

# The *Mycobacterium tuberculosis* Phagosome Interacts with Early Endosomes and Is Accessible to Exogenously Administered Transferrin

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## Summary

Previous studies have demonstrated that the *Mycobacterium tuberculosis* phagosome in human monocyte-derived macrophages acquires markers of early and late endosomes, but direct evidence of interaction of the *M. tuberculosis* phagosome with the endosomal compartment has been lacking. Using the cryosection immunogold technique, we have found that the *M. tuberculosis* phagosome acquires exogenously added transferrin in a time-dependent fashion. Near-maximal acquisition of transferrin occurs within 15 min, kinetics of acquisition consistent with interaction of the *M. tuberculosis* phagosome with early endosomes. Transferrin is chased out of the *M. tuberculosis* phagosome by incubation of the infected macrophages in culture medium lacking human transferrin. Phagosomes containing latex beads or heat-killed *M. tuberculosis*, on the other hand, do not acquire staining for transferrin. These and other findings demonstrate that *M. tuberculosis* arrests the maturation of its phagosome at a stage at which the phagosome interacts with early and late endosomes, but not with lysosomes. The transferrin endocytic pathway potentially provides a novel route for targeting antimicrobials to the *M. tuberculosis* phagosome.

*Mycobacterium tuberculosis* is an intracellular pathogen that parasitizes human mononuclear phagocytes. Throughout its life cycle in these host cells, this bacterium resides and multiplies in a membrane-bound phagosome that resists acidification and fusion with lysosomes (1–4). In previous studies, using quantitative immunoelectron microscopy, we have examined the composition and maturation of the *M. tuberculosis* phagosome, focusing on known markers of the endocytic pathway (4). These studies revealed that the *M. tuberculosis* phagosome exhibits delayed clearance of MHC class I molecules, relatively intense staining for MHC class II molecules and the endosomal marker transferrin receptor, and relatively weak staining for the lysosome-associated membrane glycoproteins CD63, LAMP-1, and LAMP-2 and the lysosomal acid protease cathepsin D. Like *M. tuberculosis*, wild-type *Legionella pneumophila* inhibits phagosome-lysosome fusion and phagosome acidification (5, 6). However, in contrast to the *M. tuberculosis* phagosome, the *L. pneumophila* phagosome rapidly clears MHC class I molecules and excludes MHC class II molecules as well as all endosomal-lysosomal markers studied (4, 7, 8). In contrast to phagosomes containing either live *M. tuberculosis* or *L. pneumophila*, phagosomes containing heat-killed *M. tuberculosis* or polystyrene beads fuse with lysosomes and stain intensely for lysosome-associated membrane glycoproteins and cathepsin D (4).

These findings suggested that *M. tuberculosis* retards the maturation of its phagosome along the endocytic pathway and resides in a compartment with endosomal, as opposed to lysosomal, characteristics. However, direct evidence in support of this hypothesis, and more specifically, direct evidence that the *M. tuberculosis* phagosome acquires endosomal markers by virtue of interaction with endosomes rather than by other mechanisms, was lacking. In an effort to obtain such evidence, we have studied the origin of transferrin receptors present on the *M. tuberculosis* phagosome.

Transferrin receptors are synthesized in the host cell endoplasmic reticulum and transported via the Golgi complex to the host cell surface. There, they bind their natural ligand, iron-saturated transferrin (holotransferrin), after which the receptor-ligand complexes are internalized in coated vesicles that fuse with early endosomes. Acidification of the early endosome to pH 6.5–6.0 causes release of iron from the transferrin, thereby yielding iron unsaturated transferrin (apotransferrin). From the endosomal compartment, the apotransferrin-transferrin receptor complexes are rapidly recycled to the plasma membrane (9, 10). Transferrin and the transferrin receptor do not traffic through the lysosomal compartment (9, 10).

