EXAMINATION BY ELECTRON MICROSCOPY OF THE INTERACTION BETWEEN PERITONEAL PHAGOCYTES AND CORYNEBACTERIUM OVIS

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PLATES XXVII-XXXI

Various species of laboratory animals exhibit different degrees of resistance to Corynebacterium ovis (C. pseudotuberculosis), the causative agent of caseous lymphadenitis in sheep. Guinea-pigs are highly susceptible to the organism, the disease being characterised by internal abscess formation and progressing invariably to death (Dickinson and Bull, 1931), whereas rabbits are naturally resistant. Mice can be readily infected with C. ovis and, although susceptible to the lethal effect of the exotoxin, resemble sheep in their regular ability to control and eliminate infection (Jolly, 1965a and b). Normal mouse macrophages have been shown by in-vitro and in-vivo techniques to be sensitive to the necrotising action of C. ovis, whereas macrophages from previously infected mice are more resistant (Jolly, 1965b; Hard, 1969a).

C. ovis possesses a toxic surface lipid the existence of which was first postulated on the basis of extraction studies (Bloch, 1950; Carne, Wickham and Kater, 1956). The lipid coat can be recognised as an electron-dense layer surrounding single bacteria or clumps of organisms; it lies exterior to and detached from the cell wall (Hard, 1969b). The lipid appears to play a role in the pathogenesis of the organism and Jolly (1966) suggests that it may provide a protective barrier against the antibacterial mechanisms of the host. In the present study, an experimental model of peritoneal infection (Hard, 1969a) has been used in conjunction with electron microscopy to investigate the interaction between this facultative intracellular parasite and the host phagocytes. The host-parasite relationships have been compared in the mouse, rabbit and guinea-pig.

MATERIALS AND METHODS

Bacteria. Virulent C. ovis strain 137C/2 was used in the phagocytosis experiments. The derivation of this strain, and its preparation as a single-cell suspension, has been described by Jolly (1965a). It was cultured from lyophilised stock for 24 hr aerobically at 37°C on tryptose agar slopes. An attenuated strain, 133A, was used for the preparation of mice resistant to peritoneal infection with C. ovis (Hard, 1969a).

Phagocytosis system. C57BL or Sydney White mice of 18-25 g weight received intraperitoneal injections of graded concentrations of strain 137C/2 suspended in foetal calf serum

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or hyperimmune sheep serum. The latter was obtained from sheep that received subcutaneous injections two to three times weekly of formalised or live suspensions of the 137C/2 strain for 8 wk. At intervals varying from 5 min to 10 days after treatment, mice were killed by cervical dislocation, the skin over the ventral abdomen and thorax was reflected forwards, and 1·5 ml of cold, freshly prepared fixative (see Electron microscopy below) were injected intraperitoneally. After gentle massage, the contents were withdrawn and 0·4 ml transferred to polythene microcentrifuge tubes and held in suspension at 4°C for 1 hr. Centrifugation at 450g for 10 min. produced a well-packed pellet of approximately 1 μl which was suitable for subsequent processing. Some pellets were incubated in tests for acid phosphatase activity by the improved Gomori (1950) technique with omission of the acid rinse.

*C. ovis* was also injected into the peritoneal cavity of 4–6-wk-old New Zealand × Himalaya rabbits and young short-haired guinea-pigs. Injection into unstimulated animals yielded infected peritoneal populations of polymorphonuclear neutrophil leucocytes (PMN) in rabbits and macrophages in guinea-pigs, whereas infected guinea-pig PMN were obtained by administering the bacteria 3 hr after stimulation with intraperitoneal peptone. The peritoneal contents of both species were fixed *in vivo* and harvested in the same way as for the mouse cells. For in-vitro studies, mononuclear cell exudates were evoked in the rabbit and guinea-pig by intraperitoneal injection of peptone, harvested in heparinised Tyrode's nutrient medium 4–5 days later, centrifuged at 300g for 10 min. to remove phagocytes laden with intracytoplasmic peptone and resuspended in nutrient medium with 5 per cent. foetal calf serum in plastic test-tubes. After the addition of bacteria the tubes were incubated with gentle agitation at 37°C in a humidified atmosphere of 5 per cent. CO₂ in air. After further centrifugation, rabbit and guinea-pig cells were resuspended in cold fixative for 1 hr and transferred to microcentrifuge tubes for electronmicroscopic preparation.

