THE PARTICULATE HYDROLASES OF MACROPHAGES

II. Biochemical and Morphological Response to Particle Ingestion*

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The preceding article (1) delineated the nature and properties of the hydrolase-containing cytoplasmic granules of the BCG-induced alveolar macrophage. These organelles represented a spectrum of morphological types which were considerably denser than mitochondria, were stained with vital dyes, and contained large amounts of hydrolytic enzymes. These enzymes exhibited the property of "latency," were presumably surrounded by a semipermeable membrane, and could be liberated in a soluble form by mechanical trauma. Since the macrophage is an actively phagocytic cell, it was of some interest to examine the fate of these organelles and their contents following the uptake of particulate material. This communication will describe both biochemical and morphological events following the ingestion of microorganisms by BCG-induced alveolar macrophages.

Materials and Methods

The harvesting and preparation of BCG-induced alveolar macrophages, and enzymatic and chemical methodology were described in the preceding article (1).

Heat-killed *Escherichia coli* K-12 was employed as the particle for all experiments involving the redistribution of the particulate hydrolases. Organisms were grown in penassay broth for 18 hours, collected by centrifugation, and washed twice with large volumes of saline. The bacteria were then heated at 80°C for 30 minutes in a water bath, washed twice with saline, and resuspended in the same medium to the appropriate density.

Experiments involving morphological observations were made with zymosan (yeast cell walls). This was obtained from Standard Brands, Inc., New York (lot OB298) as a dry powder. The material was suspended in saline, boiled for 1 hour, and then washed three times and suspended in saline at a concentration of 1 mg/ml.

Biochemical Redistribution.—The general plan of these experiments was to add various multiplicities of particles to macrophages and to incubate the mixtures at 38°C. At different time intervals after mixing, the suspensions were centrifuged at low speed and the extra-

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cellular fluid removed. The macrophages were then washed, homogenized, and the homogenate centrifuged at 15,000 *g* to obtain a pellet and supernatant fluid. Each of these fractions was then frozen and thawed six times and assayed for enzymatic activity.

BCG-induced rabbit macrophages were washed twice in phosphate buffered saline and resuspended in buffer containing 10 per cent fresh normal rabbit serum and 100 mg per cent glucose. The cells were dispensed to glass tubes and heat-killed bacteria added. The final cell concentration was maintained at 35×10^6/ml in a total volume of 4.0 ml. The number of bacteria/macrophage varied from 2 to 20. The tubes were then stoppered and rotated end over end at 38°C (rotator model ST, New Brunswick Scientific Company, New Brunswick, New Jersey). Under these conditions more than 90 per cent of the cells were phagocytic and more than 95 per cent of the bacteria had been ingested after 30 minutes of rotation. Control tubes which did not contain bacteria were always included in each test.

At time intervals from zero time (immediately after addition of the particles) to 180 minutes, aliquots were removed and quickly centrifuged at 1000 RPM for 6 minutes (International, type I). The cell-free supernatant fluid was carefully removed by means of a Pasteur pipette and constituted the "medium" fraction. The cell pellets were then washed once in ice cold 0.25 M sucrose and resedimented at 1,2000 RPM for 5 minutes. No significant loss of cell-associated enzyme occurred during this step. The resuspended cells were then quantitatively transferred to individual microhomogenizers and homogenized for a 4 minute period at 0 to 4°C by means of a motor-driven teflon pestle. The homogenate was next transferred to 10 × 78 mm lusteroid tubes and centrifuged at 15,000 *g* for 15 minutes in a high speed, angle head, rotor (Lourdes, model 9RA). This resulted in a "15 *g*" pellet and a 15 *g* supernatant fluid. Each fraction was taken to volume with 0.25 M sucrose, frozen and thawed six times in a dry ice–alcohol bath, and assayed for enzymatic activity.

Vital Staining of Normal and Phagocyting Alveolar Macrophages.—Macrophages were suspended to a final concentration of 20×10^6/ml in phosphate buffer, ph 7.5, containing 10 per cent fresh normal rabbit serum and 100 mg per cent of glucose. Washed particles of zymosan were added and the tubes rotated for 15 to 20 minutes at 38°C. Macrophages without particles were prepared in an identical fashion. After incubation, small drops of the suspension were placed on glass slides which had previously been coated with a thin film of neutral red (1 mg/ml in absolute ethanol). Coverslips were then applied and the preparations immediately examined with the oil immersion lens of the light microscope.

