Characterization of the *Mycobacterium tuberculosis* Phagosome and Evidence that Phagosomal Maturation Is Inhibited

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Summary

We have used the cryosection immunogold technique to study the composition of the *Mycobacterium tuberculosis* phagosome. We have used quantitative immunogold staining to determine the distribution of several known markers of the endosomal-lysosomal pathway in human monocytes after ingestion of either *M. tuberculosis, Legionella pneumophila*, or polystyrene beads. Compared with the other phagocytic particles studied, the *M. tuberculosis* phagosome exhibits delayed clearance of major histocompatibility complex (MHC) class I molecules, relatively intense staining for MHC class II molecules and the endosomal marker transferrin receptor, and relatively weak staining for the lysosomal membrane glycoproteins, CD63, LAMP-1, and LAMP-2 and the lysosomal acid protease, cathepsin D. In contrast to *M. tuberculosis*, the *L. pneumophila* phagosome rapidly clears MHC class I molecules and excludes all endosomal-lysosomal markers studied. In contrast to both live *M. tuberculosis* and *L. pneumophila* phagosomes, phagosomes containing either polystyrene beads or heat-killed *M. tuberculosis* stain intensely for lysosomal membrane glycoproteins and cathepsin D. These findings suggest that (*a*) *M. tuberculosis* retards the maturation of its phagosome along the endosomal-lysosomal pathway and resides in a compartment with endosomal, as opposed to lysosomal, characteristics; and (*b*) the intraphagosomal pathway, i.e., the pathway followed by several intracellular parasites that inhibit phagosome-lysosome fusion, is heterogeneous.

Tuberculosis is a global health problem of escalating proportions. Each year, approximately 8 million new cases of pulmonary tuberculosis occur worldwide and 3 million people die of this disease. The incidence of tuberculosis in the United States, which had been declining since the turn of the century, has been rising since 1985, due in large part to the AIDS epidemic (1, 2). Compounding this public health dilemma, multi-drug resistance strains of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, have emerged. New strategies for the prevention and treatment of tuberculosis are urgently needed. This requires a vastly improved understanding of the fundamental interactions of *M. tuberculosis* with host cells.

M. tuberculosis is an intracellular pathogen of human mononuclear phagocytes. Inside these host cells, the organism resides in a membrane-bound phagosomal compartment that resists fusion with lysosomes (3) and is only mildly acidified (4). Beyond this, relatively little is known about the composition of the *M. tuberculosis* phagosome or the interaction of the phagosome with other organelles of the host cell. In this study, we have used the cryosection immunogold technique to characterize the *M. tuberculosis* phagosome and its interaction with the host cell, focusing on markers of the endosomal-lysosomal pathway. We have compared the *M. tuberculosis* phagosome with phagosomes containing an inert particle, polystyrene beads, and phagosomes containing the intracellular bacterial pathogen *Legionella pneumophila*, which has many features in common with *M. tuberculosis*. We shall show that the *M. tuberculosis* phagosome is unique in its composition, differing both from polystyrene bead phagosomes, which fuse with lysosomes, and from *L. pneumophila* phagosomes, which do not fuse with lysosomes. Specifically, *M. tuberculosis* phagosomes, but not phagosomes containing polystyrene beads or *L. pneumophila*, show prolonged staining for the plasma membrane marker β_2-microglobulin, and acquire relatively large amounts of HLA-DR and the endosomal marker transferrin receptor. We shall propose that *M. tuberculosis* retards the maturation of its phagosome along the endolysosomal pathway and resides in a compartment with endosomal, as opposed to lysosomal, characteristics.

Materials and Methods

Reagents. Mouse mAbs anti-CD63 (IgG1), antitransferrin receptor (IgG1), anti-MHC class I heavy chain (IgG1, clone IOT2), and anti-HLA-DR (IgG2b, clone BL2) were purchased from AMAC, Inc. (Westbrook, ME), and anti-β_2-microglobulin (IgG1, clone BM-63) from Sigma Chemical Co. (St. Louis, MO). Mouse mAb to LAMP-1 (H3B3, IgG1) and LAMP-2 (H4B4, IgG1) were obtained from the Hybridoma Bank of the University of Iowa (Iowa City, IA). Mouse mAb to human cathepsin D (1C11, IgG1) was

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obtained from Triton Diagnostics (Alameda, CA). Isotypic mouse myeloma control proteins were obtained from Cappel Organon-Technica (West Chester, PA). Goat anti-mouse IgG gold conjugate (8 nm) was purchased from Sigma Chemical Co. Goat anti-rabbit IgG gold conjugate (15 nm) was purchased from Amersham Corp. (Arlington Heights, IL). Antibodies to lipoarabinomannan (LAM)[1] were prepared by immunizing rabbits subcutaneously with 40 μg of purified LAM in IFA three times at 2-wk intervals. LAM was a gift from Dr. Patrick Brennan (Colorado State University, Fort Collins, CO). Ficoll-Hypaque was purchased from Pharmacia (Piscataway, NJ); glutaraldehyde from Polysciences, Inc. (Warrington, PA); polyvinyl alcohol, polyvinylpyrolodone, and paraformaldehyde from Sigma Chemical Co.; RPMI and Dulbecco's PBS from GIBCO-BRL (Gaithersburg, MD); and recombinant human IFN-γ from Upstate Biotechnology, Inc. (Lake Placid, NY).

BSA-colloidal gold of homogeneous size distribution (20–25 nm) was prepared by the method of Frens (5).

Bacteria. *M. tuberculosis* Erdman strain (ATCC 35801), a highly virulent strain, was obtained from the American Type Culture Collection (Rockville, MD). The organism was passaged through guinea pig lung to maintain virulence as described previously (6). Briefly, guinea pigs were infected by exposure to aerosolized *M. tuberculosis*, and killed after 4 mo. The heavily infected lungs were then removed aseptically and homogenized in 10 ml 7H9 containing 2% BSA (Sigma Chemical Co.) with a mortar and pestle and 90-mesh Norton Alundrum (Fisher Scientific Co., Pittsburgh, PA). Aliquots of the lung homogenate were flash frozen at −70°C in cryovials (Nunc, Roskilde, Denmark). Before an experiment, a vial of guinea pig lung homogenate was rapidly thawed at 37°C, and the bacteria were cultured on 7H11 agar at 37°C, 5% CO_2, 100% humidity. 7 d later, bacteria were scraped from several plates into 10 ml of RPMI containing 15% human serum. A suspension containing predominantly single bacilli was prepared by sonicating the bacteria in a water bath (model 9; Astrason, Plainview, NY) for 60 s, sedimenting any remaining clumps of organisms by centrifugation at 20 *g* for 10 min, and removing a 1–2-ml aliquot of the single bacillus suspension at the top of the tube. The concentration of organisms was determined by measurement of optical density at 540 nm and by counting in a Petroff-Hauser chamber. Viability of the organisms was determined by plating serial dilutions of the infecting inoculum on 7H11 agar.

L. pneumophila, Philadelphia 1 strain, was grown in embryonated hens' eggs, harvested, tested for viability and contaminants, and stored at −70°C, as described (7). The egg yolk grown *L. pneumophila* were cultured one time only on charcoal yeast extract agar, harvested after 4 d of growth, and used immediately.

Preparation of Monocytes and Labeling of Lysosomes. Human mononuclear cells were isolated from fresh heparinized blood of purified protein derivative-negative healthy donors using Ficoll-Hypaque density gradients. Mononuclear cells (3 × 10^6/ml) were plated in 75 cm^2 tissue culture flasks (12 ml/flask; Falcon; Becton Dickinson, Lincoln Park, NJ) in RPMI containing 15% autologous human serum. Monocytes were allowed to adhere to the plastic for 90 min at 37°C in 5% CO_2- 95% air, washed three times with RPMI, and incubated in RPMI containing 15% autologous serum for 5 d. In experiments examining the distribution of MHC class II molecules, monocytes were incubated with IFN-γ (50 U/ml) to increase the expression of MHC class II molecules and hence to facilitate the study of their distribution.

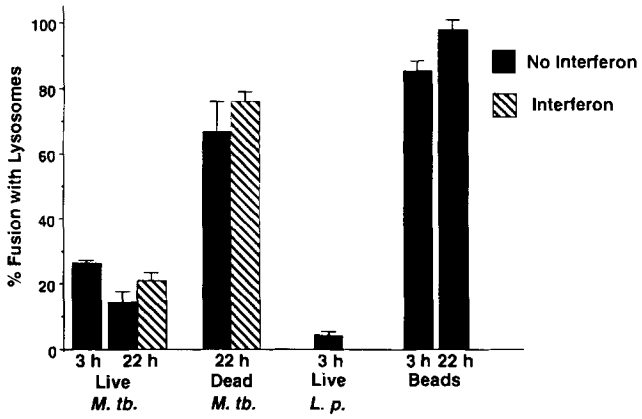

Figure 1. Extent of fusion of *M. tuberculosis*, *L. pneumophila*, and polystyrene bead phagosomes with BSA-gold labeled secondary lysosomes. Human monocytes in monolayer culture for 5 d were first incubated with BSA-gold to label their lysosomal compartments and then incubated with *M. tuberculosis*, *L. pneumophila*, or polystyrene beads. Monocytes were fixed after 3 or 22 h and examined by electron microscopy. For each condition, the data shown represent the mean ± SD of counts from at least 20 cells on each of at least two electron microscopy grids.

Before monocytes were incubated with *M. tuberculosis*, *L. pneumophila*, or polystyrene beads, monocyte lysosomes were labeled with BSA conjugated to colloidal gold (BSA-gold) (8). Monocytes in monolayer culture were incubated with BSA-gold (20–25 nm, diluted to a final OD_{520} of 0.5) in RPMI containing 15% autologous serum for 8–12 h at 37°C, washed four times with RPMI, and incubated overnight at 37°C in RPMI containing 15% autologous serum.