**Electron microscopy.** Cell pellets were fixed for 1 hr in a mixture of 5·6 per cent. glutaraldehyde and 0·9 per cent. osmium tetroxide in 0·2M S-collidine buffer, pH 7·4, at 4°C (Trump and Bulger, 1966). Samples for histochemical localisation were fixed in 1·3 per cent. glutaraldehyde. The technique for manipulating the pellets in microcentrifuge tubes for uranyl acetate staining and subsequent preparation to the embedding stage has been previously described (Hard, 1969b). Sections cut on an LKB ultratome were stained on the grid with lead citrate and examined in a Phillips EM200 electron microscope.

**RESULTS**

In parallel with the material processed for conventional electron microscopy, representative samples of peritoneal cells were prepared for cytochemical localisation of acid phosphatase and this facilitated the initial identification of lysosomal hydrolase-containing structures in the phagocytes. The ultrastructural appearance of *C. ovis* believed to represent the living organism is described in a previous report (Hard, 1969b) and the assumed correlation was further monitored by a companion study on the kinetics of intraperitoneal survival of *C. ovis* (Hard, 1969a).

**Studies with normal mouse phagocytes**

Five minutes after intraperitoneal injection into a mouse, bacteria are found about to be engulfed by macrophage pseudopods, and some are already within phagosomes and within phagolysosomes (figs. 1 and 2). Bacteria within vacuoles at or near the periphery of phagocytes are surrounded by a wide, electron-lucent space. These vacuoles were interpreted as phagosomes, representing the newly ingested organism together with a quantity of extracellular medium before the contribution of lysosomal hydrolytic enzymes. At
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this stage, the surface lipid of C. ovis is identifiable as an electron-dense layer outside the bacterial cell wall. Organisms found deeper within the macrophage cytoplasm are associated with vacuoles in which the limiting membrane is more tightly apposed to the ingested organism leaving only a relatively narrow, intervening peribacillary space. This space is filled with a dense, finely granular, acid phosphatase-positive material identical in appearance to the matrix of lysosomes adjacent to the phagocytic vacuoles. In some cells a continuity between the limiting membranes of these granules and vacuoles or discontinuity in juxtaposed membranes of the two structures is seen (figs. 3 and 4). At this stage, it is difficult to visualise the electron-dense layer of surface lipid surrounding C. ovis. However, a distinct, clear band of similar width to that which characteristically separates lipid from bacterial cell wall separates the dense peribacillary material from the organism. The presence of this halo implies that the lipid layer is intact, though the latter may be largely obscured by discharged lysosomal contents of similar electron density. The limiting membranes of phagolysosomes possess a crenate outline. In samples examined at 10 and 15 min. after injection of C. ovis, macrophages possess few intact lysosomes. In these early stages the cytoplasm immediately surrounding phagolysosomes is sometimes devoid of structure but for floccular or granular material. Similar structureless cytoplasm is found occasionally in pseudopods near ingested bacteria (fig. 1).

From 5 min. after the intraperitoneal challenge, mouse macrophages containing a number of bacteria are observed in various stages of degeneration in samples of peritoneal exudate. The early stages are characterised by dilatation of both the nuclear envelope and the cisternae of the granular endoplasmic reticulum (figs. 5 and 6). As normal organelles disappear, the cleared cytoplasm contains dilated vesicles, residual membranes studded with osmiophilic particles, floccular material and sparse microfilaments. Mitochondria and intact lysosomes retain a more or less unaltered appearance until a late stage in the degenerative process. The nucleus becomes homogeneous and coarsely granular, but sometimes there are large oval densities at the periphery. Within these cells, phagolysosomes containing viable bacteria are often surrounded by layers of myelin-like membranes (fig. 6). In the late degenerative stage the plasma membrane is ruptured, and altered mitochondria, granule-studded vesicles, vacuoles and membrane fragments, together with bacteria, are often released into the extracellular space. Throughout this process, all bacteria retain a morphological appearance consistent with viability, the surface lipid remaining intact and separated from the cell wall by the characteristic clear band. Necrotic phagocytes are frequently observed to be undergoing ingestion by scavenging macrophages. After that event, bacteria originally contained within the dead phagocyte can no longer be recognised.