There are three possible sources of the transferrin receptors observed on the *M. tuberculosis* phagosome. First, the receptors may be derived by fusion of the phagosome with

vesicles coming from the *trans*-Golgi network. Second, the receptors may be derived from the plasma membrane during phagocytosis and be retained on the phagosome. Third, the receptors may be derived from phagosome interaction with early endosomes. To distinguish the first two possibilities from the third one, we administered transferrin exogenously to *M. tuberculosis*-infected macrophages and examined phagosomes for the presence or absence of transferrin. We reasoned that if transferrin receptors are derived from *trans*-Golgi network or the plasma membrane during phagocytosis, then a pulse of transferrin administered after phagocytosis should not be delivered to the phagosome. On the other hand, if transferrin receptors are derived from phagosome-endosome interaction, exogenously administered transferrin should be delivered to the *M. tuberculosis* phagosome.

In this article, we show that exogenously administered transferrin is delivered to the *M. tuberculosis* phagosome, hence demonstrating directly that the *M. tuberculosis* phagosome interacts with endosomes.

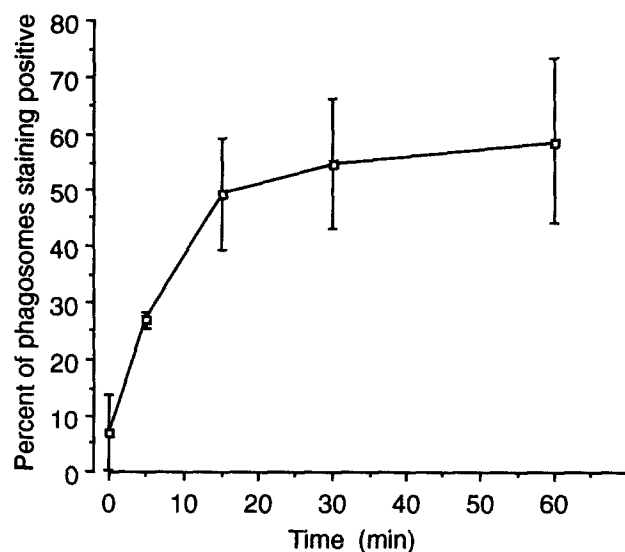
## Materials and Methods

**Reagents and Antibodies.** Human holotransferrin was purchased from Sigma Chemical Co. (St. Louis, MO). Solutions of transferrin were ultracentrifuged at 150,000 *g* for 90 min at 4°C to remove multimeric aggregates before incubation with monolayers. Ficoll-Hypaque was purchased from Pharmacia Biotech Inc. (Piscataway, NJ); glutaraldehyde was from Polysciences (Warrington, PA); polyvinyl alcohol, polyvinylpyrrolidone, and paraformaldehyde were from Sigma Chemical Co.; and RPMI and Dulbecco's PBS was from Gibco Laboratories (Santa Clara, CA). BSA-colloidal gold and mannan-colloidal gold of homogeneous size distribution (20–25 nm) were prepared by the method of Frens (11).

Mouse mAb to human CD63 (IgG<sub>1</sub>) was purchased from Amac, Inc. (Westbrook, ME); isotypic mouse myeloma control Ig was from Cappel Organon Teknika (Durham, NC); goat anti-mouse IgG gold conjugates (5 nm) were from Sigma Chemical Co.; and goat anti-rabbit IgG gold conjugates (10 nm) were from Amersham Corp. (Arlington Heights, IL). Rabbit polyclonal antibodies to human transferrin were raised by immunizing rabbits three times, 3 wk apart, with 1 mg of holotransferrin in Syntex adjuvant (12). The first immunization was supplemented with 100 µg of *N*-acetylmuramyl-L-alanyl-D-isoglutamine. The resulting antibodies had a titer of >1:10,000 to human transferrin by ELISA and did not cross-react with bovine, goat, or rabbit transferrin or with *M. tuberculosis* antigens.

**Bacteria.** *M. tuberculosis* Erdman strain (ATCC 35801), a highly virulent strain, was obtained from American Type Culture Collection (Rockville, MD). The organism was passaged through guinea pig lung to maintain virulence, a single-cell suspension was obtained, and viability was assayed as described previously (4). Viability ranged from 67 to 84% in these experiments. Under the conditions of these experiments, *M. tuberculosis* multiplied in macrophage monolayers with a doubling time of ~17 h.