Infection of Monocytes and Localization of Antigens by Immunogold Staining of Cryosections. Monocytes in monolayer culture were infected by incubating them with *M. tuberculosis* (1 × 10^6/ml to 2 × 10^7/ml) or *L. pneumophila* (2 × 10^8/ml) in RPMI containing 15% fresh autologous human serum for 90 min at 37°C. As additional controls, monocytes were also incubated with heat-killed *M. tuberculosis* or polystyrene beads. *M. tuberculosis* were heat killed by boiling in RPMI for 10 min. Polystyrene beads (1 μm spheres, 2% solids; Polysciences, Inc.) were diluted 1:1,000 in suspensions of *M. tuberculosis* or *L. pneumophila*, and monocytes were then coincubated with polystyrene beads and one of these two bacteria. Infected mono-

[1] *Abbreviations used in this paper:* LAM, lipoarabinomannan; NGS, normal goat serum.

Figure 2. Cryosection immunogold staining for CD63 molecules in monocytes infected with *L. pneumophila* (*A*) or *M. tuberculosis* (*B*). Human monocytes were cultured for 5 d and labeled with BSA-gold. The monocytes were then incubated with either *L. pneumophila* and polystyrene beads or *M. tuberculosis* and polystyrene beads. The monocytes were fixed after 3 h (*L. pneumophila*, *A*) or 1 d (*M. tuberculosis*, *B*) and cryosections were stained for CD63 by immunogold. In these representative micrographs, the polystyrene bead (*B*) phagosome contains abundant CD63 immunogold (*arrowheads*) and BSA-gold (*arrows*) in both panels. In contrast, the *L. pneumophila* (*Lp*) phagosome (*A*) contains negligible amounts of BSA-gold and had minimal staining for CD63. CD63 is absent from one *M. tuberculosis* (*Mt*) phagosome and present at an intermediate level on a second phagosome (*B*). Neither *M. tuberculosis* phagosome contains BSA-gold. (*A*) ×35,200; (*B*) ×36,200.

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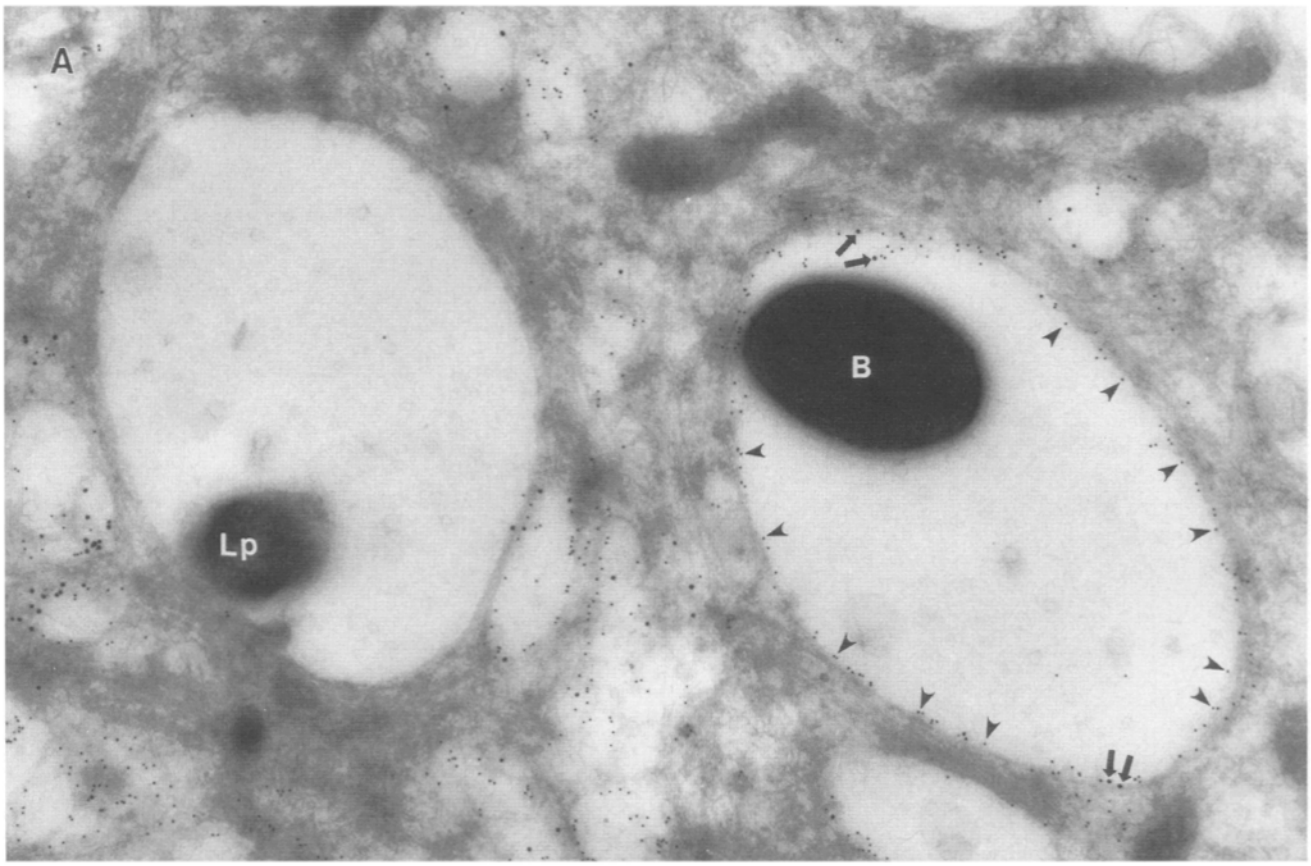