In other experiments macrophages were supravitally stained with neutral red before the addition of particles. Macrophages were prepared in phosphate-buffered saline, ph 7.5, at a concentration of 20×10^6/ml. Neutral red suspended in 0.25 M sucrose (1 mg/ml) was then added (1 part neutral red to 9 parts macrophage suspension) and the tubes allowed to stand at room temperature for 1 to 3 minutes. The cells were then sedimented at 500 RPM, washed once with buffer, and resuspended in the phagocytosis medium which contained 10 per cent rabbit serum. Zymosan was then added, and phagocytosis was allowed to proceed at 38°C for 15 minutes. Slide preparations were made and examined as above.

Histochemical Demonstration of Acid Phosphatase in Normal and Phagocyting Macrophages.—BCG-induced alveolar macrophages were suspended in balanced salt solution containing 10 per cent fresh normal rabbit and 100 mg per cent glucose to a concentration of 20×10^6/ml. One ml aliquots were dispensed to 10 × 76 mm glass tubes, and 0.1 ml of a suspension of zymosan (1 mg/ml) was added. Control tubes without particles were prepared simultaneously. The mixtures were then rotated end over end for 15 minutes at 38°C in a constant temperature room. After this period of phagocytosis, in which the majority of particles had been ingested, small drops of the suspension were placed on acid-cleaned, warm, glass slides. Usually 4 drops were placed on each slide; *i.e.*, two phagocyting and two controls. The slides were then placed in a moist chamber and incubated for 15 minutes to allow the cells to adhere

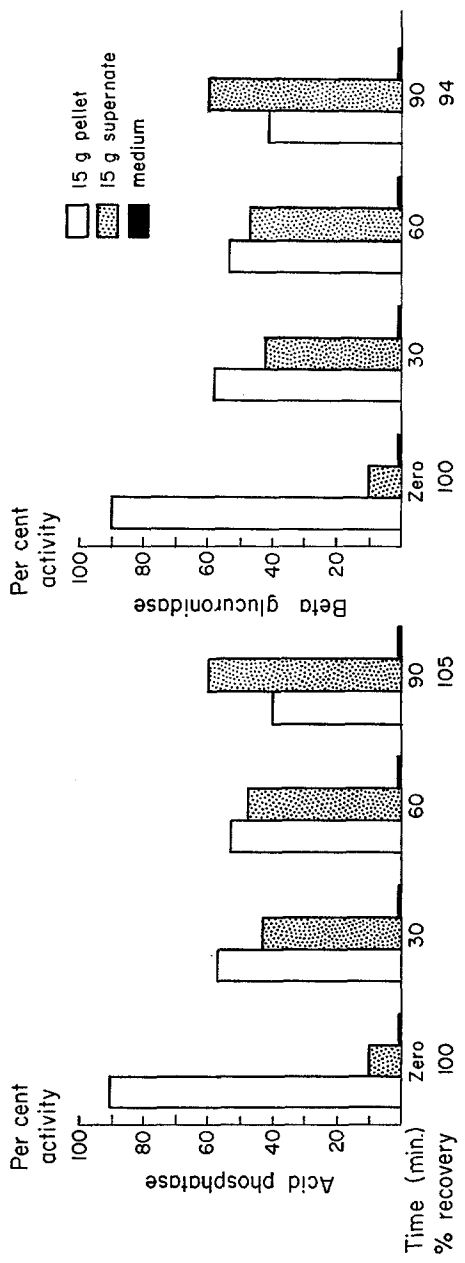
and spread out. The slides were then flash dried with a jet of air, and this resulted in uniform circles of cells which were visible on the gross. They were then immediately placed in 4 per cent neutral formalin containing 1 per cent $CaCl_2$, fixed for 30 minutes at 4°C, and then rinsed in 0.88 M sucrose containing 1 per cent gum acacia for 30 minutes (2). After this step they were incubated in the Gomori medium (3) containing β-glycerophosphate at ph 5.0 for 10 to 15 minutes at 38°C. This time period was sufficient to give deep staining of the cytoplasmic particles. The incubation medium was freshly prepared and never older than 24 hours at the time of use. Development was performed by the usual procedure including a 1 minute rinse in 1 per cent acetic acid.