**Preparation of Human Monocyte-derived Macrophages and Labeling of Lysosomes.** Human mononuclear cells were isolated from fresh heparinized blood of purified protein derivative (PPD)-negative healthy donors using Ficoll-Hypaque density gradients. Mononuclear cells ( $3 \times 10^6$ /ml) were plated in Falcon 75-cm<sup>2</sup> tissue culture flasks (12 ml/flask) in RPMI containing 15% heat-



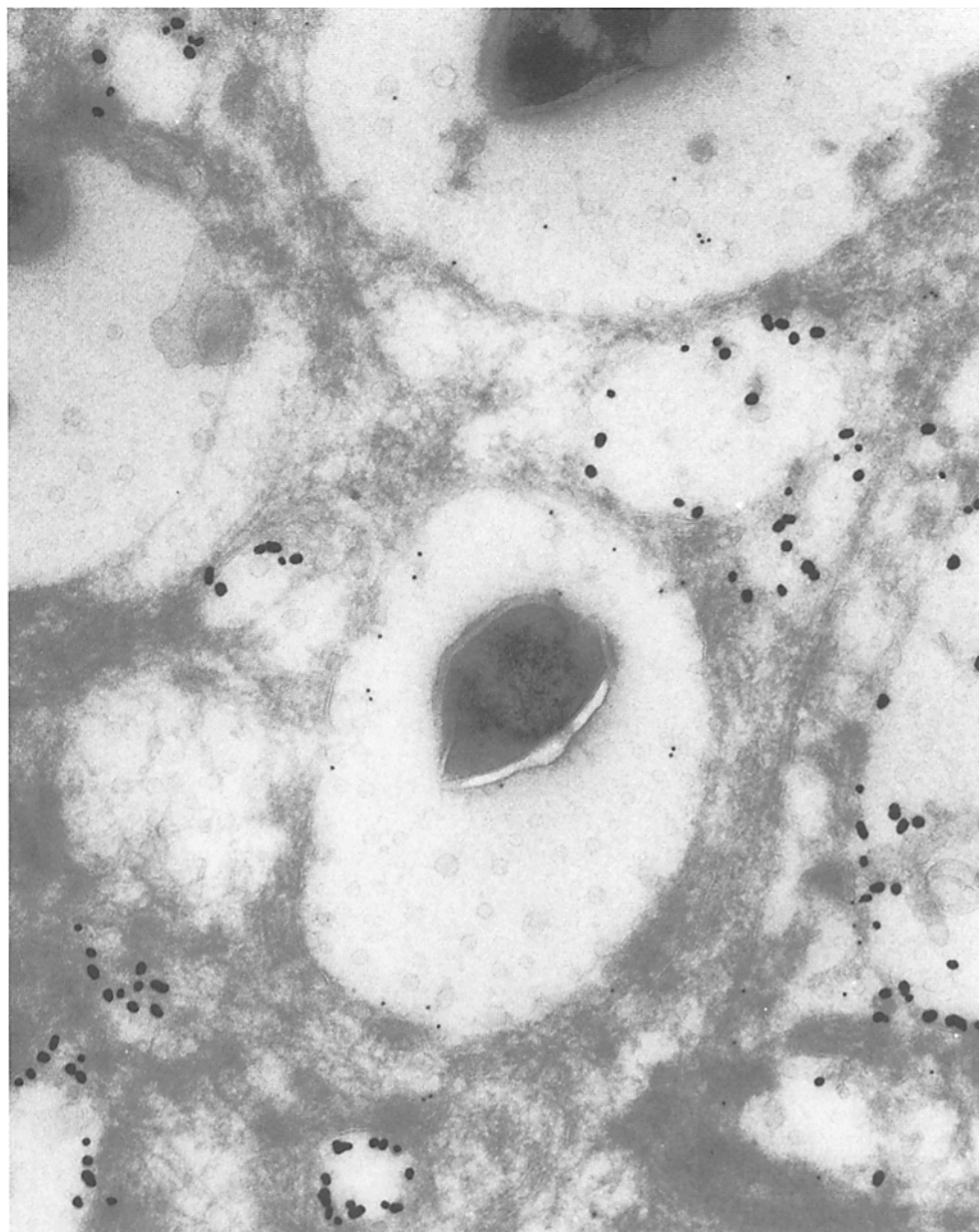
**Figure 1.** Kinetics of delivery of human transferrin to *M. tuberculosis* phagosomes. Human monocyte-derived macrophages were infected with *M. tuberculosis* for 3 d in culture medium lacking human transferrin, washed with transferrin-free culture medium, and incubated with or without human transferrin for 0–60 min as indicated. The monocytes were then fixed, prepared for electron microscopy, stained for transferrin by immunogold, and the number of gold particles per phagosome was enumerated. A phagosome was considered to stain positive if it contained two or more immunogold particles. Results are the mean  $\pm$  SD for at least two grids on which at least 20 cells were counted per grid.