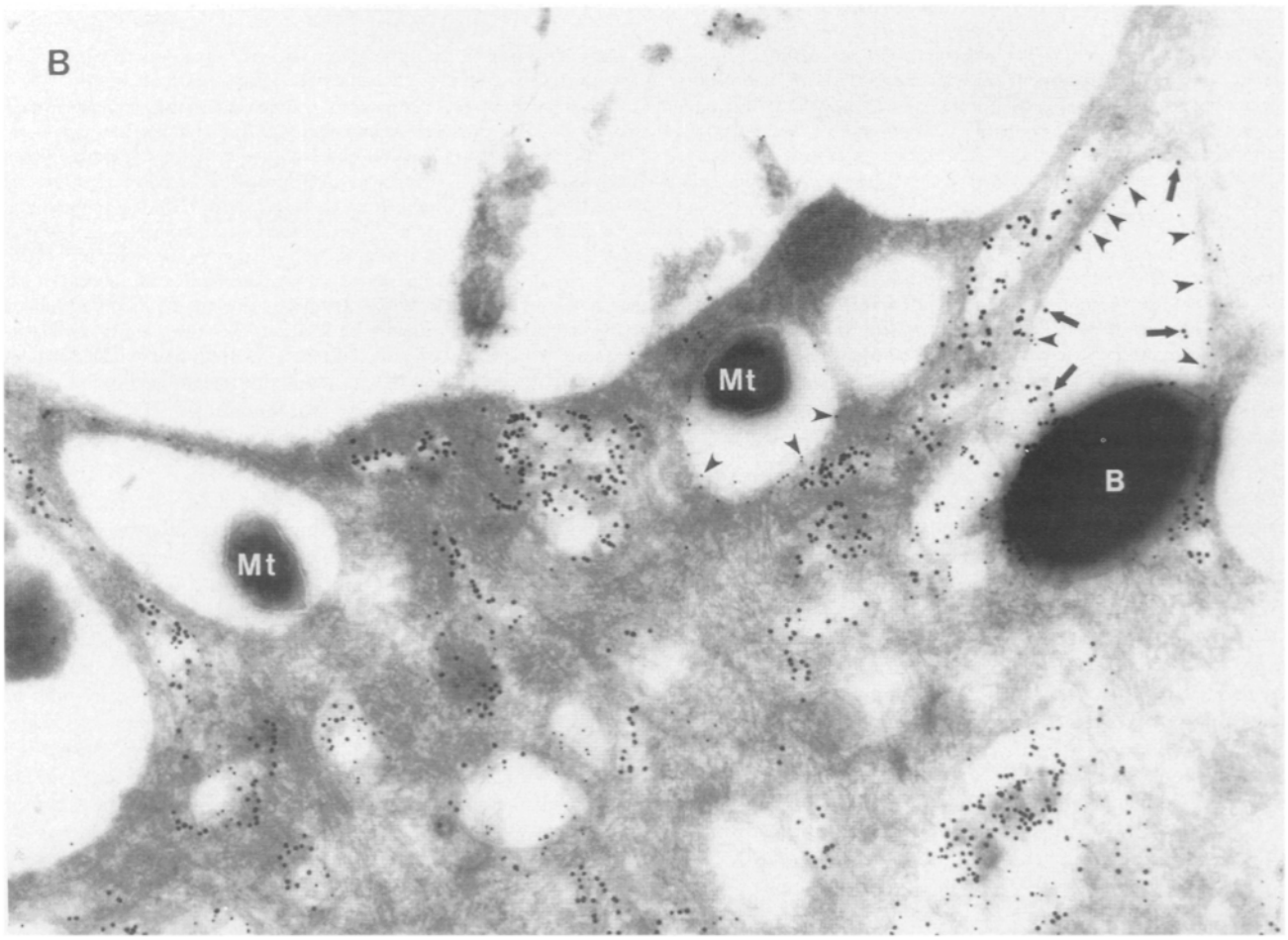

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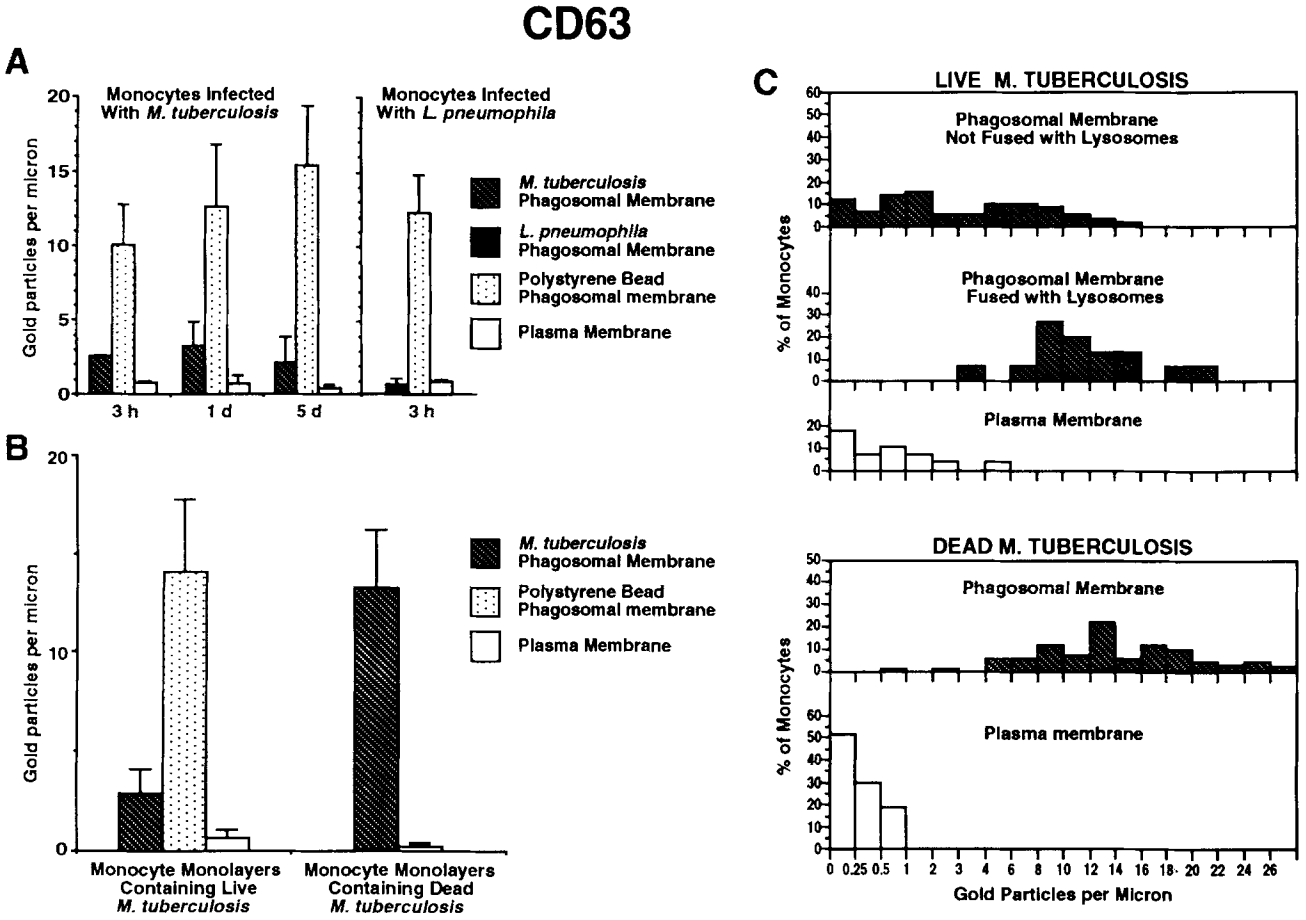

Figure 3. Expression of CD63 molecules on *M. tuberculosis, L. pneumophila*, and polystyrene bead phagosomes as assessed by cryosection immunogold staining. (*A*) Human monocytes were incubated with *M. tuberculosis* or *L. pneumophila*, together with polystyrene beads, and fixed at 3 h, 1 d, or 5 d after phagocytosis. Cryosections were stained for CD63 by immunogold and the number of gold particles was enumerated on the plasma membrane and on the *M. tuberculosis, L. pneumophila*, or polystyrene bead phagosomal membranes. Data are the mean number (± SD) of gold particles per micron of membrane for at least 15 monocytes on each of at least two electron microscopy grids. (*B*) Expression of CD63 molecules on live or heat-killed *M. tuberculosis* phagosomes and polystyrene bead phagolysosomes 1 d after phagocytosis. Human monocytes were incubated with live *M. tuberculosis* together with polystyrene beads (*left*) or with heat-killed *M. tuberculosis* (*right*) and fixed after 1 d. Cryosections were stained for CD63 by immunogold and the number of gold particles was enumerated on the plasma membrane and phagosomal membrane. Data are the mean number (± SD) of gold particles per micron of membrane for at least 15 monocytes on each of at least two electron microscopy grids. (*C*) Frequency distribution of CD63 staining on phagosomes containing live or heat-killed *M. tuberculosis*. Human monocytes were incubated with live or heat-killed *M. tuberculosis* and fixed after 1 d. Cryosections were stained for CD63 by immunogold and the number of gold particles was enumerated on the of plasma membrane and phagosomal membrane. For live *M. tuberculosis*, the results for phagosomes that had fused or not fused with BSA-gold are shown separately. Phagosomes containing dead *M. tuberculosis* fused extensively with the BSA-gold labeled compartment, and since no differences were apparent in CD63 staining between dead *M. tuberculosis* phagosomes that had fused and those that had not, the results for the two groups are presented together.

cytes were washed three times with RPMI to remove nonadherent bacteria and beads and then incubated for an additional 3 h–5 d with or without 50 U/ml IFN-γ in RPMI containing 15% autologous serum. The infected monocytes were then fixed with 4% paraformaldehyde, 0.01% glutaraldehyde in 0.15 M sodium cacodylate, pH 7.30, for 2 h at 4°C. The monocytes were washed twice with 0.15 M sodium cacodylate, pH 7.3, incubated with 10 mM glycine-HCl in cacodylate buffer for 30 min to quench aldehyde groups, and scraped from the culture flasks with cell scrapers (Costar Corp., Cambridge, MA) into cacodylate buffer containing 0.1% BSA. The monocytes were pelleted by centrifugation at 1,000 rpm for 10 min, and embedded in 10% gelatin at 37°C. The gelatin was solidified at 0°C and the embedded cell pellet was trimmed with scalpels at 0°C and infiltrated with 2.3 M sucrose and 20% polyvinylpyrrolodone at 4°C overnight. Cryosections were obtained at −90°C. Sections were collected on 2.3 M sucrose, transferred to formvar-coated nickel grids, and incubated with 50% normal goat serum (NGS) and 0.1% fish skin gelatin in 0.9% NaCl in 0.05 M Hepes, pH 7.40, for 1 h at 4°C to block nonspecific antibody binding. Sections were incubated with 20 μg/ml mouse monoclonal primary Ig (or 2.5 μg/ml in the case of mouse mAb to cathepsin D) or control isotypic myeloma Ig diluted in Hepes buffer containing 50% NGS and 0.1% fish skin gelatin overnight at 4°C, washed with Hepes buffer, and incubated with goat anti-mouse Ig conjugated to 8 nm colloidal gold (Sigma Chemical Co.) for 90 min at room temperature. For double labeling of sections for

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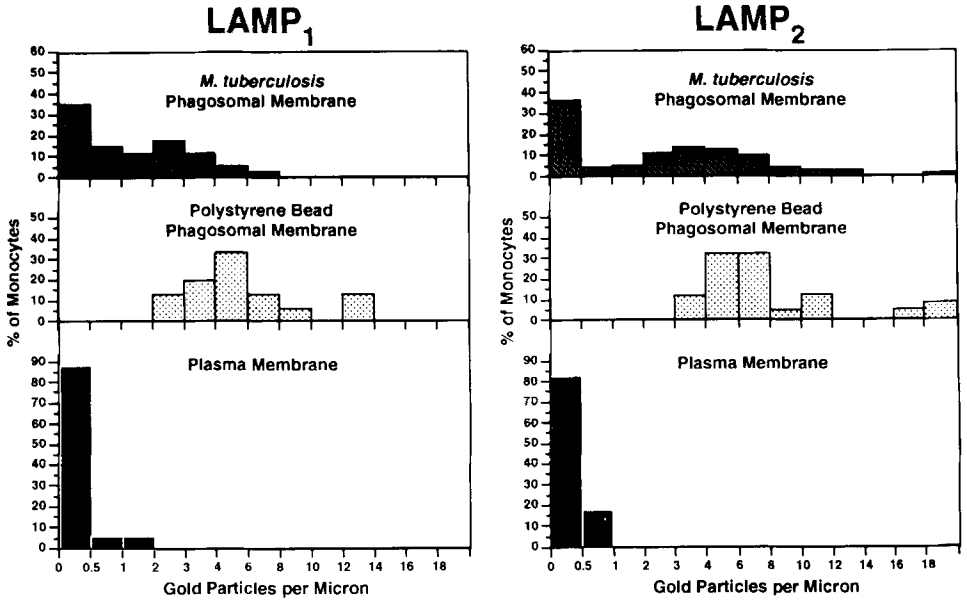


Figure 4. Frequency Distribution of LAMP-2 and LAMP-2 staining on *M. tuberculosis* phagosomes. Human monocytes were infected with *M. tuberculosis* and fixed 1 d later. Cryosections were stained for LAMP-1 or LAMP-2, and gold particles per micron of phagosomal membrane and per micron of plasma membrane were enumerated. The frequency distribution of LAMP-1 and LAMP-2 staining closely resembles that of CD63 staining.

mycobacterial LAM, a 1:1,000 dilution of rabbit antibody against LAM was included in the primary antibody incubation, and a 1:20 dilution of goat anti–rabbit Ig conjugated to 15 nm colloidal gold (Amersham, Arlington Heights, IL) was included in the second antibody incubation step. Sections were washed again with Hepes buffer, postfixed in 2% glutaraldehyde for 5 min, washed in distilled water, and embedded in 2% polyvinyl alcohol containing 0.3% uranyl acetate. Sections were viewed with a transmission electron microscope (model 100 CX II; JEOL, Ltd., Tokyo, Japan). Photomicrographs of consecutive infected monocytes were made at magnifications of 7,200 to 14,000. The number of gold particles per micron of membrane or per square micron of subcellular compartment was enumerated directly from the negatives using a translucent digitizer tablet (model 2210; Numonics Corp., Montgomeryville, PA) and Sigma-Scan software program (Jandel Scientific Co., Corte Madera, CA).

