***Mycobacterium*-containing phagosomes are accessible to early endosomes and reflect a transitional state in normal phagosome biogenesis**

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The success of *Mycobacterium* as a pathogen hinges on its ability to modulate its intracellular environment. *Mycobacterium avium* reside in vacuoles with limited proteolytic activity, maintain cathepsin D in an immature form and remain accessible to internalized transferrin. Artificial acidification of isolated phagosomes facilitated processing of cathepsin D, demonstrating that pH alone limits proteolysis in these vacuoles. Moreover, analysis of IgG-bead phagosomes at early time points during their formation indicates that these phagosomes also acquire LAMP 1 and cathepsin D prior to the accumulation of proton-ATPases, and are transiently accessible to sorting endosomes. This suggests that the anomalous distribution of endosomal proteins in *M.avium*-containing vacuoles results from their arrested differentiation in an early transitional stage through which all phagosomes pass.

Keywords: endosome/macrophage/*Mycobacterium*/phagocyte/phagosome

Introduction.

Mycobacterium spp. cause persistent infections within the phagocytes of their vertebrate hosts. Earlier studies indicated that the vacuoles inhabited by most mycobacterial species display a marked reluctance to fuse with host cell lysosomes (Armstrong and Hart, 1971; de Chastellier *et al.*, 1993; Xu *et al.*, 1994). Despite this apparent lack of communication between mycobacterial vacuoles and the endocytic network, recent studies employing labeling of surface-exposed, terminal *n*-acetyl glucosamine residues (de Chastellier *et al.*, 1995) or complexing of GM1 ganglioside with biotinylated cholera toxin B subunit (Russell *et al.*, 1996) have demonstrated that these vacuoles are actually highly dynamic compartments that are readily accessible to certain macrophage surface components. Furthermore, the maintenance of this regulated fusion correlates directly with the intracellular survival of the bacilli. In *Mycobacterium tuberculosis*, *M.avium* and *M.leprae* infections, activation of the host macrophage leads to fusion of the bacilli-containing compartments with lysosomes and culminates in death of the bacteria (Sibley *et al.*, 1987; de Chastellier *et al.*, 1993).

Previous characterization of mycobacterial vacuoles in

resting macrophages has demonstrated that while the vacuoles assimilate certain lysosomal constituents, such as LAMP 1 and cathepsin D (Sturgill-Koszycki *et al.*, 1994; Clemens and Horwitz, 1995), they do not exhibit the accumulation of proton-ATPases normally responsible for acidification of endosomes and phagosomes. This anomalous acquisition of lysosomal constituents sits uneasily with models of phagosome biogenesis which restrict maturation to sequential fusion with pre-existing endosomal and lysosomal compartments possessing significant numbers of proton-ATPase complexes (Desjardins *et al.*, 1994; Beron *et al.*, 1995). This discrepancy highlights current inconsistencies regarding the source, and site of incorporation into the endocytic network, of proton-ATPases (Schmid *et al.*, 1989; Fuchs *et al.*, 1994; Xie *et al.*, 1994). Although it is already known that the restricted development of mycobacterial vacuoles correlates with the bacterium's ability to survive within macrophages (Hart and Armstrong, 1974; de Chastellier *et al.*, 1993), the mechanism behind the limited-fusion capacity of these vacuoles and the effect on the hydrolytic capacity of the compartments has not been determined. Moreover, it is unclear if mycobacterial vacuoles represent compartments unique to that organism–host interaction, or reflect a stage of 'normal' phagosome biogenesis which is subverted by the infecting pathogen.

Results

***Mycobacterium avium* vacuoles acquire and retain cathepsin D in its immature form**

To resolve the anomalous distribution of the endosomal constituents LAMP 1 and the proton-ATPase within mycobacterial vacuoles, we examined the intracellular routing of certain endosomal markers to these vacuoles. In a previous immunoelectron microscopy study, Clemens and Horwitz detected cathepsin D in *M.tuberculosis*-containing vacuoles in human macrophages (Clemens and Horwitz, 1995). The lysosomal hydrolase cathepsin D is synthesized as a 51–55 kDa proenzyme that emerges from the *trans*-Golgi network and is delivered to the early endosomal system (Rijnboutt *et al.*, 1992; Delbrueck *et al.*, 1994). From this point onwards, the enzyme is subjected to cleavage(s) at the amino-terminus that result in a 48 kDa intermediate form that is processed further by an internal cleavage to yield a mature, two chain (31 and 17 kDa) proteinase (Richo and Connor, 1991). Since processing of cathepsin D requires exposure to an acid environment, the form(s) of the enzyme found in phagosomes provides a readout of the route taken through the cell prior to delivery to those compartments.