inactivated fetal bovine serum (HI-FBS)<sup>1</sup>. Monocytes were allowed to adhere to the plastic for 90 min at 37°C in 5% CO<sub>2</sub>–95% air, washed three times with RPMI, and incubated in RPMI containing 15% HI-FBS for 5 d to obtain transferrin receptor-rich monocyte-derived macrophages.

In some experiments, macrophage lysosomes were labeled with BSA or mannan conjugated to colloidal gold (BSA-gold or mannan-gold) 1 d before fixation (13). The BSA-gold or mannan-gold (20–25 nm) was diluted to a final OD at 520 nm of 0.5 with RPMI containing 15% HI-FBS. Macrophages were incubated with the gold conjugates for 8 h at 37°C, washed four times with RPMI, and incubated overnight at 37°C in fresh culture medium.

**Infection of Macrophages, Incubation with Transferrin, and Localization of Antigens by Immunogold Staining of Cryosections.** Macrophages in monolayer culture were infected by incubating them with *M. tuberculosis* ( $0.5$ – $1 \times 10^6$ /ml) in RPMI containing 15% autologous human serum for 90 min at 37°C. As one control, macrophages were incubated with heat-killed (100°C  $\times$  10 min) *M. tuberculosis* ( $2 \times 10^7$ /ml). As a second control, macrophages were coincubated for 90 min at 37°C with *M. tuberculosis* and polystyrene beads (1-µm spheres, 2% solids; Polysciences) diluted 1:1,000. After phagocytosis, macrophages were washed three times with RPMI to remove nonadherent bacteria and beads and then incubated for an additional 1–3 d in RPMI containing 15% HI-FBS. Macrophage monolayers were washed three times with RPMI and incubated twice for 15 min at 37°C in serum-free RPMI (to wash out competing fetal bovine transferrin). Monolayers were

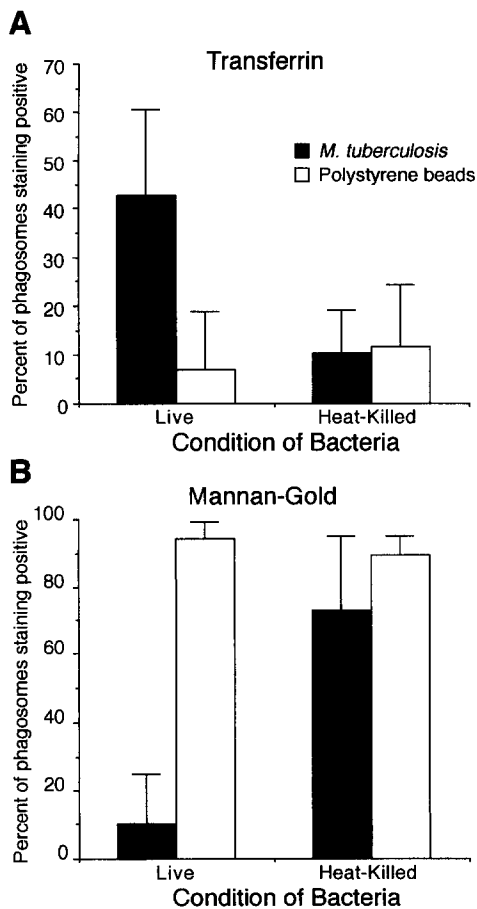
<sup>1</sup>Abbreviations used in this paper: HI-FBS, heat-inactivated fetal bovine serum; HRP, horseradish peroxidase.



