# PHAGOCYTOSIS OF BACTERIA BY HETEROPHIL LEUKOCYTES

## Acid and Alkaline Phosphatase Cytochemistry

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Prominent characteristics of heterophil* leukocytes include their numerous cytoplasmic granules and their capacity to phagocytose particulate matter. Recent studies have contributed significantly to the understanding of the nature of the cytoplasmic granules and have indicated that these granules play a role in the process of phagocytosis. Cohn and Hirsch [1] have demonstrated biochemically that nonspecific acid and alkaline phosphatases and a variety of other hydrolytic enzymes are found in the granule fraction obtained from peritoneal exudate leukocytes. Histochemical evidence for the presence of some of these enzymes in exudate leukocytes has been observed at the light microscope level.[2,3]

Ultrastructural localization of nonspecific acid and alkaline phosphatase in particular granule types has recently been accomplished in the heterophils of rabbit bone marrow and peripheral blood.[4,5] Acid phosphatase activity was demonstrable in relatively large and adielectronic granules which appeared to correspond in their distribution with the histochemically defined azurophil granules.[6] Another type of granule showing acid phosphatase activity in these cells has been characterized as a small, pleomorphic, dense granule, more frequently encountered in mature cells. In addition, these studies tentatively distinguished a population of cytoplasmic granules of intermediate size and lower density, more abundant in mature heterophils, which evidenced nonspecific alkaline phosphatase activity but have thus far shown no indication of acid phosphatase activity with established methods.

Cohn and Hirsch [7] have demonstrated biochemically that during bacterial phagocytosis by exudate leukocytes, acid hydrolases are lost from the granule fraction but are recoverable from the supernatant.[7] The more recently devised phase contrast cinematography of Hirsch, demonstrating granule lysis around phagocytosed particles, further implicates these

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* The general term heterophil is employed in lieu of the term neutrophil which conventionally designates the analogous cell type in humans.

granules as a source of catabolic enzymes needed for the degradation of ingested material.[8] Ultrastructural studies by Lockwood and Allison have revealed configurations suggesting fusion of granule membrane with the membranes of phagocytic vacuoles.[9] These studies, confirmed by Zucker-Franklin and Hirsch,[10] in addition, showed granule-like material in the phagocytic vacuoles.

The work of Spitznagel and Chi, based on fast green staining of basic protein in leukocyte granules, has also indicated that phagocytosed bacteria are coated by this granule constituent.[11] However, the presence of hydrolytic enzyme activity characteristic of leukocyte granules has not been demonstrated within the phagocytic vacuoles of these cells. The present study affords more direct evidence for the role of heterophil granules in phagocytosis, confirming the morphologic relationships involved and, furthermore, revealing by cytochemical means the presence of acid and alkaline phosphatases in the cytoplasmic granules and subsequently within phagocytic vacuoles.

## Material and Methods

Leukocytic ingestion of *Staphylococcus albus* was studied in an *in vitro* system modeled after that of Cohn and Hirsch (see ref. 7, p. 1016). Glycogen-induced rabbit peritoneal exudate leukocytes and washed viable bacteria were mixed in Hanks's solution, modified by the addition of 0.01 per cent bovine serum albumin, 0.0056 M glucose, and 10 per cent fresh rabbit serum. The medium was rotated slowly at 37° C. in siliconized centrifuge tubes; aliquots were removed at intervals of 2, 5, 10, 20, 30, and 60 minutes, chilled, and centrifuged. For cytochemical determinations, the pellets were fixed 90 minutes in sodium cacodylate-buffered 6.25 per cent glutaraldehyde[12] followed by rinsing in cold 7.5 per cent sucrose, freezing, and sectioning in the cryostat.[13,14] Rinsed cryostat sections cut at 40 $\mu$ were then incubated 15 to 60 minutes at 37° C. in the Barka and Anderson modification[15] of Gomori's acid phosphatase medium,[16] or incubated in a comparable fashion in Gomori's alkaline phosphatase medium[16] with subsequent conversion of the calcium phosphate reaction product to lead salt by a 5-minute exposure to 2 per cent $PbNO_3$. Following the cytochemical procedures, rinsed sections were then fixed 45 minutes in Millonig's[17] solution prior to ethanol dehydration, embedding in Maraglas,[18] and ultramicrotomy.