To determine the forms of cathepsin D in *M.avium*- and IgG-bead-containing phagosomes, bone marrow-derived murine macrophages were incubated together with IgG-

Fig. 1. Immunoblot of *M. avium*- and IgG-bead-containing phagosomes with anti-cathepsin D antibody demonstrating the restricted processing of cathepsin D in the *M. avium*-containing vacuoles, and the subsequent proteolysis of cathepsin D on acidification of isolated vacuoles. SDS-PAGE (12%) gels were run with 60 min IgG-bead-containing phagosomes (lane 1), and *M. avium*-containing phagosomes isolated 9 days (lane 2) and 60 min post-infection (lanes 3 and 4). Lane 4 contains 60 min *M. avium*-containing phagosomes following incubation in 50 mM acetate buffer pH 4.5, with 0.05% Nonidet P-40 at 37°C for 10 min. Vacuole preparations shown in lanes 3 and 4 were isolated in the absence of proteinase inhibitors. The samples were normalized for protein content prior to electrophoresis.

coated beads or *M. avium*, washed and left to internalize bound particles, prior to isolation of phagosomes (Chakraborty *et al.*, 1994; Sturgill-Koszycki *et al.*, 1994; Russell *et al.*, 1996). We have shown previously that 60 min post-internalization the IgG-bead vacuoles were known to be below pH 5.5, whilst the *M. avium* vacuoles had equilibrated to pH 6.3 (Sturgill-Koszycki *et al.*, 1994). SDS-PAGE and immunoblot analysis of the forms of cathepsin D in *M. avium*- and IgG-bead-containing vacuoles yielded markedly differing results (Figure 1). Cathepsin D in IgG-bead phagosomes was present in both the 48 kDa immature and 31/17 kDa mature forms. In contrast, cathepsin D in mycobacterial vacuoles was retained almost exclusively in its 48 kDa immature form, even in vacuoles isolated 9 days following infection, indicating limited processing of the enzyme.

These results demonstrate two important points in our appreciation of the properties of the mycobacterial phagosome. First, the retention of the high molecular weight 'early' form of cathepsin D bears testimony to the limited proteolytic capacity of these vacuoles; second, because cathepsin D is present in mycobacterial vacuoles in this immature form, it indicates that the enzyme has not been exposed to an acidic, proteolytic environment prior to its delivery to the vacuoles.

Contamination of vacuole preparations was assessed by quantitation and analysis of cross-contamination of phagosomes internalized by unlabeled macrophages mixed with an equivalent number of macrophages labeled metabolically with [³⁵S]methionine prior to processing (Chakraborty *et al.*, 1994; Russell *et al.*, 1996). The percentage contamination for the preparations detailed above varied between 3 and 10%, dependent on the isolation protocol. Two-dimensional SDS-PAGE analysis (data not shown) demonstrated that there was no clear selection or bias towards a subset of contaminants.

Fig. 2. (A) Graph showing the cysteine proteinase activity in *M. avium* vacuoles isolated 2 h post-infection. The activity was measured by cleavage of the fluorogenic substrate cbz-Phe-Arg-methyl- β -naphthylamide in the presence and absence of the cysteine proteinase inhibitors E64 and cbz-Phe-Tyr(Obut)-CHN₂ at 10 μ M. (B) and (C) Immunoblot of SDS-PAGE (12%) gels run with cell homogenates (lanes 1) and 2 day *M. avium*-containing vacuoles (lanes 2) probed with rabbit anti-cathepsin B (B) and anti-cathepsin L (C) antibodies. The lanes were normalized for protein content, and both cysteine proteinases could be detected in the mycobacterial vacuole preparations although the amount of cathepsin L appeared markedly less than that of cathepsin B.