RESULTS

Phagocytic Properties of BCG-Induced Alveolar Macrophages.—Studies were conducted on the ability of alveolar macrophages to ingest microorganisms in a variety of *in vitro* systems. Coverslip preparations of cells maintained on a warm stage incubator remained sessile for periods up to 14 hours and did not demonstrate ameboid motion or pseudopod formation. Under these conditions the addition of a variety of particles resulted in only an occasional ingestion. In contrast, cells suspended in fluid media and agitated in the presence of particles, exhibited prompt and efficient phagocytosis. Kinetic studies of the type described previously (4) were performed employing strains of viable *Escherichia coli* and *Staphylococcus albus* as test organisms. These experiments will not be described in detail, but indicated that under these conditions both microorganisms were rapidly ingested and inactivated within the cell. Within 20 minutes after rotation was started the macrophages had ingested 95 per cent of the inoculum. These data were quantitatively similar to previous results employing rabbit polymorphonuclear (PMN) leucocytes or peritoneal macrophages in a similar system. However, in contrast to these cells the phagocytic activity of the alveolar macrophage was blocked with cyanide (10^{-2} M), 2, 4-dinitrophenol (10^{-4} M) as well as inhibitors of glycolysis; *i.e.*, iodoacetate (10^{-4} M). This has been reported previously for normal guinea pig alveolar macrophages (5).

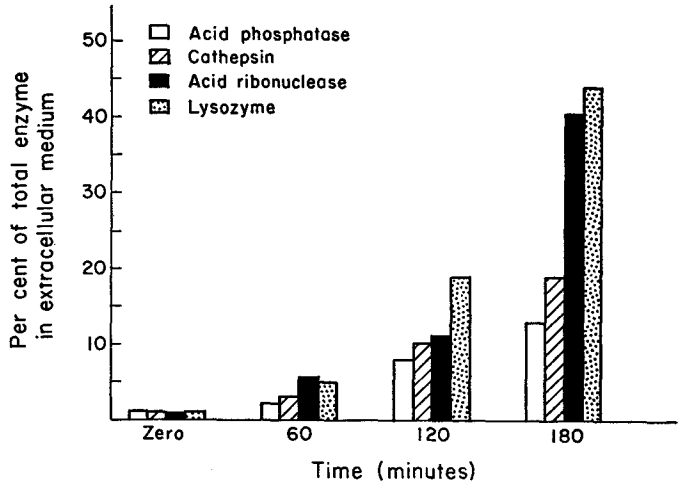
Redistribution of Macrophage Hydrolases Following Particle Ingestion.—Once the conditions for efficient phagocytosis had been determined it was possible to examine the fate of macrophage hydrolases following the phagocytic event. Previous studies (6) employing PMN leucocytes suggested that shortly after ingestion, granule-associated enzymes were liberated within the cell in a soluble form. These events were temporally related to disruption of the granule as visualized on stained smears and under the phase contrast microscope (7, 8).

A similar experiment performed with BCG-induced alveolar macrophages is presented in Text-fig. 1. Immediately after the addition of 15 heat-killed *E. coli*/macrophage (To), 90 to 95 per cent of acid phosphatase, β glucuronidase, cathepsin, and lysozyme were localized in the "15 *g*" pellet which had previously been shown to contain the hydrolase particles. The remainder was in



TEXT-FIG. 1. The influence of phagocytosis on the intracellular distribution and recovery of four particle-bound hydrolases of BCG-induced alveolar macrophages.

the soluble fraction, and only traces of activity were assayable in the extracellular medium. Thirty minutes after the addition of bacteria, when the bulk of the organisms was intracellular, there was a marked change in the localization of intracellular enzyme. This was characterized by a decrease in particle-bound enzyme and a concomitant increase in soluble enzyme. During the next 60 minutes this trend continued but at a much slower rate. After 90 minutes approximately 60 per cent of the total activity was present in the soluble phase. Control preparations, without particles, demonstrated that 90 per cent or more of these enzymes were still particle-bound after 90 minutes