**Figure 2.** Cryosection immunogold staining for transferrin in a human macrophage infected with *M. tuberculosis*. The macrophage was infected with *M. tuberculosis* and pulsed with transferrin for 1 h as in Fig. 1. Lysosomes in the macrophage were prelabeled by incubating the cells with 20 nm BSA-gold followed by an overnight chase. The *M. tuberculosis* phagosome has acquired abundant transferrin immunogold staining (10-nm gold particles) but has not fused with the BSA-gold-labeled compartments.  $\times 61,000$ .

then incubated with 1 mg/ml human holotransferrin in RPMI containing 10% autologous human serum. The dose of holotransferrin used (1 mg/ml) is similar to the level of transferrin normally present in human serum (3 mg/ml;  $\sim 40\%$  iron saturated). Infected macrophages were incubated for 0–3 h at  $37^\circ\text{C}$  and fixed with 4% paraformaldehyde, 0.01% glutaraldehyde in 0.15 M sodium cacodylate, pH 7.3, for 2 h at  $4^\circ\text{C}$ . The fixed macrophages 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 macrophages were pelleted by centrifugation at 1,000 rpm for 10 min and embedded in 10% gelatin at  $37^\circ\text{C}$ . The gelatin was solidified at  $0^\circ\text{C}$ , and the embedded cell pellet was trimmed with scalpels at  $0^\circ\text{C}$  and infiltrated with 2.3 M sucrose, 20% polyvinylpyrrolidone at  $4^\circ\text{C}$  overnight. Cryosections were obtained at  $-90^\circ\text{C}$ . Sections were

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 7.4, for 1 h at  $4^\circ\text{C}$  to block nonspecific antibody binding. Sections were incubated with preimmune serum or rabbit anti-human transferrin diluted 1:500 in HEPES buffer containing 50% NGS and 0.1% fish skin gelatin overnight at  $4^\circ\text{C}$ , washed with HEPES buffer, and incubated with goat anti-rabbit Ig conjugated to 10 nm colloidal gold (Sigma Chemical Co.) for 90 min at room temperature. For double labeling of sections for CD63, mouse mAb (20  $\mu\text{g}/\text{ml}$ ) or control isotypic myeloma Ig were included in the primary antibody incubation, and a 1:9 dilution of goat anti-mouse Ig conjugated to 5 nm colloidal gold (Amersham Corp.) 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



**Figure 3.** Delivery of transferrin and mannan-gold to phagosomes containing live or dead *M. tuberculosis* or latex beads. Monocyte-derived macrophages were coincubated with latex beads and either live or heat-killed *M. tuberculosis*. After 2 d, macrophage secondary lysosomes were labeled by incubation of the cells with mannan-gold for 8 h. The macrophage monolayers were then washed, and early endosomes were labeled by incubation of the cells with human holotransferrin for 1 h. The monolayers were fixed immediately, cryosectioned, and evaluated for transferrin immunogold staining (A) or mannan-gold staining (B) as described in Fig. 1.

microscope (model 100 CX II; JEOL USA, Peabody, MA). A latex bead phagosome or *M. tuberculosis* phagosome was considered to stain positive for transferrin if it contained two or more transferrin immunogold particles. In some experiments, photomicrographs of consecutive infected macrophages were made at magnifications of 7,200 to 14,000, and the number of gold particles per micrometer of membrane was enumerated directly from the negatives using a translucent digitizer tablet (model 2210; Numonics Corp., Montgomeryville, PA) and software program (Sigma-Scan; Jandel Scientific Co., Corte Madera, CA).