Acidification of isolated *M. avium* phagosomes facilitates further processing of cathepsin D

The processing of cathepsin D in mammalian cells requires an acidic environment and additional proteolytic activity (Richo and Connor, 1991; Larsen *et al.*, 1993). The presence of the lysosomal cysteine proteinases cathepsins B and L in isolated *M. avium*-containing phagosomes was demonstrated both by enzymatic activity (Figure 2A) and by immunoblot (Figure 2B). The immunoblots indicate that cathepsins B and L are also present in early processed forms of 33 and 37 kDa respectively.

When *M. avium*-containing phagosomes were incubated at 37°C in acetate buffer, pH 4.5, cathepsin D was processed to lower molecular weight forms (Figure 1). In control preparations, isolated mycobacterial vacuoles

incubated under comparable conditions at 37°C in phosphate-buffered saline (PBS) showed minimal conversion of cathepsin D to its mature form (data not shown). Although we cannot state categorically that processing is due to cysteine proteinases within the isolated mycobacterial vacuoles rather than a low level of contamination, these results suggest that it is the elevated pH of the mycobacterial vacuole that is responsible for the limited processing of cathepsin D.

Early IgG-bead phagosomes exhibit a similar paucity of proton-ATPases and acquisition of immature cathepsin D

The presence of LAMP 1 and cathepsins D, B and L in *M.avium* phagosomes suggests that the vacuoles remain accessible to the endosomal continuum; however, the vacuoles fail to accumulate proton-ATPases and retain a relatively high pH (Sturgill-Koszycki *et al.*, 1994). Moreover, the presence of cathepsin D in an immature form suggests that the enzyme has emerged relatively recently from the cell's synthetic pathway, or at least has not passed through an acidic environment prior to its arrival in mycobacterial vacuoles. These data raise questions about the intracellular origins of phagosomal constituents, and it is unclear if this anomalous accumulation of endosomal constituents represents a phenomenon peculiar to *Mycobacterium* or whether it is the expression of an early remodeling event common to all phagosomes.

To test this, synchronously formed IgG-bead phagosomes were isolated at various time points following internalization, subjected to SDS-PAGE and analyzed by immunoblot. The results of this experiment, shown in Figure 3, reveal that within 3–5 min following phagocytosis the IgG-bead phagosomes acquire 51–55 kDa procathepsin D. The identity of the 51–55 kDa polypeptide as the proenzyme form was confirmed by immunoblotting with an affinity-purified rabbit antibody prepared against a synthetic peptide corresponding to the first 20 amino acids of the murine cathepsin D proenzyme sequence (data not shown). At later time points, the total amount of cathepsin D within the isolated IgG-bead phagosomes has increased, and the majority appears to have been processed to lower molecular weight forms. However, at these later time points, we cannot discriminate between conversion of proenzyme acquired during phagosome formation versus fusion with endosomes/lysosomes containing ready-processed cathepsin D. Analysis of the rates of acquisition for the other lysosomal constituents LAMP 1 (Figure 3A) and both the E subunit (Figure 3C) and 110 kDa transmembrane accessory protein (Figure 3D) of the proton-ATPase reveal that both LAMP 1 and procathepsin D accumulate more rapidly than the proton-ATPase complex in the IgG-bead phagosomes. Furthermore, the processing of cathepsin D to its mature form correlates with the accumulation of proton-ATPases in these vacuoles.

The data indicate that the initial source(s) of endosomal constituents accessed by forming IgG-bead phagosomes contains LAMP 1, cathepsins B, L and D yet lacks significant levels of proton-ATPases.