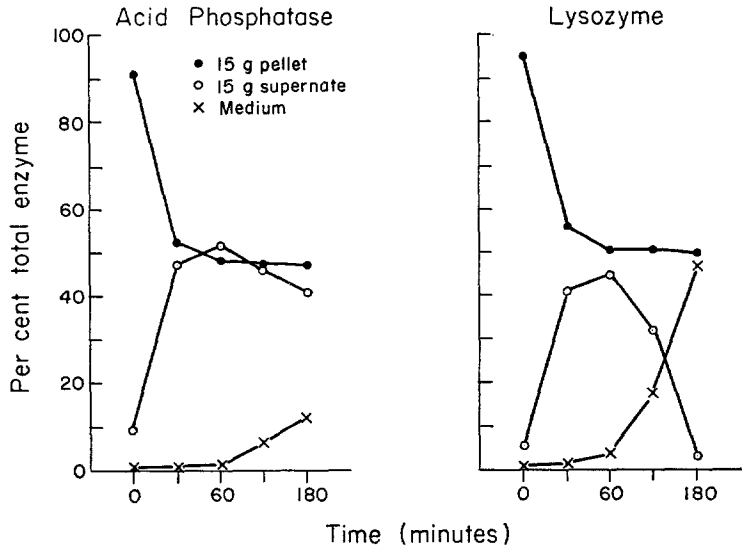
TEXT-FIG. 2. The differential release of acid phosphatase, cathepsin, acid ribonuclease, and lysozyme into the extracellular medium following the ingestion of heat-killed *Escherichia coli*.

of rotation. It was of interest that during the course of these and other experiments the total amount of enzyme in the preparations (sum of the three fractions) remained constant for at least 180 minutes. These findings, of course, depended upon the complete activation of the particulate fraction by freezing and thawing. In addition, each of these enzymes as well as acid ribonuclease, aryl sulfatase, and acid deoxyribonuclease exhibited the same temporal pattern of liberation into the soluble phase.

It was noted that following intracellular solubilization, certain enzymes accumulated in the extracellular medium. Text-fig. 2 illustrates one experiment carried out for 180 minutes in which the percentage of total enzyme in the extracellular medium is plotted. For the 1st hour, even though intracellular redistribution had taken place, less than 5 per cent of the total enzymes had escaped into the medium. During the next 2 hours of incubation large amounts

of both acid ribonuclease and lysozyme were liberated, whereas acid phosphatase and cathepsin remained associated with the cell. At 180 minutes almost half the total cell content of lysozyme and ribonuclease was free in the extracellular medium in a soluble form. Soluble intracellular and extracellular enzyme were fully active and did not demonstrate latency.

Although the nature of this differential liberation is not clear, it is unlikely that it resulted from the total disruption of the cell. First, microscopic observations under phase contrast showed the presence of cells with intact limiting membranes, and second, it would be difficult to explain the cell association of



TEXT-FIG. 3. The temporal relationship between soluble intracellular and extracellular enzyme following phagocytosis.

acid phosphatase and cathepsin under these circumstances. Irrespective of mechanism it seemed clear that the origin of intracellular enzyme was the soluble intracellular pool. Text-fig. 3 illustrates this point with the enzymes acid phosphatase and lysozyme. After the initial intracellular solubilization which accompanied the phagocytic event, the particle-bound complement of enzyme remained constant. Thereafter, the increased activity of the medium fraction was accompanied by a similar loss in soluble enzyme. In the case of lysozyme the soluble intracellular pool fell almost to zero. A similar result was obtained with acid ribonuclease but is not presented.

When alveolar macrophages were incubated with small numbers of heat-killed bacteria (1 to 2 bacteria/macrophage) less striking results occurred. In this case, intracellular soluble enzyme increased 10 to 20 per cent in a temporal sequence similar to that with higher multiplicities. The liberation of enzyme

into the extracellular phase occurred again, but was quantitatively lower than in the previous experiments.