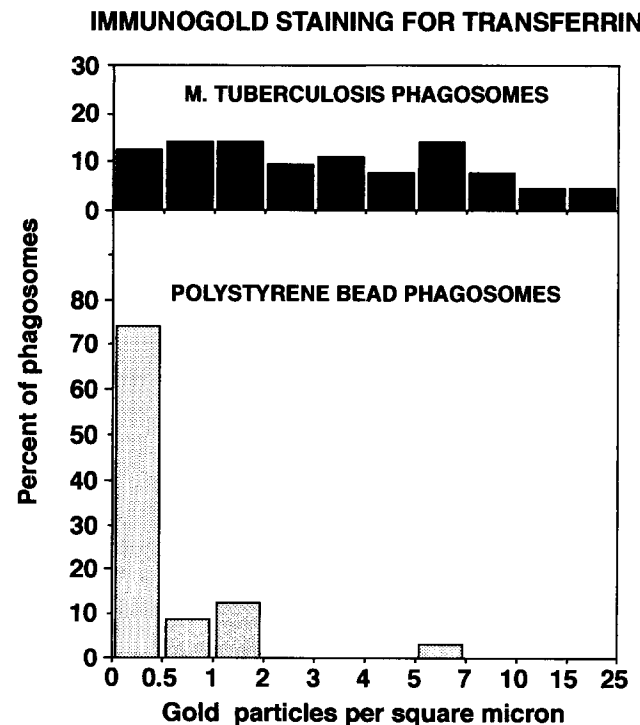
## Results

*Exogenously Administered Transferrin Is Delivered to the M. tuberculosis Phagosome.* To determine whether exogenously administered transferrin is delivered to the *M. tuberculosis* phagosome, we coincubated human monocyte-derived macrophages with *M. tuberculosis* and polystyrene beads and cultured the infected macrophages for 3 d in RPMI contain-

ing HI-FBS. The macrophages were pulsed for 0–60 min with human holotransferrin (1 mg/ml), fixed, and cryosectioned, and the sections stained specifically for human transferrin using immunogold. In control macrophages not incubated with human transferrin, only 7% of *M. tuberculosis* phagosomes stained for transferrin (Fig. 1), a level consistent with the background level of staining. In macrophages incubated with transferrin, up to 60% of *M. tuberculosis* phagosomes acquired staining for transferrin, depending on the length of incubation (Figs. 1 and 2). Incorporation of transferrin into the *M. tuberculosis* phagosome was evident at 5 min and reached a plateau at 15–30 min of incubation, kinetics of labeling consistent with interaction of the *M. tuberculosis* phagosome with early endosomes. Levels of transferrin staining of the *M. tuberculosis* phagosome at 3, 6, and 12 h were similar to that at 1 h.

Whereas transferrin was delivered to phagosomes containing live *M. tuberculosis*, transferrin was not delivered to phagosomes containing either polystyrene beads or heat-killed *M. tuberculosis* (Fig. 3 A). In contrast, mannan-gold particles added to the culture medium after phagocytosis accumulated in phagosomes containing polystyrene beads and heat-killed *M. tuberculosis* but did not accumulate in phagosomes containing live *M. tuberculosis* (Fig. 3 B).

*M. tuberculosis* phagosomes that stained for transferrin also



**Figure 4.** Distribution of staining for transferrin on phagosomes containing either *M. tuberculosis* or latex beads. Human monocyte-derived macrophages were coincubated with *M. tuberculosis* and latex beads, and after 3 d, incubated for 3 h with human holotransferrin. The macrophages were then fixed, cryosectioned, and stained for transferrin. The number of transferrin immunogold particles per micrometer of membrane was enumerated for both *M. tuberculosis* phagosomes and polystyrene bead phagosomes.

typically stain for the lysosome-associated membrane glycoprotein CD63, although staining for this marker was less intense than we have observed in phagosomes containing latex beads (4). The presence of both markers in these phagosomes suggested that these *M. tuberculosis* phagosomes interact with both early and late endosomal compartments, although we cannot rule out the possibility that the lysosome-associated membrane glycoprotein is derived directly from the trans-Golgi network, an interaction that has been proposed by Sturgill-Koszycki and coworkers (14).