Extensive macrophage degeneration is observed between 1 and 6 hr and even cells containing only a few bacteria display incipient signs of necrosis. Organisms within non-degenerate phagocytes are closely surrounded by a crenate, phagolysosome membrane. Dense amorphous material invariably occupies the narrow, peribacillary space and is always separated from the
bacterial cell wall by an intact lipid layer. By 5½ hr, PMN migrating into the peritoneal cavity are relatively abundant and many contain ingested bacteria. The response of the organisms to the ingestive process of these phagocytes presents the same appearance as described in the normal mouse macrophage. PMN seem to be as susceptible to the necrotising action of C. ovis and like the affected macrophages are ingested when degenerate by scavenging mononuclear cells.

Degenerate macrophages and PMN are less frequent at 20 hr but recovery is evident by 3-4 days. However, intracellular organisms were observed up to the end of the present study at 10 days and all retained an appearance consistent with viability. They were always found within the tight variety of phagolysosome with crenate membrane and narrow, amorphous peribacillary space. The clear zone between dense, peribacillary material and bacterial cell wall remains entire, indicating that the surface lipid layer is intact (figs. 7 and 8). Persistent C. ovis organisms tend to be elongate in form (fig. 8) and some display division septa.

Studies with immune mouse macrophages

When virulent C. ovis is injected into mice previously immunised with the attenuated strain 133A, several differences are noted. Because of the greater numbers of lysosomes within immune macrophages (Hard, 1969c), coalescence of these organelles with phagosomes is more frequently seen. In addition, the amorphous material within the peribacillary space is greater in quantity than in normal cells. The lysosomes of immune macrophages characteristically contain oval, electron-lucent zones that are probably artefacts, but appear to remain intact for a period after their discharge into the phagolysosome (fig. 4). Cell necrosis is less frequent than in the non-immune peritoneal cavity, but it occurs in immune macrophages that ingest many organisms. Regardless of the stage of examination and the increased amount of acid phosphatase-positive, peribacillary dense material, ingested bacteria retain an appearance of viability with an intact lipid layer and clear band, and are associated with phagolysosomes of the tight variety. By 24 hr, degenerate macrophages are rare and organisms are extremely difficult to find. No bacteria are seen at 48 hr and thereafter.

Studies with mixtures of mouse phagocytes and immune serum

When C. ovis is suspended in hyperimmune sheep serum before injection, changes associated with bacterial death are observed after phagocytosis by normal and immune mouse macrophages and by PMN. Degenerating serum-treated organisms are particularly frequent within immune macrophages at 5½ hr (fig. 9). Altered bacteria are usually less dense and there is a contraction of the internal contents away from the cell wall which is often distorted. The dense lysosomal material occupying the peribacillary space in these instances is confluent with the bacterial cell wall and the characteristic intervening halo wholly or partly obliterated. It thus appears that some removal of the surface lipid occurs in bacteria undergoing degeneration. The degradation of
organisms is often associated with a widening and decrease in density of the peribacillary space, so that the phagolysosome membrane is no longer tightly invested upon the ingested organisms; nor is it so scalloped in outline.

**Studies with rabbit and guinea-pig phagocytes**

Phagocytosis of *C. ovis* by rabbit and guinea-pig phagocytes follows the same course as in the mouse. In the unstimulated rabbit peritoneal cavity there is rapid engulfment and destruction of bacteria by the resident PMN. By 2 hr, most ingested bacteria are obviously degenerate and found within large phagolysosomes surrounded by a wide peribacillary space containing clumps of amorphous material. In most instances, neither the bacterial surface lipid layer nor the intervening clear halo is visible, the dense lysosomal material being adherent to the vastly altered or disrupted cell wall. The bacterial cytoplasm is vacuolated or structureless and often contracted away from the distorted cell-wall remnants. The limiting membranes of phagolysosomes containing degenerate organisms are not crenate. Guinea-pig PMN degrade virulent *C. ovis* also, but less strikingly than rabbit PMN; viable bacteria are found within these guinea-pig phagocytes at 2 hr.