For morphologic investigations, parallel aliquots of phagocytosing leukocytes were fixed 90 minutes in cacodylate-buffered 6.25 per cent glutaraldehyde followed by 45 minutes' fixation in Millonig's solution, and additional specimens were fixed 90 minutes in Millonig's solution alone. These tissues were then processed for electron microscopy, with the omission of the cytochemical procedures. Similar morphologic and cytochemical studies were performed on exudate leukocytes in the absence of bacteria, both prior to and following 60 minutes' incubation in modified Hanks's solution. Bacteria alone were studied similarly.

Thin sections cut on a Porter-Blum MT-1 microtome with glass knives were collected on 65 mesh copper grids covered with a carbonized collodion film. Staining procedures included Karnovsky's "A" lead stain,[19] Reynold's lead citrate solution,[20] uranyl acetate solution,[21] and various combinations of uranyl and lead methods. Specimens were examined and micrographed in either an RCA EMU-2A or an RCA EMU-3G electron microscope.

## Results

Heterophils comprised approximately 90 per cent of the peritoneal exudate cells, the remainder consisting of mononuclear cells and occasional eosinophils. Each of these cell types was observed to contain phagocytosed bacteria. However, the observations reported below pertain only to heterophil leukocytes unless otherwise specified.

The granule population appeared to include the 3 generally distinguishable granule types previously observed in normal rabbit bone marrow and blood.[4,5] Relatively large and adielectronic granules (Figs. 1 and 2), comparable to those characterized as azurophil granules in developing and circulating cells, seemed more numerous in the cells of the peritoneal exudates than in those of buffy coat preparations from the blood of normal rabbits. Small, pleomorphic, dense granules were frequently noted in both heterophils and mononuclear cells. Both granule types showed localized deposition of reaction product following incubation for acid phosphatase (Figs. 3 and 5). Acid phosphatase reaction product was also observed occasionally in the Golgi lamellas of mononuclear cells and heterophils. Intermediate-sized, generally lower density granules (Fig. 2), assumed to correspond to the preponderant type in normal circulating heterophils, showed no evidence of acid phosphatase activity, but appeared to contain nonspecific alkaline phosphatase (Fig. 6).

Phagocytosis occurred promptly, and the actual engulfment of bacteria by pseudopodia appeared to proceed as reported by others.[22] In aliquots removed after 2 minutes' incubation, many leukocytes contained ingested bacteria, and numerous granules of phagocytosing cells were as yet dispersed within the cytoplasm. However, some cytoplasmic granules were already observed in close approximation to the phagocytic vacuoles. Configurations indicating fusion of granule membrane with phagosome membrane were seen in several instances (Fig. 1). Material of density and texture comparable to granule substance was frequently encountered coating ingested bacteria or lying free within the vacuole, occasionally retaining the form of granules (Figs. 1 and 2). These findings confirmed morphologic evidence reported by others.[9,10]

Following 60 minutes' incubation of bacteria and leukocytes, most heterophil profiles showed numerous ingested bacteria and few or no cytoplasmic granules. This depletion of granules presumably resulted from the contribution of granule material to phagocytic vacuoles; no other mechanism accounting for granule loss was observed. The absence of all granules from some cells at later incubation times supported the impression that each of the 3 general types of cytoplasmic granule par-

ticipated in the phagocytic process through association and fusion with phagocytic vacuoles. Peritoneal exudate leukocytes, incubated similarly except for the absence of bacteria, showed no evidence of granule depletion.

Acid phosphatase reactive granules were frequently observed immediately adjacent to phagocytic vacuoles and, in some instances, fusion of these structures was apparent (Fig. 5). Heavy deposition of reaction product was noted in some phagocytic vacuoles (Figs. 3 to 5). This presumptive acid phosphatase activity in phagocytic vacuoles substantiated the indirect evidence cited above[7,8] which suggested transfer of acid hydrolases from leukocyte granules to the site of ingested particles.

Similarly, granules showing alkaline phosphatase reaction product deposition occurred in close association with phagocytic vacuoles. Alkaline phosphatase reaction product was visualized within these vacuoles, providing evidence consistent with the suggested fusion of such granules with phagosomes (Fig. 6).

The validity of the positive cytochemical findings was strengthened by the absence of reaction product from leukocyte granules in tissue incubated without added substrate, with the inclusion of 10 per cent formalin in the incubation medium, or after heat inactivation of enzymes.

Nonspecific acid or alkaline phosphatase reaction product was observed coating bacterial cell walls only within phagocytic vacuoles. Bacteria found extracellularly in the phagocytosis system, or processed independently for cytochemical study, were generally free of reaction product, the only notable exception being the occasional association of precipitate with bacterial nucleoids.