The Morphological Fate of Macrophage Organelles Following Particle Ingestion.—From the previous biochemical results it seemed clear that following the uptake of heat-killed bacteria a large portion of the particle-bound hydrolytic enzymes were liberated within the cell in a soluble form. If this data were analogous to the previous results with polymorphonuclear leucocytes, then disruption of hydrolase-containing particles should occur in proximity to the newly formed phagocytic vacuole. In order to study these events within the macrophage a different technology was required. This was necessitated by: (*a*) the inability of the cell to ingest efficiently on a glass surface and (*b*) the lack of characteristically stainable granules when employing Romanowsky stains. Two techniques were finally employed which depended on separate properties of the hydrolase-containing particles. The first was the histochemical demonstration of acid phosphatase and the second was supravital staining with neutral red. Each of these activities was related to the isolated granules as obtained from macrophage homogenates (1).

The morphology of control and phagocyting alveolar macrophages as demonstrated by the Gomori procedure for acid phosphatase is shown in Fig. 1. Normal cells showed intensely staining granules which varied in size and which corresponded in number and location to the centrosphere region as seen with phase optics and to the neutral red–positive granules. Under the conditions employed, a 10 to 15 minute incubation period was sufficient to give adequate staining. When freshly prepared Gomori substrate was employed, no staining of the nucleus was noted. It should be pointed out that some variations occurred in the staining intensity of different preparations as well as among different cells. The nature of this effect is unknown, but the variations made it mandatory to compare control and phagocyting cells on the same slide.

Cells which had phagocytosed zymosan presented a markedly different appearance depending upon the number of yeast cell walls ingested. Fig. 1 *b* illustrates a typical preparation. Extracellular zymosan particles, which are not illustrated, were never stained by the Gomori procedure, indicating that non-specific adsorption of the reaction product did not occur. In contrast, intracellular zymosan was surrounded by the lead sulfide precipitate which in most cases outlined the particle. In addition, cells which contained zymosan lost their fine particulate cytoplasmic staining. This loss of cytoplasmic staining was usually related to the number of intracellular yeast cell walls and to the size of the macrophage. Fixation of these cells usually took place about 30 minutes after the addition of particles, 15 minutes of which was required for adherence to glass in the settle slide preparations. Examination of the same slides with phase optics showed a marked reduction in granules corresponding to the loss of acid phosphatase–positive structures.

Studies were next conducted using neutral red as the indicator. Normal

alveolar macrophages when exposed to neutral red immediately take up this basic dye and segregate it into granules. The mechanism of the staining reaction is not clear but two possibilities exist. First, that the dye enters by diffusion and is then preferentially bound by acid groups contained within the granules of the centrosphere, and secondly, that it enters through micropinocytotic vacuoles which then fuse with preexisting granules. In either case the macrophage granules demonstrate affinity for this dye, since isolated granules are readily stained, whereas mitochrondria remain unstained.

In the first group of studies macrophages were allowed to ingest zymosan, and small drops of the suspension were placed on slides coated with neutral red. Control cells (Figs. 2 *a* and 2 *b*) exhibited numerous red granules localized to the centrosphere region and the absence of dye in the nucleus as well as in the peripheral portions of the cytoplasm. Extracellular zymosan remained unstained and demonstrated a yellowish-green color when viewed under the light microscope. Macrophages which had ingested zymosan showed brilliant staining of the ingested zymosan and a loss of neutral red–positive granules (Figs. 2 *c* and 2 *d*). Under these conditions, neutral red was taken up by the cell and preferentially localized about the phagocytosed particle. It was of interest that some intracellular zymosan which remained in the peripheral cytoplasm, and not within the centrosphere regions did not stain with neutral red. With the passage of time, neutral red disappeared from about the zymosan leaving a degranulated macrophage containing greenish yeast cell walls.

In the next group of experiments, macrophages were stained supravitally with neutral red, washed, and then allowed to ingest yeast cell walls. Examination of these preparations showed exactly the same morphological appearance. Again, there was disappearance of neutral red–positive granules with localization about the phagocytosed organism. In this instance, an intracellular redistribution of neutral red had taken place with final localization to the newly formed phagocytic vacuole.