Assessment of Intracellular Growth of M. tuberculosis in Monolayers of Human Monocytes. Monocytes were cultured in 2 cm^2 tissue culture wells for 5 d in RPMI with 15% autologous human serum and infected with *M. tuberculosis* at a multiplicity of infection of one bacterium for every two monocytes. Monocyte monolayers were washed with RPMI and incubated in fresh medium containing 15% autologous serum. CFU of *M. tuberculosis* were determined in the culture supernatant fluid and the monolayer daily for 5 d using the method described by Hirsch et al. (9). Briefly, the supernatant fluid was removed and retained, and then the monocyte monolayer was washed with RPMI and lysed by adding 0.1% SDS in sterile distilled water. Serial dilutions of the tissue culture supernatant fluid and the lysed monolayer were cultured on 7H11 agar for 2 wk at 37°C in 5% CO_2, and CFU enumerated.

Results

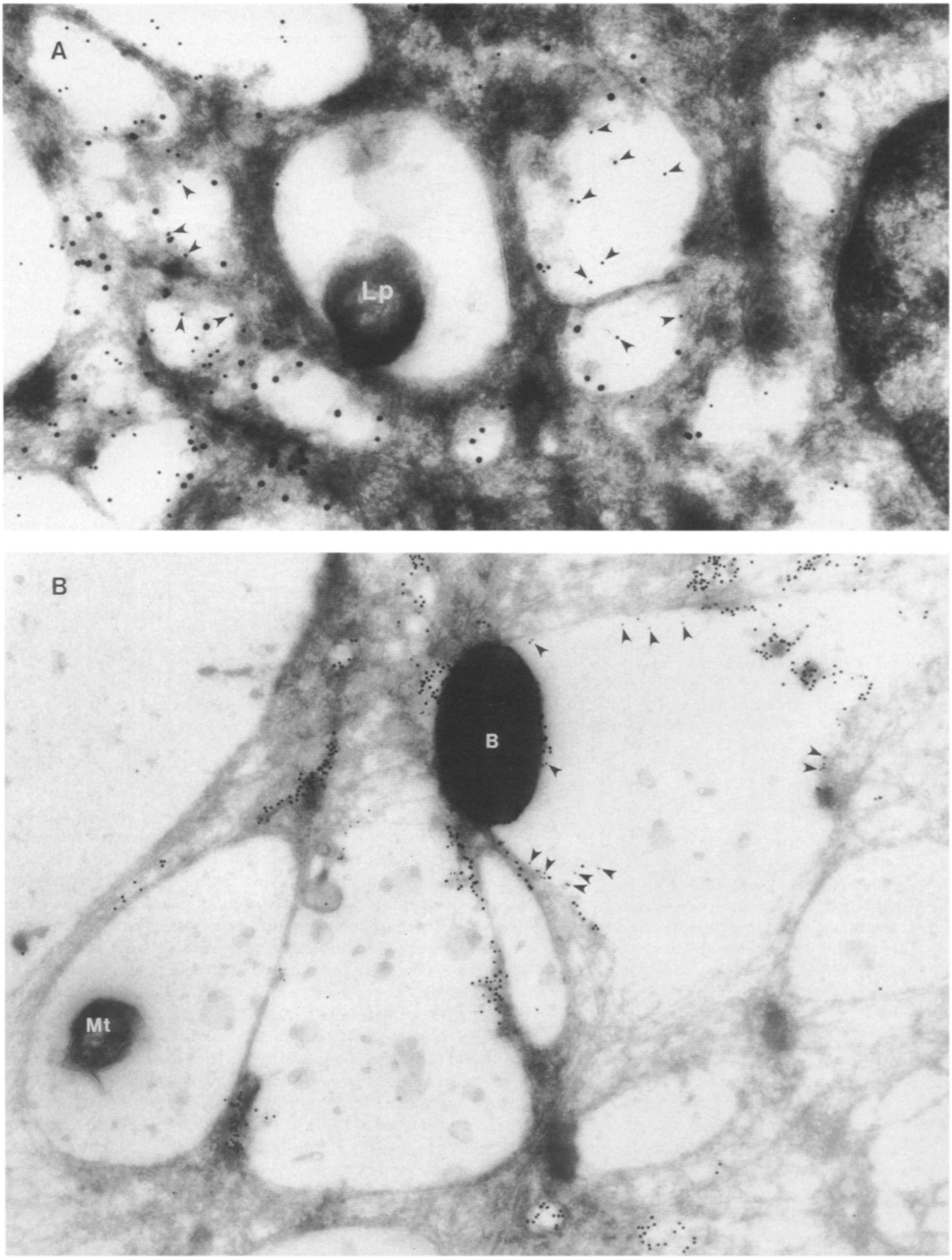
Virulence, Viability, Single Cell Status, and Intracellular Growth of the M. tuberculosis Inoculum. The *M. tuberculosis* strain used in these studies was the highly virulent Erdman strain. To ensure virulence of the organism, we passaged it through guinea pigs by aerosolization (6) and recovered it from the lungs of the infected animals.

Viability of *M. tuberculosis* is a major consideration in studies of this type because live and dead organisms interact differently with mononuclear phagocytes (3). Therefore, we carefully monitored the viability of our inoculum. Viability ranged from 30 to 75%. As discussed below, we believe that nonfusion of the phagosome with BSA-gold labeled lysosomes is a distinguishing characteristic of live *M. tuberculosis* intracellularly.

In our experience, single cell organisms interact differently with monocytes than clumps of organisms (see below). We therefore used single cell suspensions in these studies. Our technique for preparing single cells yielded a suspension containing 90–95% single organisms.

Monocytes cultured in vitro for 5 d and then incubated with *M. tuberculosis* for 90 min at 37°C at a multiplicity of infection of 1 CFU per two monocytes ingested 11.5% of the infecting inoculum. Subsequently, the bacteria multiplied with a doubling time of ~1 d. Growth of *M. tuberculosis* in monocyte cultures appeared to be exclusively intracellular; no growth was observed when *M. tuberculosis* was added to culture medium without monocytes. During the first few days after infection, relatively few organisms were released by monocytes and potentially available to infect other cells in the culture. At 1 and 3 d after infection, CFU in the culture medium was 4.5 and 20%, respectively, of the number associated with the monolayer. By day 5, considerable lysis of the monolayer had occurred, such that the number of bacteria present in the culture supernatant fluid exceeded the number remaining in the monolayer.

M. tuberculosis Phagosomes Resist Fusion with BSA-gold Labeled Lysosomes. Armstrong and Hart (3) demonstrated that phagosomes containing viable *M. tuberculosis* organisms inhibit phagosome-lysosome fusion whereas phagosomes containing inert particles fuse with lysosomes. However, other investigators (10) have reported a conflicting result. To assess the extent of fusion of *M. tuberculosis* phagosomes with lyso-



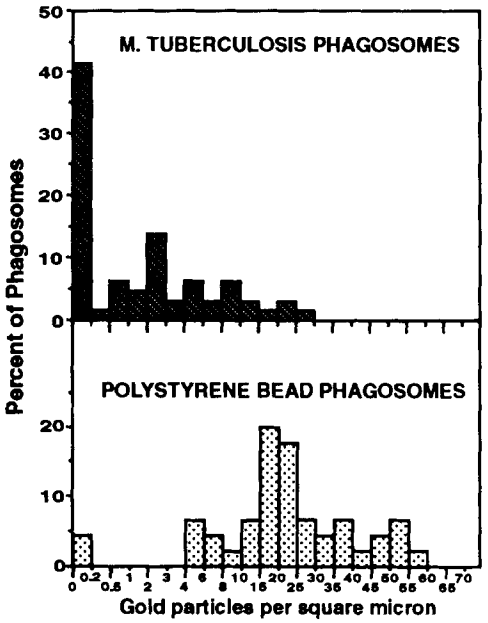


Figure 6. Frequency distribution of cathepsin D staining on *M. tuberculosis* phagosomes. Human monocytes were incubated with *M. tuberculosis* and polystyrene beads, and fixed 1 d later. Cryosections were stained for cathepsin D and gold particles per square micron of phagosome were enumerated.

somes, we cultured monolayers of human monocytes for 5 d and labeled their lysosomes with BSA-colloidal gold (20 nm) (8). We then incubated the monocytes with live *M. tuberculosis*, heat-killed *M. tuberculosis*, or live *L. pneumophila*, together with polystyrene beads or with polystyrene beads alone. After 3 h or 1 d, we fixed the monolayers and prepared them for electron microscopy. Phagosomes containing either live *M. tuberculosis* or live *L. pneumophila* showed very little fusion with BSA-gold labeled lysosomes (Fig. 1). In contrast, phagosomes containing heat-killed *M. tuberculosis* or polystyrene beads showed a high level of fusion with the BSA-gold labeled lysosomes both 3 h and 1 d after phagocytosis. The extent of fusion with the BSA-gold labeled compartment was not substantially altered by treatment of the monocytes with IFN-γ (50 U/ml) before and after phagocytosis (Fig. 1). Thus, our data support the conclusion of Armstrong and Hart (3) that live *M. tuberculosis* inhibits phagosome-lysosome fusion.