***Mycobacterium avium*-containing vacuoles retain access to the recycling endosomal network**

Previous analysis of glycosphingolipid trafficking in infected macrophages had suggested that mycobacterial

Fig. 3. Immunoblot of IgG-bead-containing phagosomes isolated at different time points following internalization demonstrating the differential acquisition of (A) LAMP 1, (B) cathepsin D, (C) proton-ATPase E subunit and (D) proton-ATPase 110 kDa accessory protein. Cathepsin D in the 3 min phagosome is predominantly in its proenzyme form, and precedes acquisition of detectable levels of the proton-ATPase. The samples were normalized for protein content prior to electrophoresis.

vacuoles were accessed readily by certain plasmalemma-derived markers (Russell *et al.*, 1996). To elucidate the accessibility of mycobacterial vacuoles to the early/sorting endosomal apparatus, macrophages were infected with *M.avium* 120 min or 9 days prior to the addition of 10 µg/ml of digoxigenin-labeled transferrin. Transferrin has been used as a marker of the sorting endosomal machinery because it is internalized ligated to its receptor, and, after losing its bound iron, is recycled to the cell surface without penetrating deeper into the endosomal system (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). Immunoblot analysis of isolated mycobacterial vacuoles revealed that transferrin had entered both populations of mycobacterial vacuoles (Figure 4A) whilst there was minimal delivery of transferrin to the 120 min IgG-bead phagolysosomes. Internalization and delivery of labeled transferrin to the mycobacterial vacuoles was confirmed by immunoelectron microscopy (Figure 5A). The digoxigenin-transferrin signal detected within the mycobacterial vacuoles was low, as illustrated in the distribution graph of *M.avium*- versus IgG-bead-containing phagosomes

Fig. 4. (A) and (B) Immunoblot of isolated vacuoles following incubation of macrophages with digoxigenin-labeled transferrin, demonstrating delivery to mycobacterial vacuoles and non-delivery to IgG-bead phagolysosomes. Particle-loaded macrophages were incubated with 10 $\mu\text{g/ml}$ of digoxigenin-labeled, iron-loaded transferrin for 45 min prior to lysis and isolation of 120 min IgG-bead phagosomes (lanes 1 and 2) and 120 min (lanes 3 and 4) and 9 day (lane 5) mycobacterial vacuoles. Digoxigenin-transferrin was competed with a 10-fold excess of unlabeled transferrin (lanes 2 and 4) to demonstrate that uptake was receptor specific. The gels were probed with (A) anti-LAMP 1 (1D4B) to demonstrate the relative amounts of vacuolar material, and (B) mouse monoclonal anti-digoxigenin. (C) Control preparations of cell homogenate (lane 1) and isolated *M. avium*-containing vacuoles (lanes 2 and 3) from *M. avium*-infected cells (lane 2) incubated with digoxigenin-transferrin mixed 1:1 with control cells and *M. avium*-infected cells (lane 3) mixed 1:1 with cells incubated with digoxigenin-transferrin. The gel was probed with mouse monoclonal anti-digoxigenin. Equivalent amounts of protein were loaded in each well. The level of cross-contamination with digoxigenin-transferrin during isolation of *M. avium* phagosomes is almost below the level of detection.

(Figure 5B). However, calculation of the number of gold particles per μm of vacuolar membrane demonstrates clearly the enrichment of digoxigenin-transferrin in mycobacterial (1.2×10^{-1} gold particles/ μm) versus IgG-bead (5.3×10^{-3} gold particles/ μm) phagosomes.

Control experiments were conducted to verify both the specificity of uptake and trafficking, and the contamination of mycobacterial vacuole preparations with transferrin from another source. Vacuole preparations from cells incubated in digoxigenin-transferrin in the presence of a 10-fold excess of unlabeled transferrin showed no detectable digoxigenin-transferrin, despite loading of comparable amounts of total protein (Figure 4A). To confirm that the transferrin was in the mycobacterial vacuoles, infected macrophages were scraped and combined with macrophages that had been incubated in labeled transferrin to steady-state prior to vacuole isolation. The resultant mycobacterial vacuole preparations did not contain detectable levels of labeled transferrin (Figure 4C).

Further experiments were conducted to determine the rate of clearance of transferrin from mycobacterial vacuoles. Macrophages were infected with *M. avium* for 16 h, incubated with 10 $\mu\text{g/ml}$ digoxigenin-transferrin for 10 min on ice, washed and placed in warm medium for the time points shown in Figure 6. Under these conditions, delivery of digoxigenin-transferrin to mycobacterial vacuoles showed a marked increase from 0 to 5 min, followed by a rapid decline from 5 to 15 min. The rate

of egress of iron-depleted digoxigenin-transferrin from mycobacterial vacuoles mirrored closely the overall loss of labeled transferrin from the total macrophage homogenate. Although it is feasible that some digoxigenin-transferrin re-acquired iron and was re-internalized by the macrophages, the excess of holo-transferrin in 10% fetal calf serum (FCS) present throughout the chase period minimized this potential source of background. In contrast, the identical experiment conducted with macrophages that had been fed IgG-beads 60 min previously failed to show any transferrin in IgG-bead vacuoles (data not shown).