In rabbit and guinea-pig macrophages the organisms first become associated with phagolysosomes of the tight, crenate variety. In some cases the clear zone between bacterial surface lipid and cell wall is obliterated and the amorphous material is in contact with the bacterial cell surface. Under these circumstances, the organisms often display early signs of degeneration (fig. 10). By 3 hr, altered bacteria are found in some phagolysosomes in which the peribacillary space is wide. Such organisms display a focal or complete loss of surface lipid. Degeneration of the host cell is observed only in cases where very large numbers of *C. ovis* have been ingested. Bacteria contained in the subsequent cell debris usually are degenerate.

**DISCUSSION**

The ultrastructural findings support the evidence of in-vivo and cell-culture studies that mouse macrophages are susceptible to the necrotising action of *C. ovis*. Following peritoneal infection with virulent *C. ovis* in susceptible unsensitised mice, viable bacteria can be cultured from the abdominal cavity in numbers approaching the infecting dose for at least 7 days. In contrast, the mouse immunised by repeated injections of an attenuated strain is able to exert an enhanced bactericidal action on virulent challenge organisms, for the peritoneal cavity becomes sterile within 3 days, there being first a rapid fall in numbers to 1 per cent. of the challenge inoculum by 24 hr (Hard, 1969a). Hence viable bacteria were detectable by electron microscopy within macrophages of normal mice for up to 10 days, but rarely after 24 hr in immune mice. Although clearance from the immune mouse is rapid, electron-microscopic examination indicated that the individual immune macrophage, despite its increased lysosomal complement of hydrolytic enzymes (Hard, 1969c and 1970), was only a little more capable of killing *C. ovis* than was the normal
macrophage. In both groups of mice it appeared that bacterial death might occur commonly upon the reingestion by scavenging macrophages of the organisms inside degenerate phagocytes. The increased hydrolytic activity of immune macrophages possibly facilitates a more rapid clearance of organisms in concert with the unfavourable environment of degradation products from the degenerating phagocyte. Furthermore, in-vitro dye-exclusion studies have shown that immune mouse cells are almost twice as resistant to the cytotoxic effect of \textit{C. ovis} as are normal cells (Hard, unpublished observations) and the electron-microscopic findings supported this.

Once the virulent organisms were coated with hyperimmune serum they became visually susceptible to the lytic action of individual macrophages from both normal and sensitised mice. This is consistent with the observation that bacterial clearance is more rapid when organisms suspended in immune serum are injected into the peritoneal cavity of normal mice although such treatment does not necessarily alter the ultimate incidence of abscess formation (Hard, unpublished observations).

Ingestion of \textit{C. ovis} by phagocytes, the fusion of lysosomes and vesicles with phagolysosomal membranes, and extrusion of enzymic contents into the peribacillary space, followed the same course as has been described in preceding studies with various bacteria (North and Mackaness, 1963\textit{a} and \textit{b}; Lockwood and Allison, 1964; Zucker-Franklin and Hirsch, 1964; Dumont and Sheldon, 1965; Gordon, Miller and Bensch, 1965). As concluded by previous authors, the coalescence of lysosomes and vesicles with the limiting membrane of the phagocytic vacuoles appeared to result in the characteristic, scalloped outline of these structures. The presence of altered, structureless cytoplasm immediately surrounding the phagocytic vacuole shortly after its formation has also been recorded after ingestion of \textit{Staphylococcus aureus} by Kupffer cells (Horn \textit{et al.}, 1969), of \textit{Listeria monocytogenes} (North and Mackaness, 1963\textit{a}) and at the base of the food cup in phagocytising amoebae (Christiansen and Marshall, 1965). In the latter case it has been suggested that this cytoplasmic alteration is associated with the interiorisation of the engulfed vacuoles and this may be correlated with the occasional observation in the present study of macrophage pseudopods composed of similar cytoplasm (fig. 1). The tight phagolysosome, i.e., a crenate vacuole with a relatively narrow peribacillary space occupied by hydrolytic enzymes, was typically associated with viable \textit{C. ovis} organisms, even 10 days after infection. However, when degenerate \textit{C. ovis} organisms were observed, they were often found in more spacious, clearer phagolysosomes, the limiting membrane of which was usually smooth in outline. This may suggest that once digestion of the phagocytosed material has begun, lysosomal bombardment of the vacuole is inhibited. Leake and Myrvik (1966), tracing the host-parasite interaction with \textit{Mycobacterium smegmatis} in rabbit cells, also observed that intracellularly degraded bacteria in PMN were associated with spacious phagolysosomes, whereas intact bacteria relatively resistant to alveolar macrophages were found within phagolysosomes of the tight variety in the latter cells. This may be a common feature of the host-parasite relationship, because somewhat similar observations have been recorded after phagocytosis of
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Staphylococcus (Brewer, 1963), M. lepraemurium (Allen, Brieger and Rees, 1965) and latex beads (Korn and Weisman, 1967).