Although our procedure for preparing *M. tuberculosis* yielded a predominantly single cell suspension, some clumps of *M. tuberculosis* remained and were phagocytosed by monocytes. Clumps of *M. tuberculosis* showed a higher level of fusion with BSA-gold labeled lysosomes than single mycobacteria. For example, in one experiment, 53.4% of phagosomes containing clumps of *M. tuberculosis* fused with BSA-gold labeled lysosomes, whereas only 20.4% of phagosomes containing single *M. tuberculosis* fused at 3 h after phagocytosis.

The extent of fusion of BSA-gold with polystyrene bead phagosomes was not substantially altered by infection of the same monocytes with either live *L. pneumophila* or live *M. tuberculosis*. For example, in one experiment, the percentage of polystyrene bead phagosomes fused with BSA-gold labeled lysosomes at 3 h after phagocytosis was 72% in uninfected monocytes, 81.4% in *L. pneumophila*–infected monocytes, and 85.4% in *M. tuberculosis*–infected monocytes. Conversely, in this experiment and in subsequent experiments, the appearance and staining characteristics of phagosomes containing either *M. tuberculosis* or *L. pneumophila* was not altered by coingestion of polystyrene beads by the monocytes.

At 5 d after phagocytosis, *M. tuberculosis* phagosomes showed almost no fusion with the BSA-gold compartment (only 1 of 42 phagosomes were BSA-gold positive). In contrast, polystyrene bead phagosomes showed nearly 100% fusion with secondary lysosomes labeled with BSA-gold. The very low rate of fusion of *M. tuberculosis* at 5 d likely reflects the very high viability of organisms evident at this point after infection consequent to multiplication of the live bacteria and degradation of the dead bacteria in the original inoculum. Consistent with this reasoning, when monocytes were infected with live *M. tuberculosis* at a multiplicity of infection of 1:2 (bacterium/monocyte), 50% of the monocytes contained *M. tuberculosis* organisms 5 d later. In contrast, when heat-killed *M. tuberculosis* were added to monocytes at a bacterium to monocyte ratio of 5:1 (10 times greater than the ratio used with live *M. tuberculosis*), only 7% of the monocytes were found to contain morphologically recognizable *M. tuberculosis* organisms after 5 d.

In marked contrast to the results recently reported by McDonough et al. (10), we did not observe *M. tuberculosis* free in the cytoplasm at any time after infection (from 3 h to 5 d).

M. tuberculosis Phagosomes Contain Relatively Low Levels of Lysosomal Membrane Glycoproteins. We next examined the immunogold staining of *M. tuberculosis* phagosomes for the lysosome-associated membrane glycoproteins CD63, LAMP-1, and LAMP-2, and the acid protease, cathepsin D. These markers have previously been described on late endosomes and lysosomes (11–15). As expected, phagolysosomes containing polystyrene beads and heat-killed *M. tuberculosis* stained intensely for these markers (Figs. 2–6). In contrast, phago-

Figure 5. Immunogold staining for cathepsin D in monocytes incubated with *L. pneumophila* (*A*) or *M. tuberculosis* (*B*) and polystyrene beads. Human monocytes were cultured and lysosomes labeled with BSA-gold as described in Fig. 2. The monocytes were fixed 3 h after infection with *L. pneumophila* (*Lp*) (*A*) or 1 d after infection with *M. tuberculosis* (*Mt*) (*B*), and cryosections were stained for cathepsin D by immunogold. (*A*) In this representative *L. pneumophila*–infected cell, cathepsin D (10 nm gold particles, *arrowheads*) and BSA-gold (20 nm gold particles) are absent from the *L. pneumophila* phagosome but abundant in adjacent cytoplasmic vesicles. ×58,700. (*B*) In this representative *M. tuberculosis*–infected cell, both cathepsin D (10 nm gold particles, *arrowheads*) and BSA-gold (20 nm gold particles) are abundant in the polystyrene bead (*B*) phagolysosome. In contrast, cathepsin D is markedly reduced and BSA-gold is absent from the *M. tuberculosis* (*Mt*) phagosome. ×34,300.

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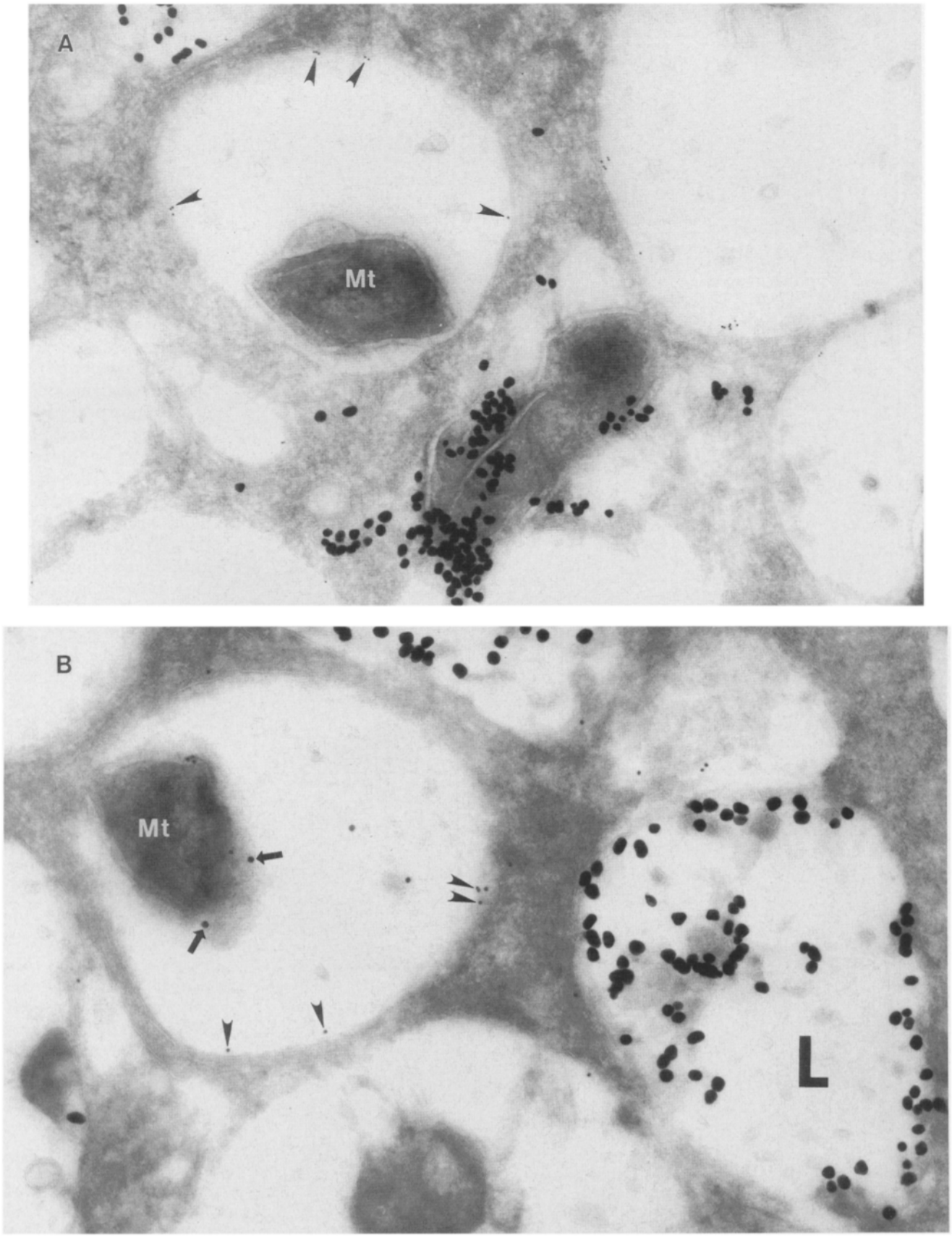