## Discussion

The positive cytochemical and morphologic findings presented afford direct evidence that nonspecific phosphatases are transferred from cytoplasmic granules of rabbit heterophils to newly formed phagocytic vacuoles. The results confirm and extend previous biochemical and morphologic studies which suggested such a mechanism; they further provide the first definitive evidence for hydrolytic enzymes in the phagocytic vacuoles of these cells. The selective localization of phosphatase reaction product to particular sites with generally accepted procedures[12–14] and the absence of reaction product in control specimens support the validity of the cytochemical methods.

It should be emphasized that under these conditions most phagocytic vacuoles were free of demonstrable reaction product, despite heavy deposition in cytoplasmic granules. From the frequent appearance of the granule-like material in the phagocytic vacuoles, a higher incidence of

phosphatase-positive phagosomes might be expected. Some of the vacuoles would presumably lack acid phosphatase if included granule material were derived only from intermediate-sized, lower density, alkaline phosphatase-positive granules. Parallel reasoning could account for a proportion of the vacuoles lacking alkaline phosphatase activity. More importantly, alterations in the physical state of granules accompanying their accession to the phagosome may render the enzymes more susceptible to solubility loss or fixative inactivation. It should be noted that the present interpretation is based on the positive findings of this study and is not dependent upon negative cytochemical results of inconclusive significance.

In tissues such as liver,[23] kidney,[13] and thyroid,[24] membrane-limited organelles, rich in hydrolases, are considered to be instrumental in the compartmentalized degradation of incorporated or intrinsic substrate material. Rabbit exudate heterophils apparently constitute a dramatic example of this phenomenon, with features uniquely adapted to their role in phagocytosis. The predominant cytoplasmic constituent of these cells consists of preformed specific organelles rich in hydrolase activity. The ready availability of these hydrolytic enzymes in heterophils permits the effective degradation of phagocytosed material without prerequisite synthesis of these enzymes. This potentiality, coupled with the capacity of glycogen-rich heterophils for anaerobic metabolism, might account, in part, for the ability of these cells to function effectively in the often metabolically unfavorable circumstances of acute inflammation.

## Summary

Ultrastructural and cytochemical studies on rabbit exudate heterophil leukocytes indicate the occurrence of nonspecific acid and alkaline phosphatases in particular cytoplasmic granules. Phagocytosis of staphylococci by these cells was accompanied by configurations suggesting granule fusion with newly formed phagocytic vacuoles. The apparent transfer of granule-like material into phagocytic vacuoles coincided with the appearance of presumptive acid and alkaline phosphatase activities around some phagocytosed bacteria. Each of 3 apparently distinct granule types evidently participated in this process. Continued phagocytic activity resulted in the depletion of both acid and alkaline phosphatase-containing granules from the cell.

## References

1. Cohn, Z. A., and Hirsch, J. G. The isolation and properties of the specific cytoplasmic granules of rabbit polymorphonuclear leucocytes. *J. Exper. Med.*, 1960, **112**, 983–1004.