In view of the similarities between the degranulation of PMN leucocytes and macrophages analogous studies were performed with rabbit peritoneal exudate PMN leucocytes. Normal leucocytes readily took up neutral red which stained the specific granules. Following phagocytosis by supravitally stained cells, the same redistribution of neutral red was observed, which was in all cases identical to the results with macrophages.

DISCUSSION

Both the biochemical and morphological results are consistent with the concept of granule lysis as a concomitant of the phagocytic event. Following particle ingestion each of the hydrolases studied was liberated in a non-particulate form and recovered in the high speed supernatant fluid from the homogenized cell. This intracellular rearrangement was related temporally to the

kinetics of phagocytosis and quantitatively to the number of particles taken in by the macrophage. These results could be correlated with morphological observations in which there was a loss of both acid phosphatase and neutral red–positive granules from the cytoplasm. Although the histochemical and biochemical data are not strictly comparable, it appears that the granule contents are liberated not into the general cytoplasm but into the vicinity of the phagocytic vacuole. This was suggested by the localization of acid phosphatase and neutral red avid material in the area immediately adjacent to the zymosan. A similar result with acid phosphatase has been described by Essner (9) and the fusion of peroxidase-positive granules with preexisting acid phosphatase–positive structures by Straus (10). Since all phagocyted material is surrounded by a membrane system or phagocytic vacuole it is into this structure that granule contents are probably liberated. This presumably occurs by fusion of the granule with the phagocytic vacuole and subsequent resegregation of the hydrolases. Whether all of the granule hydrolytic enzymes enter the phagosome is not certain, since soluble enzyme in the cytoplasm might not be apparent by the histochemical methods employed. If this mechanism is correct, then one must assume that the newly formed phagosome is ruptured during the homogenization of the cell with the liberation of soluble enzymes. In this regard, it seems probable that the newly formed phagocytic vacuole has properties which are somewhat different from the preexisting granules. This is suggested by the differential leakage of lysozyme and acid ribonuclease into the extracellular phase, a process which could alter not only the specific activity of enzymes in the whole cell but also the relative proportion of hydrolases in the granules and residual bodies.

As mentioned in the preceding article (1) some of the granules of the centrosphere region may represent phagocytic vacuoles remaining from previous endocytic events, whereas others may be "primary lysosomes." In view of the marked degranulation which follows the uptake of large numbers of microorganisms, both types may presumably discharge into the new phagosome. It is therefore not surprising to find the wide variety of morphological types when viewed with the electron microscopy, since the contents of previously formed pinocytic and phagocytic vacuoles may intermingle with freshly ingested material. Such heterogeneity of granules necessitated the use of a large test object; *i.e.*, zymosan, which was clearly distinguished under the light microscope.

The fate of the macrophage following phagocytosis is largely unknown. Certainly, under *in vivo* circumstances the cell survives for long periods as demonstrated in rabbit ear chamber preparations (11). This, however, may depend upon the type of particle, its inherent toxicity, and the number ingested. Although the PMN leucocyte shows no indication of specific granule resynthesis, certain indirect evidence suggests that the macrophage may have

this ability. Suter and Hullinger (12) and Thorbecke *et al.* (13) report that macrophage acid phosphatase may increase under the stimulus of either bacterial endotoxin or tubercle bacilli. Whether this new enzyme is segregated into granules is unknown and requires more definite correlative studies of the ultrastructure and biochemistry of the phagocyting cell under *in vitro* conditions.

Once a foreign substance has been taken into the cytoplasm of the macrophage and segregated within a membrane system, its fate may depend in part upon its susceptibility to macrophage enzymes. Certain inert substances such as carbon are retained at tattoo sites for many years (14), whereas others are probably degraded in short order (4). Such storage of material may be reflected in the accumulation of macrophages at local sites such as tuberculous and lepromatous lesions with the formation of sessile epithelioid cells. In addition to particulate objects the macrophage may also incorporate proteins, including specific antibody, into similar vacuoles by a process of pinocytosis. Such intracellular antibody interacting with soluble or particulate antigens could very well influence their subsequent intracellular disposition.