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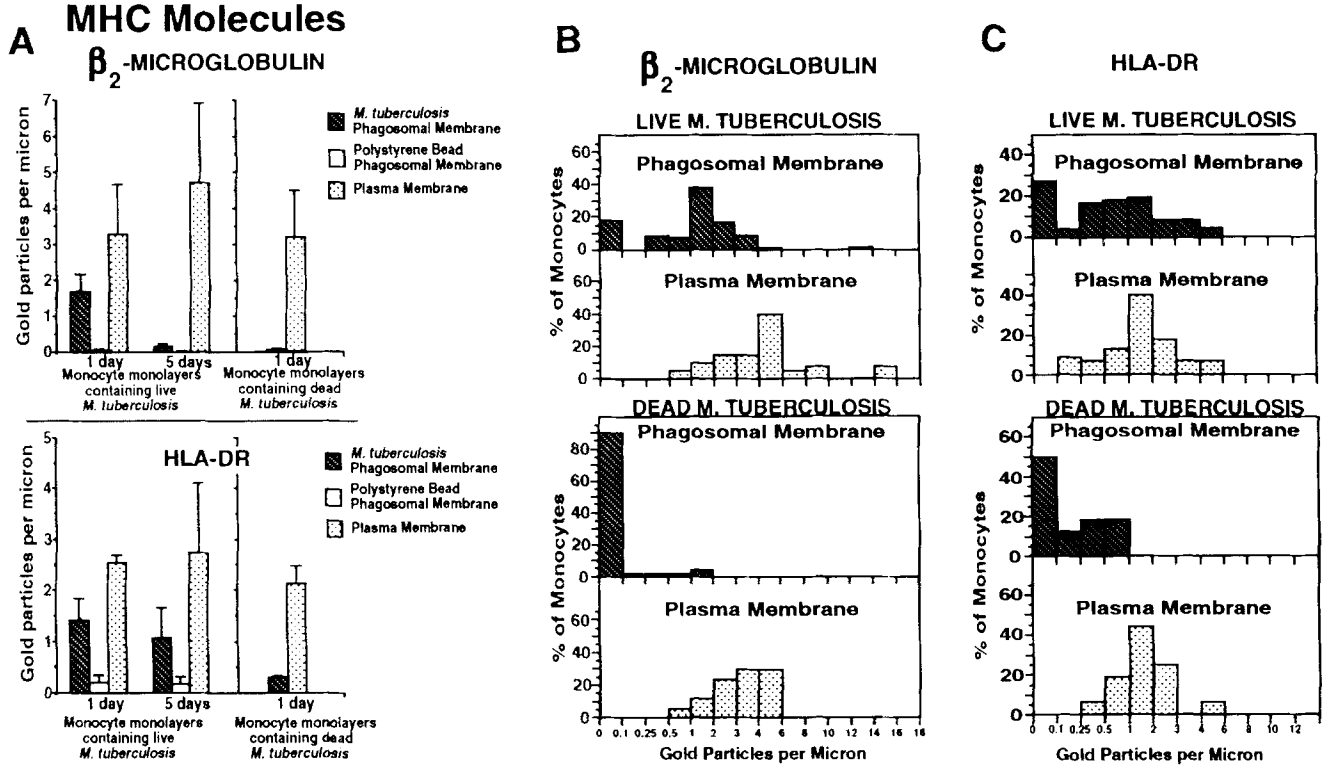

Figure 8. Expression of MHC molecules on *M. tuberculosis* phagosomes at 1 and 5 d after phagocytosis. (*A*) IFN-γ–treated monocytes were incubated with live *M. tuberculosis* and polystyrene beads (*left*) or with dead *M. tuberculosis* (*right*) and fixed 1 or 5 d later. Cryosections were stained by immunogold for β_2-microglobulin (*top*) or HLA-DR (*bottom*). The number of gold particles was enumerated on *M. tuberculosis* phagosomes, polystyrene bead phagosomes, and the plasma membrane. Data are the mean number ± SD of gold particles per micron of membrane for at least 20 monocytes on each of at least two electron microscopy grids. Frequency distribution of staining for β_2-microglobulin (*B*) and HLA-DR (*C*) on the plasma membrane and the phagosomal membrane of monocytes infected with live (*top*) or dead (*bottom*) *M. tuberculosis*. IFN-γ–treated human monocytes were incubated with live or dead *M. tuberculosis*, fixed 1 d later, and stained by immunogold for β_2-microglobulin (*B*) or HLA-DR (*C*). The number of gold particles per micron of membrane was determined on the phagosomal membrane and plasma membrane.

somes containing *L. pneumophila* were virtually devoid of CD63, LAMP-1, and LAMP-2 staining, with levels comparable to the low levels on the plasma membrane (Figs. 2 *A* and 3 *A*). Phagosomes containing live *M. tuberculosis* exhibited a level of staining for lysosome-associated membrane glycoproteins intermediate between that present on phagosomes containing *L. pneumophila* and that present on phagolysosomes containing polystyrene beads or heat-killed *M. tuberculosis* (Fig. 2 *B*, and Fig. 3 *A–C*). The relatively small population of phagosomes containing *M. tuberculosis* derived from a live inoculum that were fused with BSA-gold labeled lysosomes exhibited a much higher level of CD63 staining than nonfused *M. tuberculosis* phagosomes and the level of CD63 staining in such phagosomes was comparable to that of phagosomes containing dead *M. tuberculosis* (Fig. 3 *C*).

Treatment of monocyte monolayers with IFN-γ had no effect on the density of CD63 staining on phagosomes containing polystyrene beads or heat-killed *M. tuberculosis* at 3 h, 1 d, or 5 d after phagocytosis, and did not increase the density of staining on *M. tuberculosis* phagosomes.

LAMP-1 and LAMP-2 exhibited a staining pattern similar to CD63 in that the live *M. tuberculosis* phagosome had a level of staining intermediate between the high level on polystyrene bead phagosomes and the low level on the plasma membrane (Fig. 4).

Cathepsin D is a lysosomal acid protease found both in endosomes and in lysosomes (14, 15). We have found that cathepsin D shows a staining distribution similar to that for CD63, LAMP-1, and LAMP-2. Cathepsin D was uniformly abundant on polystyrene bead phagolysosomes and absent

Figure 7. Cryosection immunogold staining for MHC molecules on *M. tuberculosis* phagosomes. IFN-γ–treated human monocytes were cultured for 5d, and secondary lysosomes were labeled with BSA-gold (25 nm) particles. The monocytes were infected with *M. tuberculosis* and fixed 1 d later. Cryosections were stained for β_2-microglobulin (*A*) or for HLA-DR (*B*) with 10 nm gold particles (*arrowheads*). LAM was stained using 15 nm gold particles (*B*, *arrows*). (*A*) β_2-microglobulin is present on the *M. tuberculosis* (*Mt*) phagosomal membrane. The *M. tuberculosis* phagosome has not fused with BSA-gold, which is present in adjacent secondary lysosomes. A clump of *M. tuberculosis* organisms is present in an adjacent phagolysosome, together with BSA-gold. ×52,600. (*B*) The cell wall of the *M. tuberculosis* stains positively for LAM (*arrows*) and the phagosomal membrane stains positively for HLA-DR (*arrowheads*). BSA-gold is abundant in secondary lysosomes (*L*) but absent from the *M. tuberculosis* phagosome. ×68,300.

from *L. pneumophila* phagosomes (Fig. 5 *A*) and from the plasma membrane. Cathepsin D was absent or present at low levels on the majority of live *M. tuberculosis* phagosomes (Fig. 5 *B* and Fig. 6).

Whereas CD63, LAMP-1, LAMP-2, and cathepsin D are found both on late endosomes and on lysosomes, the mannose-6-phosphate receptor is found on late endosomes but not on lysosomes, and therefore serves as a marker useful for distinguishing late endosomes from lysosomes (11). Unfortunately, we have not been successful in staining our cryosections with this marker because of as yet intractable problems with reactivity of the antiserum with *M. tuberculosis* antigens.

As noted above, a small subset of *M. tuberculosis* organisms from a live inoculum resides in phagosomes that fuse with BSA gold-labeled lysosomes. Such phagosomes, in contrast to the phagosomes that have not fused with lysosomes, exhibit a high level of CD63 staining, comparable to that of phagosomes containing heat-killed *M. tuberculosis*, which fuse extensively with the BSA-gold labeled compartment. Similarly, morphologically disrupted *M. tuberculosis* almost invariably reside in phagosomes that have fused with BSA-gold labeled lysosomes and have high levels of CD63. These results indicate that the small subset of *M. tuberculosis* organisms from a live inoculum that reside in phagosomes that fuse with BSA-gold labeled lysosomes are nonviable organisms in the live inoculum. Consequently, in subsequent studies of membrane markers on live *M. tuberculosis* phagosomes, we restricted the analysis to phagosomes that have not fused with BSA-gold labeled lysosomes.

M. tuberculosis Phagosomes Exhibit Delayed Clearance of MHC Class I Molecules and Contain Relatively High Levels of MHC Class II Molecules throughout the Course of Intracellular Infection. Both MHC class I and II molecules are abundant on the plasma membrane, but the two classes of molecules are distributed quite differently along the endosomal-lysosomal pathway. MHC class I molecules are either excluded during endocytosis or are rapidly recycled to the plasma membrane. Newly synthesized MHC class I molecules are routed from the *trans*-Golgi vesicles directly to the plasma membrane. Consequently MHC class I molecules are excluded from endosomes and lysosomes (16). In contrast, newly synthesized MHC class II molecules are routed from *trans*-Golgi vesicles to a specialized MHC class II–containing endosomal compartment (CIIV) (17–19). MHC class II molecules are present on the plasma membrane and gain access to many parts of the classical endocytic pathway (18–23). MHC class II molecules are excluded from late lysosomes (22, 23). At least under some conditions, however, MHC class II molecules are present on a subset of early tubular lysosomes (24).

To facilitate study of the distribution of MHC molecules on *M. tuberculosis* phagosomes, we upregulated the expression of these molecules by treating monocytes with a low level of IFN-γ before and after infection with *M. tuberculosis*. In previous studies of *L. pneumophila* phagosomes, we found that MHC class I and II molecules are rapidly excluded from *L. pneumophila* phagosomes during phagocytosis (25) and that these molecules remain at low levels on the phagosome throughout the 1-d course of infection (26). In marked contrast to these results, MHC class I and II molecules are present on live *M. tuberculosis* phagosomes at 3 h and at 1 d after phagocytosis (Figs. 7 and 8). In these experiments, we used β_2-microglobulin as a marker of MHC class I molecules. Although β_2-microglobulin may also associate with nonclassical MHC Ib molecules, we have obtained results using antibody to the MHC class I heavy chain similar to those using antibody to β_2-microglobulin (data not shown). *M. tuberculosis* phagosomes display considerable heterogeneity in the intensity of staining of phagosomes for these markers (Fig. 8, *B* and *C*). The heterogeneity of staining is present even within single monocytes infected with *M. tuberculosis*, as some phagosomes within a monocyte stain intensely whereas others show negligible staining. Phagosomes containing polystyrene beads in the same monocyte monolayer show a low level of staining for MHC molecules at 1 d after phagocytosis (Fig. 8 *A*). Similarly, phagosomes containing heat-killed *M. tuberculosis* show a low level of staining for MHC class I or II molecules at 1 d after phagocytosis (Fig. 8 *A*).