2. DANNENBERG, A. M., JR.; BURSTONE, M. S.; WALTER, P. C., and KINSLEY, J. W. A histochemical study of phagocytic and enzymatic functions of rabbit mononuclear and polymorphonuclear exudate cells and alveolar macrophages. I. Survey and quantitation of enzymes, and states of cellular activation. *J. Cell Biol.*, 1963, **17**, 465–486.
3. WULFF, H. R. Histochemical studies of leukocytes from an inflammatory exudate. V. Alkaline and acid phosphatases and esterases. *Acta haemat.*, 1963, **30**, 159–167.
4. HORN, R. G.; WETZEL, B. K., and SPICER, S. S. Fine structural localization of nonspecific acid and alkaline phosphatases in rabbit myeloid elements. (Abstract) *J. Appl. Physics*, 1963, **34**, 2517.
5. WETZEL, B. K.; HORN, R. G., and SPICER, S. S. Cytochemical localization of non-specific phosphatase activity in rabbit myeloid elements. (Letter to the editor) *J. Histochem.*, 1963, **11**, 812–814.
6. HORN, R. G., and SPICER, S. S. Sulfated mucopolysaccharide and basic protein in certain granules of rabbit leukocytes. *Lab. Invest.*, 1964, **13**, 1–15.
7. COHN, Z. A., and HIRSCH, J. G. The influence of phagocytosis on the intracellular distribution of granule-associated components of polymorphonuclear leucocytes. *J. Exper. Med.*, 1960, **112**, 1015–1022.
8. HIRSCH, J. G. Cinemicrophotographic observations on granule lysis in polymorphonuclear leucocytes during phagocytosis. *J. Exper. Med.*, 1962, **116**, 827–834.
9. LOCKWOOD, W. R., and ALLISON, F. Electronmicrographic studies of phagocytic cells. I. Morphological changes of the cytoplasm and granules of rabbit granulocytes associated with ingestion of rough pneumococcus. *Brit. J. Exper. Path.*, 1963, **54**, 593–600.
10. ZUCKER-FRANKLIN, D., and HIRSCH, J. G. Degranulation of polymorphonuclear leukocytes following phagocytosis—an electron micrographic study. (Abstract) *Blood*, 1963, **22**, 824–825.
11. SPITZNAGEL, J. K., and CHI, H-Y. Cationic proteins and antibacterial properties of infected tissues and leukocytes. *Am. J. Path.*, 1963, **43**, 697–711.
12. SABATINI, D. D.; BENSCH, K., and BARRNETT, R. J. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.*, 1963, **17**, 19–58.
13. MILLER, F. Acid Phosphatase Localization in Renal Protein Absorption Droplets. In: Electron Microscopy. Fifth International Congress for Electron Microscopy. BREESE, S. S., JR. (ed.). Academic Press, Inc., New York, 1962, p. Q-2.
14. HOLT, S. J., and HICKS, R. M. The localization of acid phosphatase in rat liver cells as revealed by combined cytochemical staining and electron microscopy. *J. Biophys. & Biochem. Cytol.*, 1961, **11**, 47–66.
15. BARKA, T., and ANDERSON, P. J. Histochemical methods for acid phosphatase using hexazonium pararonsanilin as coupler. *J. Histochem.*, 1962, **10**, 741–753.
16. GOMORI, G. Microscopic Histochemistry. University of Chicago Press, Chicago, 1952, 273 pp.
17. MILLONIG, G. Advantages of a phosphate buffer for $OsO_4$ solutions in fixation. (Abstract) *J. Appl. Physics*, 1961, **32**, 1637.
18. SPURLOCK, B. O.; KATTINE, V. C., and FREEMAN, J. A. Technical modifications in Maraglas embedding. *J. Cell Biol.*, 1963, **17**, 203–207.
19. KARNOVSKY, M. J. Simple methods for "staining with lead" at high pH in electron microscopy. *J. Biophys. & Biochem. Cytol.*, 1961, **11**, 729–732.

20. REYNOLDS, E. S. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.*, 1963, **17**, 208–212.

21. WATSON, M. L. Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. & Biochem. Cytol.*, 1958, **4**, 475–478.

22. NELSON, E.; BLINZINGER, K., and HAGER, H. Ultrastructural observations on phagocytosis of bacteria in experimental (*E. coli*) meningitis. *J. Neuropath. & Exper. Neurol.*, 1962, **21**, 155–169.

23. DEDUVE, C. Lysosomes, a New Group of Cytoplasmic Particles. In: Subcellular Particles. HAYASHI, T. (ed.). The Ronald Press Co., New York, 1959, pp. 128–159.

24. WETZEL, B. K.; SPICER, S. S., and WOLLMAN, S. H. Nonspecific acid-phosphatase activity in rat thyroid glands following TSH stimulation. (Abstract) *J. Appl. Physics*, 1963, **34**, 2525.

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[ *Illustrations follow* ]

## Legends for Figures

All figures depict portions of rabbit peritoneal exudate heterophils containing phagocytosed *Staphylococcus albus* organisms. Unless otherwise indicated, tissues were fixed in glutaraldehyde followed by Millonig's solution, and thin sections were stained with uranyl acetate followed by Reynold's lead citrate.

Fig. 1. Three bacteria are contained within a large, irregular phagocytic vacuole. Several relatively adielectronic granules (g) are included within the vacuolar membrane. The configuration of the continuous limiting membrane (arrows) suggests recent fusion of the granules with the vacuole. The clear spaces within the bacteria are probably a result of incomplete embedding. Millonig's fixative, Karnovsky "A" lead stain. × 20,000.