SUMMARY

The influence of phagocytosis on the morphological and biochemical properties of macrophage hydrolase-containing granules has been studied *in vitro*. Following the uptake of large numbers of heat-killed bacteria, an intracellular rearrangement of hydrolytic enzymes occurred. This was associated with the solubilization of 50 to 60 per cent of the total cell content of acid phosphatase, cathepsin, lysozyme, beta glucuronidase, acid ribonuclease, and acid desoxyribonuclease and with a corresponding decrease in granule-bound enzyme. With more prolonged incubation the majority of the soluble intracellular pool of acid ribonuclease and lysozyme was lost to the extracellular medium. No change in the total content of any of the hydrolases was noted during 180 minutes of incubation *in vitro*.

The morphological fate of the granules was studied by a histochemical method for acid phosphatase. After the phagocytosis of yeast cell walls there was a disappearance of acid phosphatase–positive granules and an accumulation of reaction product about the ingested particle. Experiments employing macrophages which were supravitally stained with neutral red also demonstrated the loss of neutral red–positive granules and the accumulation of the dye about the yeast cell walls.

These results strongly suggest that lysis of macrophage granules occurs following phagocytosis and that a portion of the granule contents are then resegregated within the newly formed phagocytic vacuole.

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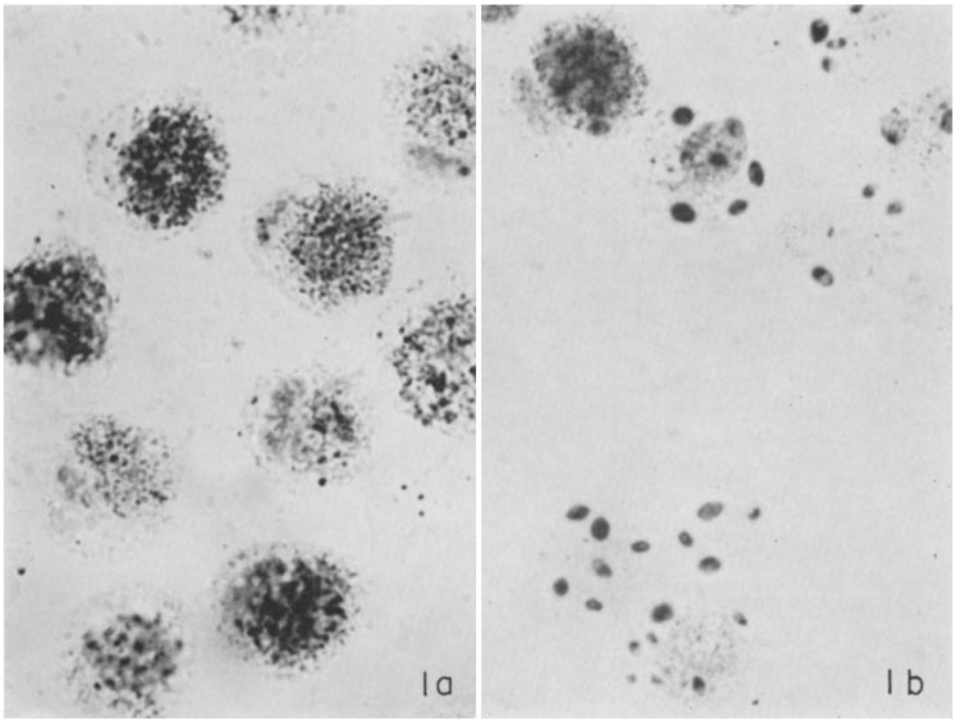
EXPLANATION OF PLATES