Live *M. tuberculosis* phagosomes continue to stain positively for HLA-DR from 1 through 5 d after phagocytosis (Fig. 8 *A*), and the level of staining remains about the same throughout this period. In contrast, live *M. tuberculosis* phagosomes lose their staining for MHC class I by 5 d after phagocytosis (Fig. 8 *A*).

In the absence of IFN-γ, expression of MHC class II molecules decreases with time in culture (27). Consequently, in our studies, expression of MHC class II molecules in the absence of IFN-γ was too low to allow reliable measurements of its level in various cell compartments. In contrast, expression of the MHC class I marker, β_2-microglobulin, was still readily detectable in monocytes not treated with IFN-γ. In such monocytes, β_2-microglobulin was present on live *M. tuberculosis* phagosomes at 3 h and 1 d after phagocytosis and absent at 5 d after phagocytosis as in IFN-γ–treated monocytes.

M. tuberculosis Phagosomes Stain Positively for the Transferrin Receptor. Our finding that *M. tuberculosis* phagosomes retain class II MHC molecules suggested that they interact with endosomes. To explore this further, we studied *M. tuberculosis* phagosomes for the presence of the transferrin receptor, an endosomal marker. Transferrin receptors on the plasma membrane are actively concentrated into coated pits and are internalized as coated vesicles that fuse with endosomes. From the endosomal compartment, transferrin receptors are rapidly recycled to the plasma membrane. Transferrin receptors are excluded from lysosomes (28, 29).

Immunogold staining for the transferrin receptor was present on a subset of live *M. tuberculosis* phagosomes at both 3 h and 1 d after phagocytosis (Figs. 9 and 10). The level of transferrin receptor staining on the *M. tuberculosis* phagosome was comparable to that on the plasma membrane of the same monocytes. A similar level of staining for the transferrin receptor was present on the *M. tuberculosis* phagosome at 5 d after phagocytosis in two out of three experiments. In contrast, immunogold staining for the transferrin receptor

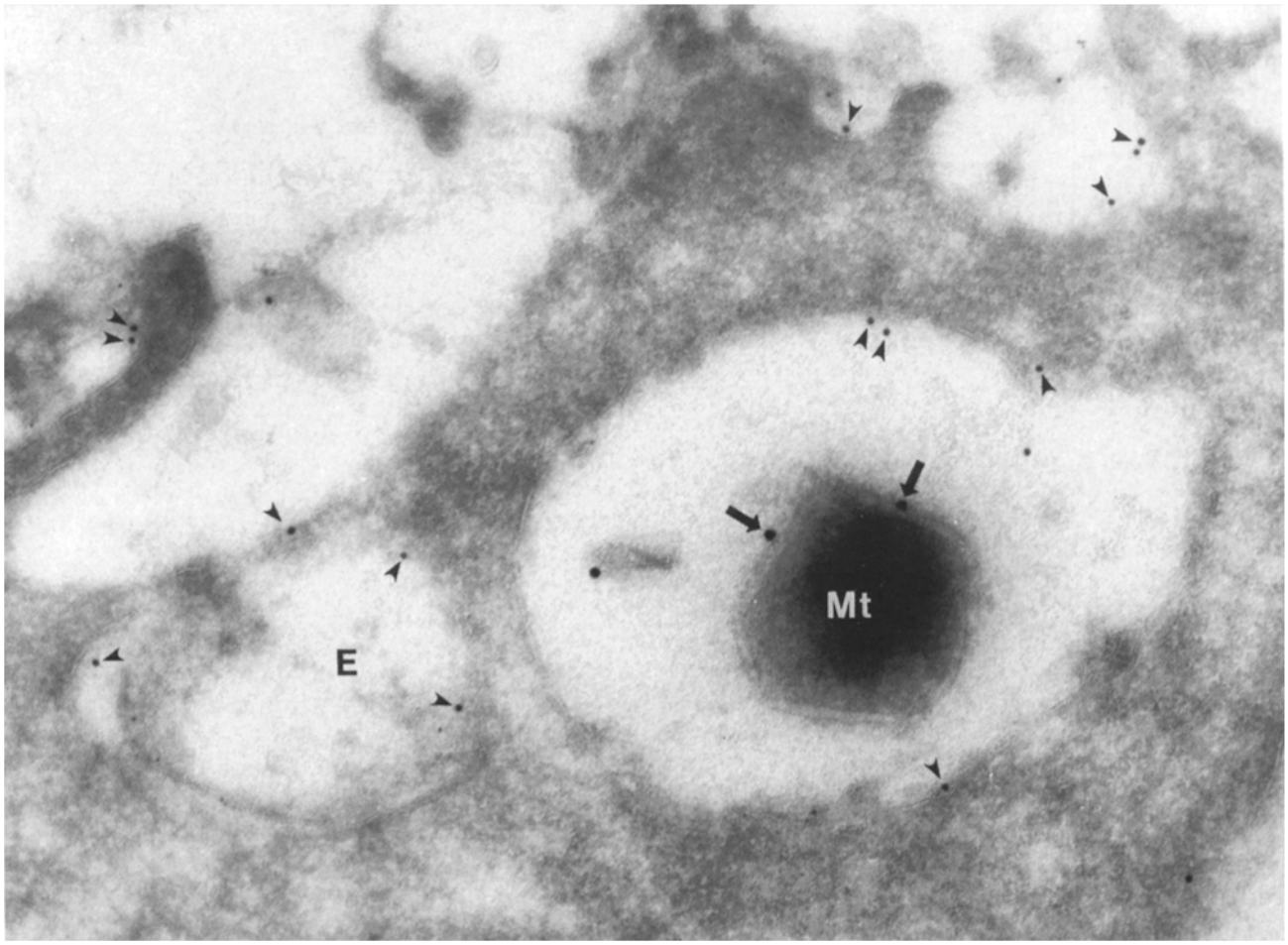

Figure 9. Cryosection immunogold staining for transferrin receptor molecules on *M. tuberculosis* phagosomes. Human monocytes were infected with *M. tuberculosis* and fixed 1 d later. Cryosections were stained for the transferrin receptor using 10 nm immunogold particles and for *M. tuberculosis* LAM using 15 nm gold particles. Secondary lysosomes were labeled with BSA-gold (25 nm). In this representative micrograph, transferrin receptor molecules (*arrowheads*) are present on the *M. tuberculosis* (*Mt*) phagosomal membrane and on an adjacent endosome (*E*). LAM is present on the cell wall of the mycobacterium (*arrows*). ×95,600.

was not observed on phagosomes containing polystyrene beads or *L. pneumophila* at either 3 h or 1 d after phagocytosis (Fig. 10).

Discussion

Little is known about the membrane composition and membrane trafficking during maturation of phagosomes containing viable intracellular parasites. Phagosomes containing inert particles have been shown to undergo a series of maturational events that mirror the maturation of endosomes to lysosomes (30, 31). This maturation is associated with a rapid recycling of plasma membrane proteins, accompanied by fusion with endosomal vesicles. Fusion of the nascent phagosome with early endosomes leads to the acquisition of early endosomal markers. With maturation, the nascent phagosome loses some of its early endosomal markers and acquires markers associated with late endosomes (30, 31), such as lysosomal membrane glycoproteins. Ultimately, the phagosome fuses with lysosomes and acquires abundant lysosomal membrane glycoproteins and lysosomal hydrolases.

We have previously shown (25) that sorting of membrane proteins occurs rapidly during phagocytosis of *L. pneumophila* and *Escherichia coli*, leading to a relative concentration of complement receptor CR3 and a relative exclusion of MHC molecules from the nascent phagosome. As the *L. pneumophila* phagosome matures further, it continues to exclude MHC class I and II molecules (26). We demonstrate here that *L. pneumophila* phagosomes also do not acquire transferrin receptor or the lysosomal membrane glycoproteins, CD63, LAMP-1, or LAMP-2, or the acid protease, cathepsin D. These results suggest that the *L. pneumophila* phagosome does not interact with the endosomal-lysosomal pathway.

