocyte hydrolases.

In other experiments it was found that normal human immunoglobulins and rabbit antiserum to *Staph. albus* and *Staph. aureus* markedly inhibited the lysis of the corresponding bacteria by human leukocyte lysates, while serum albumin and fibrinogen did not inhibit bacterial lysis. Preliminary results from our laboratory have also shown that certain synovial fluids of patients with rheumatoid arthritis and urines from patients with Hunter's disease strongly inhibited lysis of bacteria by leukocyte enzymes.

The inhibition of bacteriolysis by the macromolecular substance was also studied by electron microscopic techniques (Ginsburg *et al.*, 1974*a*; Lahav *et al.*, 1974). It was found that the presence of the inhibitors in the incubation mixtures prevents cellular breakdown to a large extent.

DISCUSSION

The data presented describe the lysis of a variety of ^{14}C-labelled bacteria by lysates obtained from different leukocyte populations and the inhibition of bacteriolysis by macromolecular substances. Several conclusions can be drawn from these experiments. It appears that PMN lysates are more efficient than monocyte or macrophage lysates in the degradation of the bacterial cells, when comparisons are made at equal protein concentrations. Although the nature of the enzymes in the PMN and macrophages which are responsible for the lysis of bacteria is still unknown it is of interest that both these extracts contained approximately the same amounts of acid phosphatase, N-acetylglucosaminidase, cathepsin D and lysozyme (to be published). Thus it is unlikely that any of these enzymes are responsible for the more efficient lytic capacity of the PMN. Lysozyme is the only enzyme which is known to attack and degrade the rigid mucopeptide structures of the cell walls. This enzyme cannot, however, cause lysis unless the complex polysaccharides and lipopolysaccharides are first removed (Ohanian and Schwab, 1967; Ginsburg *et al.*, 1973). The participation of lysozyme in bacteriolysis was recently studied using a mixture of crude trypsin, lysolecithin and lysozyme which could replace PMN enzymes in the lysis of bacteria (to be published). In the absence of lysozyme, only a very small percentage of lysis occurred. This suggests that the relative inefficiency of monocyte lysates in bringing about lysis of bacteria may be due to a lack of such 'preparing' surface-active agents or enzymes. Another possibility is that the monocyte extracts contain an inhibitor.

Although bacterial lysis *in vitro* may not fully mimic the intracellular events which take place following phagocytosis, it may nevertheless indicate what happens in many inflammatory exudates induced by proliferating virulent bacteria. It is thought that very soon after the penetration of virulent micro-organisms into the tissues PMN migrate to these sites and may be engaged in phagocytosis. Since successful phagocytosis depends on the presence of opsonic antibodies, lack of phagocytosis creates a situation in which free lysosomal enzymes of the PMN may interact with the bacterial cells extracellularly, which is analogous to the *in vitro* studies. Later on, mononuclear phagocytes replace the PMN. These cells may engulf the remaining unphagocytosed viable or non-viable bacteria as well as the broken PMN containing bacterial remnants. The long persistence of undegraded bacterial cell wall components within macrophages (Ohanian and Schwab, 1967; Ginsburg *et al.*, 1973, 1974) indicates that the sequential attack by both PMN and monocytic cells may be inefficient in degrading certain bacterial cell walls. At this point, the finding that a variety of naturally occurring macromolecular substances such as histone, leukocyte cationic proteins, heparin, chondroitin sulfate, DNA and phosphatidic acid are potent inhibitors of bacteriolysis induced by leukocyte enzymes, is of special significance. Moreover, the inhibition of bacteriolysis by immunoglobulins and by synovial fluids indicates the adverse effects such substances may have on the degradation of bacterial cell-wall constituents by the lysosomal enzymes of the leukocytes. It is postulated that a delicate balance between cationic and anionic polyelectrolytes may determine whether a micro-organism will remain intact and initiate a granulomatous reaction. These results are in accordance with previous studies (Spector *et al.*, 1970; Cohn, 1963*b*) showing that antibodies will protect bacterial cells against

degradation by macrophages. It is also of interest that histones and leukocyte cationic proteins which inhibit the degradation of bacterial cells by leukocyte enzymes are also potent bactericidal agents (Hirsch, 1958; Zeya and Spitznagel, 1968). This emphasizes the difference between the bactericidal properties of leukocytes and their degradative capacities.

The possible role played by both PMN and macrophages in the processing of bacterial constituents in relation to antibody formation must be considered. It is known that macrophage presentation increases the immunogenicity of soluble antigens. It may be even more important in the case of particulate antigens like bacterial walls, and it is possible that partial degradation by polymorph and macrophage lysosomal enzymes may be important for an immune response.