**Heterogeneity among *M. tuberculosis* Phagosomes.** The *M. tuberculosis* phagosomes that stained positive for transferrin showed a wide distribution in their relative staining intensity (Fig. 4). Moreover, even after infected macrophages were incubated for 12 h with transferrin, as many as 40% of the *M. tuberculosis* phagosomes did not acquire staining for transferrin. Of these phagosomes lacking transferrin staining, approximately half stained richly for CD63, and half had little or no CD63. In additional experiments, we compared the capacity of *M. tuberculosis* phagosomes to acquire transferrin at 1 and 3 d after phagocytosis, under conditions in which the bacteria multiplied  $\sim 1$  log between these time points in the macrophage culture. We observed transferrin acquisition by  $\sim 60\%$  of the *M. tuberculosis* phagosomes at each time point. Thus, the percentage of transferrin-positive vacuoles remains constant under conditions in which the bacteria are rapidly multiplying.

Some of the heterogeneity among the *M. tuberculosis* phagosomes may reflect differences in viability or metabolic activity of the bacteria, particularly in the case of transferrin-negative vacuoles. With respect to the 60% of phagosomes staining positive for transferrin, our data indicate that these vacuoles contained predominantly if not exclusively live mycobacteria, for several reasons. First, transferrin is not acquired by phagosomes containing killed *M. tuberculosis*. Second, the percentage of phagosomes staining positive for transferrin (60%) is substantially greater than the percentage of dead mycobacteria (15%) in our inoculum. Third, these transferrin-positive phagosomes exhibit a distinctly different morphology and staining pattern from phagosomes containing dead mycobacteria. They do not fuse with BSA- or mannan-gold-labeled lysosomes, and they acquire only low to intermediate levels of CD63, whereas phagosomes containing dead *M. tuberculosis* fuse with BSA- or mannan-gold-labeled lysosomes and stain intensely for CD63. Fourth, as noted above, the percentage of transferrin-positive phagosomes does not decrease with bacterial multiplication. This argues strongly against these vacuoles being a major repository of dead or metabolically inactive bacteria.

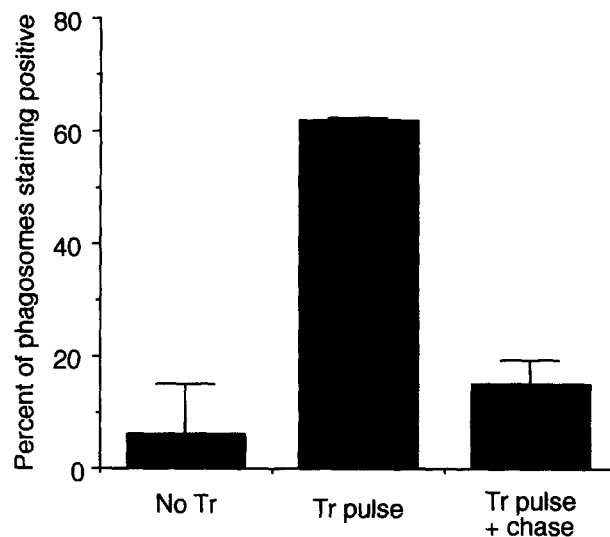
With respect to the 40% of phagosomes lacking transferrin staining, the CD63-negative phagosomes almost certainly contained live *M. tuberculosis* because these phagosomes deviate markedly from the picture seen with dead *M. tuberculosis* and latex beads. On the other hand, the 20% of the phagosomes that were transferrin negative and CD63 rich may well contain dead or metabolically inactive mycobacteria.