PLATE 87

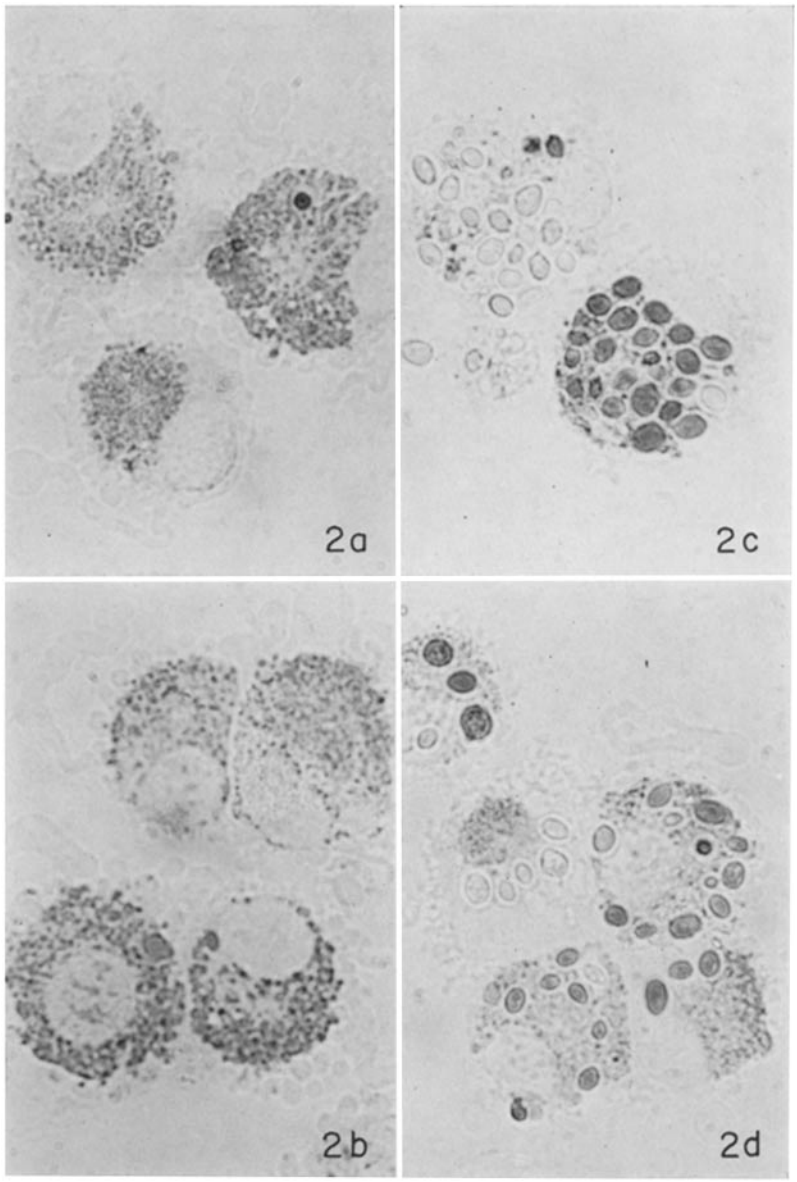
FIGS. 1 *a* and 1 *b*. The morphology of control and phagocytizing BCG-induced alveolar macrophages when stained for acid phosphatase. Fig. 1 *a*. Control cells, illustrating the numerous acid phosphatase–positive granules in the cytoplasm. Variations in size and staining intensity are apparent. Fig. 1 *b* Macrophages which have ingested yeast cell walls 30 minutes prior to fixation. Phagocyting cells demonstrate the loss of particulate cytoplasmic staining and the segregation of enzyme in opposition to the zymosan. A non-phagocyting, granulated cell is seen in the upper left-hand corner. Light microscope. Approximately × 1200.



(Cohn and Wiener: Macrophage hydrolases. II)

PLATE 88

FIGS. 2 *a* to 2 *d*. The morphology of control and phagocyting BCG-induced alveolar macrophages after staining with neutral red. Figs. 2 *a* and 2 *b*. Control, non-phagocyting cells illustrating the large number of stained granules in the perinuclear area. The nucleus remains unstained. Fig. 2 *c*. Two macrophages which have ingested many yeast cell walls and are completely degranulated. The lower cell shows dark zymosan bodies which are surrounded by neutral red. The upper cell exhibited similar staining but with time, lost its content of neutral red leaving a degranulated cell with many yellowish-green zymosan bodies. Fig. 2 *d*. Macrophages containing different numbers of zymosan particles and exhibiting various degrees of degranulation. From a color print, light microscope. Approximately × 1500.



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