Bacteria that after ingestion by mouse, rabbit and guinea-pig phagocytes retained an ultrastructural appearance consistent with viability were characterised by a halo-like, electron-lucent band immediately outside the bacterial cell wall. This appearance implied the presence of a still intact surface lipid layer which itself was probably obscured by equivalent density and continuity with the released, peribacillary lysosomal contents. However, bacteria showing degenerative changes also displayed focal or complete disappearance of the clear halo and thus loss of the surface lipid layer, as was seen in mouse cells following suspension of the organisms in immune serum or engulfment in rabbit and guinea-pig phagocytes. The consistency of these observations permits the suggestion that C. ovis lipid is of some importance in protecting the organism mechanically from the hydrolytic enzymes of the host, at least in the mouse, and possibly, by analogy, in the natural host, the sheep. Evidence is accumulating that surface lipid may constitute a defence mechanism for other organisms. When M. lepraemurium, for instance, occurs within the phagocytic vacuoles of mouse cells, there is an electron-lucent zone around the organism (Yamamoto et al., 1958; Chapman, Hanks and Wallace, 1959; Allen et al.). This zone, described as a “spacer” separating and protecting the organism from the host’s hydrolytic enzymes (Brown, Draper and Hart, 1969; Brown and Draper, 1970), has been linked with the presence of wax Ds (Draper and Rees, 1970), which is a chloroform-extractable, ether-soluble, acetone-insoluble lipopolysaccharide containing mycolic acid present in mycobacterial cell walls.

 Whereas mouse macrophages were very susceptible to the cytotoxic effect of C. ovis, rabbit and guinea-pig macrophages were able to destroy the organism and to tolerate a high individual infection rate. This difference in ability to exert a bactericidal action on C. ovis may imply an absence in the mouse and presence in rabbit and guinea-pig of enzymes able to remove the surface lipid layer of the organism. The susceptibility of mice to an abscess-forming disease can be explained by the inefficiency of mouse phagocytes in dealing with C. ovis. Similarly, the natural resistance of rabbits to a C. ovis-initiated disease is doubtless due to the rapid destructive action of the host’s PMN on the organism together with the slower effect of the macrophages. The state in the guinea-pig is less clear, for members of this species may succumb rapidly to the pyogenic effect of the organism. Although guinea-pig PMN did not possess the striking lytic properties of rabbit PMN, both guinea-pig PMN and macrophages appeared capable of destroying C. ovis with an efficiency similar to that of rabbit macrophages.

SUMMARY

The host-parasite interactions between (i) macrophages and polymorphonuclear neutrophil leucocytes (PMN) of normal and immune mice, rabbits and guinea-pigs, and (ii) the facultative intracellular parasite, Corynebacterium ovis, were examined with the electron-microscope. Normal mouse cells were incapable of exerting a significant bactericidal effect and quickly succumbed to the
necrotising action of the organism. Macrophages from immune mice were individually no more effective in killing *C. ovis*, but the organisms disappeared from the peritoneal cavities more rapidly in immune than in normal mice. Bacterial clearance appeared to be associated with the reingestion by scavenging macrophages of viable *C. ovis* within degenerating phagocytes. Suspending *C. ovis* cells in hyperimmune sheep serum rendered them susceptible to bacteriolysis by normal or immune mouse cells. Rabbit PMN exerted an extremely rapid destructive action on the organism; rabbit macrophages and guinea-pig PMN and macrophages also destroyed *C. ovis* but less effectively. In studies with all three animal species, viable organisms were found within phagolysosomes which were characterised by a relatively narrow peribacillary space and crenate limiting membrane. An electron-lucent zone always surrounded viable bacteria, and this was assumed to indicate the intact state of the surface lipid layer which was of similar density to the contiguous host enzymic material. Bacteria undergoing degeneration were often found within vacuoles with a wide peribacillary space and smooth limiting membrane; the surface lipid zone was wholly or partly obliterated in these organisms. It appears that *C. ovis* surface lipid may function as a barrier against the degradative enzymes of mouse phagocytes.