The occurrence of degradative enzymes in both polymorphs and macrophages raises the question whether the sequential attack by enzymes of these cells is needed to degrade certain bacterial cell walls. It is hoped that the results presented here may throw light on some of these problems.

References

Ayoub, E. M. and Wanamaker, L. W. (1967): The fate of group A streptococci following phagocytosis: *in vitro* phagocytic studies of isotope-labelled streptococci. *J. Immunol.*, *99*, 1099.

Clark, J. H. and Higginbotham, R. D. (1970): Reversibility of heparin inhibition of antibacterial activity of polymorphonuclear leukocyte lysosomal proteins. *Infect. Immun.*, *1*, 607.

Cohn, Z. A. (1963*a*): The fate of bacteria within phagocytic cells. I. The degradation of isotopically labelled bacteria by polymorphonuclear macrophages. *J. exp. Med.*, *117*. 27.

Cohn, Z. A. (1963*b*): The fate of bacteria within phagocytic cells. II. The modification of intracellular degradation. *J. exp. Med.*, *117*, 43.

Cohn, Z. A. and Hirsch, J. G. (1960): The isolation and properties of specific cytoplasmic granules of rabbit polymorphonuclear leukocytes. *J. exp. Med.*, *112*, 983.

Ginsburg, I. (1972): Mechanisms of cell and tissue injury induced by group A streptococci: Relation to post-streptococcal sequelae. *J. infect. Dis.*, *128*, 294, 419.

Ginsburg, I., Lahav, M. and Neeman, N. (1973): The effect of cationic and anionic polyelectrolytes and antibodies on the lysis of micrococci and streptococci by leukocyte lysates. *Israel J. med. Sci.*, *9*, 663.

Ginsburg, I., Mitrani, S., Neeman, N. and Lahav, M. (1974): Granulomata in streptococcal inflammation: Mechanisms of localization, transport and degradation of streptococci in inflammatory sites. In: *2nd Conference on the Mononuclear Phagocyte in Immunity, Infection and Pathology.* Editor: Ralph van Furth. Blackwell, Oxford. In press.

Glick, A. D., Ranhand, J. M. and Cole, R. M. (1972): Degradation of group A streptococcal cell walls by egg-white lysozyme and human lysosomal enzymes. *Infect. Immun.*, *6*, 403.

Hirsch, J. G. (1958): Bactericidal action of histone. *J. exp. Med.*, *108*, 925.

Lahav, M., Neeman, N., Adler, E. and Ginsburg, I. (1974): The effect of leukocyte hydrolases on bacteria. I. Degradation of ^{14}C-labelled streptococci and staphylococci by leukocyte lysates *in vitro*. *J. Infect. Dis.*, *129*, 528.

Lloyd, J. B., Beek, F., Griffiths, A. and Parry, L. M. (1968): The mechanism of action of acid bisazo dyes. In: *The Interaction of Drugs and Subcellular Components on Animal Cells*, pp. 171-202. Editor: P. N. Campbell. Churchill Ltd., London.

Mora, P. T. and Young, B. G. (1959): Reversible inhibition of enzymes by interaction with synthetic polysaccharide macroanions. *Arch. Biochem.*, *82*, 6.

Ohanian, S. H. and Schwab, J. H. (1967): Persistence of group A streptococcal cell walls as related to chronic inflammation of rabbit dermal connective tissue. *J. exp. Med.*, *125*, 1137.

SKARNESS, R. C. and WATSON, D. W. (1955): The inhibition of lysozyme by acidic polymers from pathogenic bacteria. *J. Bacteriol., 70*, 110.

SPECTOR, W. G., REICHHOLD, N. and RYAN, G. B. (1970): Degradation of granuloma inducing microorganisms by macrophages. *J. Path., 101*, 339.

STAHELIN, H., KARNOVSKY, M. and SUTER, E. (1956): Studies on the interaction between phagocytes and tubercle bacilli. II. The action of phagocytes upon ^{14}C-labelled tubercle bacillli. *J. exp. Med., 104*, 137.

VAN FURTH, R. C. (Ed.) (1970): *Mononuclear Phagocytes.* Blackwell, Oxford.

ZEYA, H. I. and SPITZNAGEL, J. K. (1968): Arginine rich proteins of polymorphonuclear leukocyte lysosomes. Antimicrobial specificity and biochemical heterogeneity. *J. exp. Med., 127*, 927.