**Exogenously Administered Transferrin Can Be Chased out of the *M. tuberculosis* Phagosome.** In separate experiments, we examined whether human transferrin delivered to the *M. tuberculosis* phagosome could be washed out by a chase with medium lacking human transferrin. We infected human macrophages with *M. tuberculosis*, and 3 d later incubated them with transferrin for 1 h as described above. We then either fixed the macrophages immediately or fixed them after a 3-h chase in culture medium lacking human transferrin (containing instead 10% HI-FBS). Whereas  $62 \pm 0.5\%$  of *M. tuberculosis* phagosomes in macrophages fixed immediately after incubation with human transferrin stained for transferrin, only  $24 \pm 12\%$  of *M. tuberculosis* phagosomes fixed after the 3-h chase stained for transferrin (Fig. 5).

## Discussion

Exogenously added holotransferrin does not traffick to late endosomes or lysosomes and serves as a specific marker for early endosomes. Our finding that *M. tuberculosis* phagosomes acquire exogenously added transferrin demonstrates that the *M. tuberculosis* phagosome can exchange material with the early endosomal compartment.

The capacity to interact with early endosomes is a characteristic of early phagosomes as opposed to phagolysosomes. Hence, our current findings confirm our previous observation that *M. tuberculosis* retards the maturation of its phagosome along the endocytic pathway (4). In contrast to phagosomes containing live *M. tuberculosis*, phagosomes containing latex beads and heat-killed *M. tuberculosis* mature to phagolysosomes that do not interact with early endosomes and do not acquire transferrin.



**Figure 5.** Transferrin staining of *M. tuberculosis* phagosomes pulsed with human transferrin and chased with transferrin-free medium. Human macrophages were infected with *M. tuberculosis*, incubated for 3 d, and either fixed (No Tr) or incubated with human holotransferrin for 1 h at 37°C. The latter macrophages were either fixed immediately (Tr pulse) or after a 3-h chase in medium lacking human transferrin (Tr pulse + chase). Samples were sectioned and stained, and the percentage of phagosomes staining positive for transferrin were enumerated as described above.

That transferrin can be chased out of the *M. tuberculosis* phagosome by medium lacking human transferrin suggests that the interaction of the *M. tuberculosis* phagosome with endosomes is bidirectional. In other words, the phagosome can both receive material from and give material to the early endosome. Some evidence suggesting a capacity of the *M. tuberculosis* phagosome to exchange material with the endosomal pathway has also been provided by studies of the distribution of mycobacterial antigens. We have observed the 30/32-kD major secretory proteins of *M. tuberculosis* in vesicles outside of the *M. tuberculosis* phagosome (15). Xu et al. (3) reported the presence of lipoarabinomannan in vesicles outside of the mycobacterial phagosome. Although these findings are consistent with the idea that the *M. tuberculosis* phagosome interacts with the endosomal pathway in a bidirectional fashion, the possibility remains that mycobacterial antigens reach the endocytic pathway via an extracellular route.

*Mycobacterium avium* phagosomes in mouse macrophages resemble *M. tuberculosis* phagosomes in showing inhibition of both acidification and fusion with lysosomes (2, 14). In addition, de Chastelier et al. (16) observed rapid acquisition

of horseradish peroxidase (HRP) by a small percentage of *M. avium* phagosomes with a kinetic pattern consistent with interaction of the phagosome with early endosomes. With time, most of the *M. avium* phagosomes became HRP positive. However, the overall rate of acquisition of the HRP label by *M. avium* phagosomes in mouse macrophages was slower than what we observed for acquisition of transferrin by *M. tuberculosis* phagosomes in human monocyte-derived macrophages, most likely because uptake of HRP is by a different receptor-ligand system, and possibly because of differences between *M. tuberculosis* and *M. avium* phagosomes. Nevertheless, both *M. tuberculosis* and *M. avium* phagosomes exhibit retarded maturation and retain the capacity to interact with the endocytic pathway.

Our observation that the *M. tuberculosis* phagosome interacts with endosomes is relevant both to the trafficking of mycobacterial antigens within the host cell and to the potential targeting of antimicrobials to the *M. tuberculosis* phagosome. For example, it might be possible to couple antibiotics to transferrin for direct delivery to the *M. tuberculosis* phagosome.

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