In marked contrast to the *L. pneumophila* phagosome, the *M. tuberculosis* phagosome fails to clear MHC class I molecules and continues to show positive staining for this marker for 3 d after phagocytosis. In addition, the *M. tuberculosis* phagosome shows positive staining for MHC class II and for

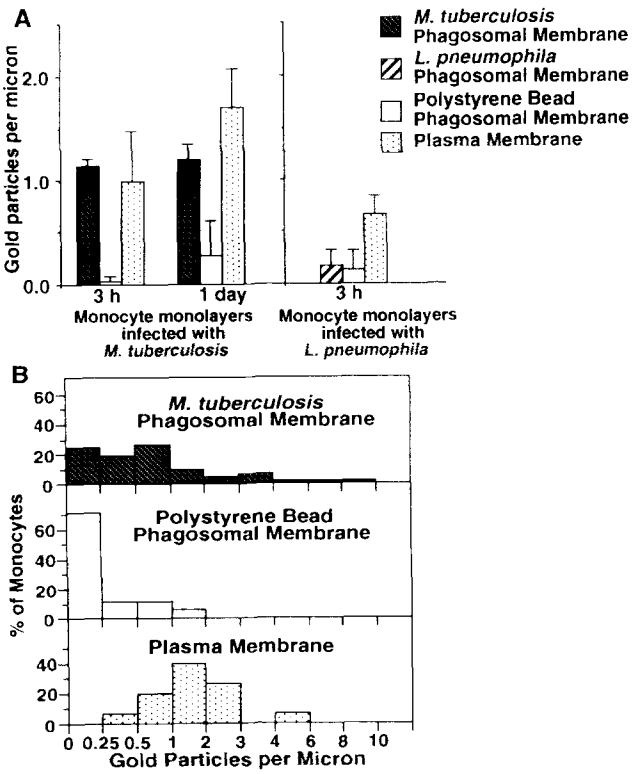

Figure 10. Expression of transferrin receptor moleculeson human monocytes infected with *M. tuberculosis* as assessed by cryosection immunogold staining. (*A*) Human monocytes were infected with either *L. pneumophila* and polystyrene beads or *M. tuberculosis* and polystyrene beads and fixed 3 h or 1 d later. Cryosections were stained for the transferrin receptor by immunogold. Transferrin receptor immunogold particles were enumerated on the plasma membrane and phagosomal membrane. Data are the mean number (± SD) of gold particles per micron of membrane for at least 15 monocytes on each of at least two microscopy grids. (*B*) Frequency distribution of staining for transferrin receptor on the plasma membrane and the phagosomal membrane of monocytes infected with *M. tuberculosis*. Human monocytes were incubated with *M. tuberculosis* and polystyrene beads, fixed 1 d later, and stained by immunogold for the transferrin receptor. The number of gold particles per micron of membrane was determined on *M. tuberculosis* and polystyrene bead phagosomal membranes and on the plasma membrane.

the transferrin receptor, an endosomal marker. Although the *M. tuberculosis* phagosome acquires some lysosomal membrane glycoproteins and cathepsin D, the staining for these markers remains less than that which is observed on phagolysosomes, consistent with acquisition of these markers from late endosomes. In addition, the *M. tuberculosis* phagosome does not fuse with secondary lysosomes labeled with BSA-gold. These results suggest that the *M. tuberculosis* phagosome interacts extensively with endosomes, but that its maturation is arrested at the phagosomal equivalent of an endosomal stage. Although MHC class II and the transferrin receptor could be present on the *M. tuberculosis* phagosome by virtue of the persistence of plasma membrane proteins, this explanation cannot account for the acquisition of low levels of lysosome-associated membrane glycoproteins and cathepsin D that we observe on the *M. tuberculosis* phagosome.

On average, the *M. tuberculosis* phagosome is markedly different from the *L. pneumophila* phagosome. However, for all markers studied, *M. tuberculosis* phagosomes demonstrated heterogeneity in staining, and as many as one third of the *M. tuberculosis* phagosomes exhibited minimal staining, despite intense staining of other structures present in the same cell. To what extent the subsets of phagosomes that stain minimally for each of the markers overlap is not known, but it is possible that they are all the same subset. Therefore, we cannot exclude the possibility that as many as one third of the *M. tuberculosis* organisms reside in a compartment similar to the *L. pneumophila* phagosome, with hypoexpression of all markers studied (MHC molecules, transferrin receptor, lysosome-associated membrane glycoproteins, and cathepsin D).

It is possible that some of the heterogeneity of *M. tuberculosis* phagosomes could be attributable to the presence of nonviable organisms, which, nonetheless, have not fused with BSA-gold labeled lysosomes. Phagosomes containing nonviable mycobacteria should contain CD63, LAMP-1, LAMP-2, and cathepsin D, but lack the other markers studied (β_2-microglobulin, HLA-DR, and transferrin receptor).

The results of our studies of phagosome-lysosome fusion are consistent with the observations of Armstrong and Hart (3), who first noted that *M. tuberculosis* phagosomes do not fuse with the lysosomal compartment. As noted, the low level of staining for lysosome-associated membrane glycoproteins and the high level of staining for MHC molecules and the transferrin receptor, also distinguish the *M. tuberculosis* phagosome from a phagolysosomal compartment.

Our results differ from those of McDonough et al. (10) who reported that *M. tuberculosis* initially enters a lysosomal compartment (one that fuses with thorium dioxide–labeled secondary lysosomes), then transfers into a nonlysosomal phagosomal compartment, and finally escapes into the cytoplasm. In contrast, we find that *M. tuberculosis* resides in a membrane-bound endosome-like compartment and does not enter a lysosomal compartment. We find that preparations of *M. tuberculosis* with a high viability show limited fusion with secondary lysosomes at 1 d after phagocytosis. In contrast, heat-killed *M. tuberculosis* show a relatively high level of fusion with BSA-gold labeled lysosomes. The discrepancy between our results and those of McDonough et al. (10) may be due to several factors. First, McDonough et al. did not state the viability of their preparation. A preparation consisting of a large percentage of dead bacteria would appear to enter a lysosomal population early after infection. With time, as the live bacteria (residing in a nonfusing phagosomal compartment) multiply and the dead bacteria in phagolysosomes fail to multiply and are degraded, the percentage of bacteria in nonfused phagosomes would increase. Second, McDonough et al. did not take steps to remove clumps of bacteria. We have found that clumps of bacteria tend to enter compartments that fuse with BSA-gold and that are relatively rich in CD63. Indeed, our data on fusion with lysosomes of phagosomes containing clumps of organisms are similar to those of McDonough et al. The higher fusogenicity of clumps of bacteria may reflect the fact that the clumps con-

tain a mixture of dead and live bacteria. Third, whereas we studied *M. tuberculosis* cultured on agar medium, McDonough et al. studied cultures of *M. tuberculosis* grown in broth culture containing the detergent Tween-80. This detergent may alter the membrane properties and the biological behavior of *M. tuberculosis*.

Also in contrast to the observations of McDonough et al. (10), we do not observe *M. tuberculosis* free in the cytoplasm. Possibly, this observation is an artifact of their dehydration and embedding procedures, which involves dehydration in organic solvents and embedding in plastic. This use of organic solvents may lead to loss of phagosomal membrane components. In contrast, our cryosection technique involves no organic solvents and consequently is less likely to result in extraction of phagosomal membrane.

Whereas we found that the *M. tuberculosis* phagosome in human monocytes contains only small amounts of LAMP-1, Sturgill-Koszycki et al. (32) have reported recently that the *Mycobacterium avium* phagosome in mouse bone marrow–derived macrophages contains as much LAMP-1 as a phagolysosome but excludes the vacuolar proton pump. However, quantitation of LAMP-1 staining was not provided, and therefore it is difficult to assess to what extent our results with *M. tuberculosis* differ from theirs with *M. avium* in this respect. In view of the lower density of lysosomal membrane glycoproteins present on the *M. tuberculosis* phagosome, we would expect to find a lower content of vacuolar proton pump as well. In any case, *M. tuberculosis* and *M. avium* are very different pathogens, and it would not be surprising if the two pathogens reside in different intracellular compartments, particularly in different host cells.

MHC class I molecules on the *M. tuberculosis* phagosome are most likely derived from the plasma membrane. Although they also could be newly synthesized molecules derived from Golgi vesicles that have fused with the *M. tuberculosis* phagosome, we would expect that this process would continue throughout the course of infection. Instead, their disappearance on the *M. tuberculosis* phagosome by day 5 is more consistent with incomplete sorting of class I MHC molecules from the phagosome after phagocytosis. The decline in class I MHC molecules on the phagosomal membrane by day 5 may reflect the natural biological half-life of MHC class I molecules, as well as a reduction in density consequent to the acquisition of new membrane by the expanding phagosome. Our finding that MHC class I persists on the *M. tuberculosis* phagosome suggests that *M. tuberculosis* inhibits sorting of membrane molecules after phagocytosis (25).

MHC class II and the transferrin receptor are endosome-associated proteins that are normally substantially cleared during maturation of phagosomes to phagolysosomes. The failure of *M. tuberculosis* phagosomes to clear these proteins suggests that *M. tuberculosis* inhibits the maturation of its phagosome along the endosomal-lysosomal pathway. Possibly, the defect in membrane sorting after phagocytosis evidenced by the persistence of MHC class I molecules underlies the proposed maturation arrest of the *M. tuberculosis* phagosome.

The presence of transferrin receptor, MHC class II, lysosome-associated membrane glycoproteins, and cathepsin D on *M. tuberculosis* phagosomes suggests that the *M. tuberculosis* phagosome interacts with endosomes in a manner that leads to the transfer of endosomal proteins to the phagosome. Interaction of endosomes with the *M. tuberculosis* phagosome may provide direct access of *M. tuberculosis* antigens to the exogenous pathway of antigen processing and presentation by MHC class II molecules. In addition, interaction of the *M. tuberculosis* phagosome with endosomes may provide nutrients to *M. tuberculosis* organisms growing intracellularly and membrane components to the expanding *M. tuberculosis* phagosome.

Intracellular pathogens follow one of three general pathways through the host cell: (*a*) the intraphagosomal pathway, followed by pathogens that reside in a phagosome that does not fuse with lysosomes; (*b*) the intraphagolysosomal pathway, followed by pathogens that reside in a phagosome that does fuse with lysosomes; and (*c*) the extraphagosomal pathway, followed by pathogens that escape the phagosome and reside free in the cytoplasm of the host cell (33). *M. tuberculosis*, *L. pneumophila* and several other organisms, including *Toxoplasma gondii* and *Chlamydia psittaci*, share the intraphagosomal pathway (33). In addition to inhibiting phagosome-lysosome fusion, these pathogens, at least in the case of *M. tuberculosis*, *L. pneumophila*, and *T. gondii*, also inhibit phagosome acidification (33). However, whereas the phagosomes occupied by these pathogens have much in common, our finding that the *M. tuberculosis* and *L. pneumophila* phagosomes interact very differently with the endolysosomal pathway demonstrates that the intraphagosomal pathway is in fact heterogeneous and that host–parasite interactions are even more diverse than previously appreciated.

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