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REFERENCES


Fig. 1.—Mouse macrophage 5 min. after intraperitoneal injection of *C. ovis*. Three bacteria are located within a phagolysosome which already contains dense material in the peribacillary space. The material is separated from the microbial bodies by a conspicuous clear space that represents the gap between the surface lipid and cell wall of *C. ovis*. Note the macrophage pseudopod (Ps) which contains structureless cytoplasm. Some phagolysosomes shortly after phagocytosis were surrounded by an area of similar cytoplasm. Electron micrograph (EM). ×18,600. (The bar in this and subsequent illustrations represents 0.5 μm.)

Fig. 2.—Mouse macrophage 5 min. after infection. The phagocyte contains eight bacteria in this section, each in separate phagolysosomes. The density and structure of the organisms is compatible with viability and a clear zone, representing an intact surface lipid layer, surrounds each bacterium. Two bacteria are in division. A large membrane-bound granule (arrow) is in continuity with one of the phagolysosomes. The macrophage shows no evidence of injury at this stage. EM. ×8400.
FIG. 3.—A dividing cell of *C. ovis* within a mouse macrophage phagolysosome at 15 min. The limiting membrane of the vacuole is characteristically scalloped (crenate) and numerous vesicles surround it. In one area (arrow) in close juxtaposition to a lysosome (L), this membrane is broken. The discontinuity does not appear to be due to oblique sectioning. Within the phagolysosome, the narrow peribacillary space contains dense amorphous material similar to that within the lysosome. The peribacillary contents are separated from the organism by a clear zone and the surface lipid layer, which is of similar density, remains intact. EM. \( \times \) 61,500.

FIG. 4.—*C. ovis* within an immune mouse macrophage at 5 min. The crenate membrane-bound phagolysosome contains more dense material within the peribacillary space than in normal cells but the organism is nevertheless surrounded by an intact lipid layer and clear band. The phagolysosome membrane is discontinuous where it is in close apposition to a lysosome (L). Oval clear areas similar to those that often characterise immune macrophage lysosomes are present within the peribacillary space. EM. \( \times \) 49,000.
Fig. 5.—A degenerate mouse macrophage containing only two organisms (B) in this section at 15 min. The cleared cytoplasm has lost much of its structure and contains dilated membranous profiles. The nuclear contents are homogeneously granular and the nuclear envelope (NE) is grossly dilated at one point. EM. $\times 15,000$.

Fig. 6.—Part of a degenerating mouse macrophage at 15 min. Four viable bacteria are contained within separate phagolysosomes. The cytoplasm contains dilated endoplasmic reticulum (arrows) and the nuclear envelope (NE) is grossly dilated. One organism (B) is surrounded by myelin-like membranes. The surface lipid of all of the bacteria remains intact. EM. $\times 24,900$. 
Fig. 7.—A viable cell of *C. ovis* persisting within a mouse macrophage at 24 hr. The surface lipid layer remains intact. The peribacillary space is exceedingly narrow and contains very little dense material; this suggests that the lysosomal complement of the macrophage is depleted. EM. \( \times 30,000 \).

Fig. 8.—A mouse polymorphonuclear neutrophil at 20 hr, contains a viable cell of *C. ovis* that retains an intact surface lipid layer. The bacillary form is elongate. The peribacillary space is relatively narrow and contains dense material, and the phagolysosome membrane is crenate. EM. \( \times 24,900 \).
FIG. 9.—An immune mouse macrophage 5½ hr after injection of *C. ovis* suspended in immune serum. The clear zone around the bacterial cell wall is absent and this indicates disappearance of the surface lipid layer. The bacterial cell wall is altered and the arrowed organism has lost its density and has a distorted form indicative of degradation. The phagolysosome membranes are less crenate than those surrounding viable bacteria. EM. ×28,300.

FIG. 10.—Guinea-pig macrophage at 1 hr. A phagolysosome (arrowed) with a wide peribacillary space contains a degenerating organism. The bacterial surface lipid and clear band are absent and phagolysosome contents are in contact with the cell wall. The bacterial cytoplasm, which is contracted away from the cell wall, is of decreased density. EM. ×49,000.
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