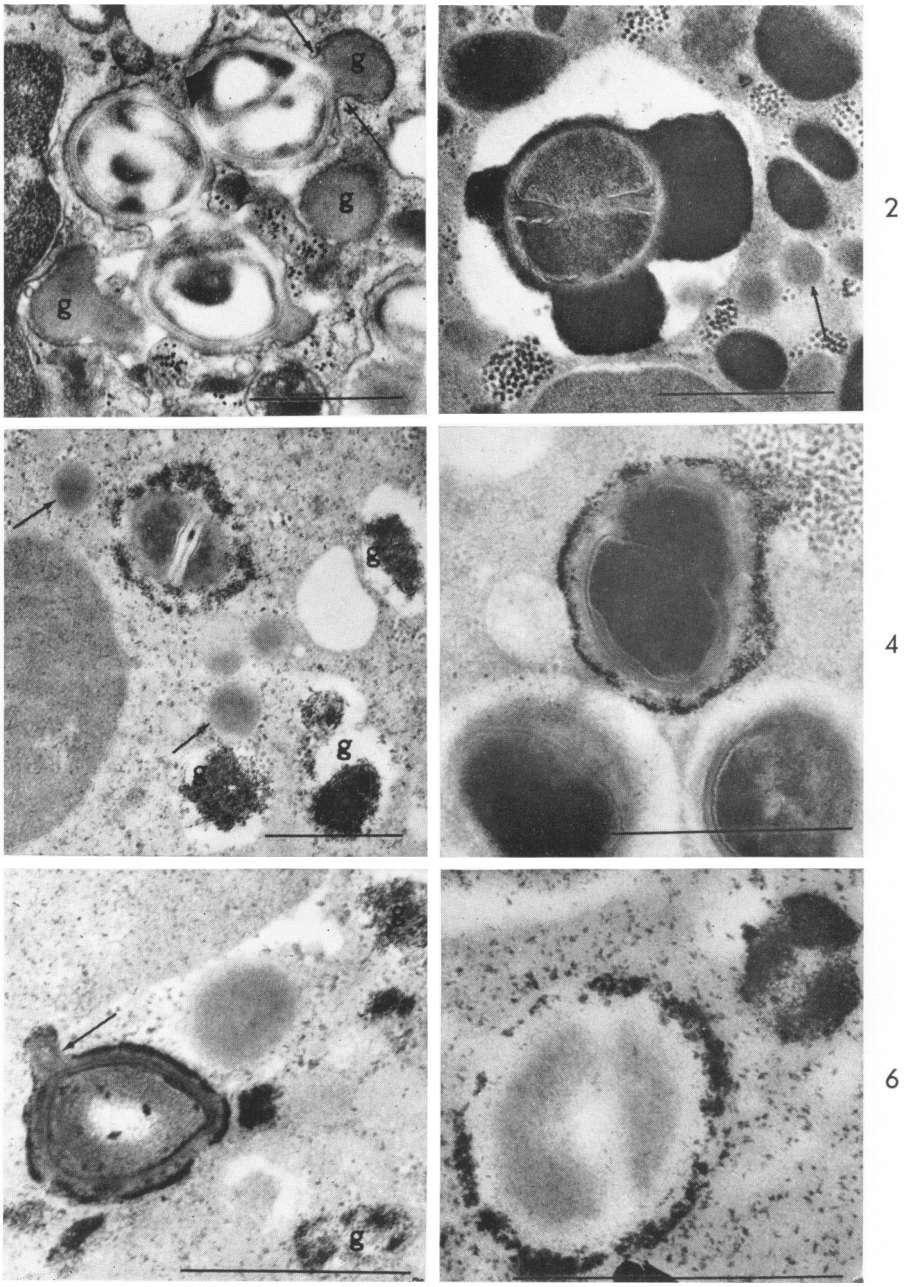
Fig. 2. An ingested bacterium is surrounded by material resembling in form and density the relatively large, adielectronic granules in the adjacent cytoplasm. Intermediate-sized, less dense granules (arrow) and abundant glycogen are also evident in the cytoplasm. × 23,000.

Fig. 3. Acid phosphatase reaction product is evident in partially extracted azurophil granules (g) and surrounds an ingested bacterium. Evident are intermediate-sized, lower density, unreactive granules (arrows). × 18,000.

Fig. 4. As in Figure 3, there is apposition of acid phosphatase-reactive material to the bacterium within one phagocytic vacuole and an absence of reaction product from two adjacent vacuoles. × 31,000.

Fig. 5. Several small, pleomorphic, cytoplasmic granules exhibit acid phosphatase reaction product deposition. Apparent fusion of this type of granule with a phagocytic vacuole is shown (arrow). Reaction product also surrounds the phagocytosed bacterium. Two azurophil granules show a deposition of reaction product (g). × 30,000.

Fig. 6. Alkaline phosphatase reaction product surrounds an ingested bacterium and appears within an intermediate-sized cytoplasmic granule. Spurious deposition of reaction product is present in the cytoplasm. Unstained section. × 42,000.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