IgG-bead phagosomes show transient access to the recycling endosomal network

While there are clear differences between endosomal and phagosomal differentiation, our hypothesis is that mycobacterial vacuoles are arrested in a transitional state in the differentiation of 'normal' phagosomes. Such a model would predict that IgG-bead phagosomes would show transient mixing with transferrin-loaded recycling endosomes which deliver the ligand to the cell surface or back to sorting endosomes (Ghosh and Maxfield, 1995). To test this, macrophages were incubated in digoxigenin-transferrin to steady-state, and IgG-bead phagosomes were isolated at the same time points shown in Figure 3. Consistent with the model, transferrin was found in IgG-bead phagosomes at 3 min, reached peak value near 5 min and was virtually undetectable at 30 min (Figure 7A). Some of the transferrin at the earliest time point may have been internalized together with the IgG-beads; however, the increase in digoxigenin-transferrin at between 3 and 5 min suggests access to a transferrin-rich intracellular compartment. Controls performed by isolation of IgG-bead phagosomes from cells mixed with macrophages incubated to steady-state with labeled transferrin demonstrated undetectable levels of contamination from non-vacuolar sources. The results further support our contention that *M. avium*-containing vacuoles represent the stabilization of a state that is transitional to the maturation of all phagosomes.

Discussion

The macrophage provides the primary defence against microbial infection and is equipped with a range of microbicidal responses; however, its ability to adhere to and internalize particles also increases its exposure to possible colonization (Russell *et al.*, 1996). Different intracellular pathogens have evolved to exploit diverse niches within their host cell. Some escape into the cytoplasm, others are immune to digestion within the lysosome and the third group, which includes pathogenic Mycobacterium species, prevents the normal maturation of their phagosome into a lysosomal compartment. Although the non-fusion of mycobacterial vacuoles with lysosomes was observed 25 years ago by Hart and colleagues (Hart *et al.*, 1972, 1987; Hart and Armstrong, 1974), the exact nature of this compartment and its relationship to other endocytic vesicles remains elusive. More recently, it has been demonstrated that while the mycobacterial vacuole is poorly acidified through its failure to accumulate proton-ATPase complexes (Sturgill-Koszycki *et al.*, 1994; Oh and Straubinger, 1996), it does acquire some lysosomal con-

Fig. 5. (A) Immunoelectron micrograph demonstrating delivery of digoxigenin-transferrin to *M. avium*-containing vacuoles. Murine bone marrow macrophages were infected 16 h previously with *M. avium*, allowed to internalize IgG-beads for 2 h, and incubated in 10 µg/ml digoxigenin-transferrin for 45 min prior to processing. Digoxigenin-transferrin was detected with mouse anti-digoxigenin/goat anti-mouse IgG (18 nm gold, arrows) and LAMP 1 was detected with ID4B/goat anti-rat IgG (6 nm gold, arrowheads). Although the label was low, digoxigenin was observed consistently within the mycobacterial vacuoles. Scale bar = 0.5 µm. (B) A graph displaying the distribution of digoxigenin-transferrin label in *M. avium* vacuoles versus IgG-bead phagolysosomes. Fifty vacuoles of each type from two independent experiments were scored.

stituents and exhibits access to certain endocytosed materials (Sturgill-Koszycki *et al.*, 1994; Xu *et al.*, 1994; Clemens and Horwitz, 1995; de Chastellier *et al.*, 1995; Russell *et al.*, 1996). This behavior appears to deviate from the normal maturation of phagosomes, yet the precise branch point and the mechanism have not been established.

Phagocytosis is a highly dynamic process that involves the remodeling of an appreciable area of plasmalemma through intracellular fusion and fission events (Clemens and Horwitz, 1992; Desjardins *et al.*, 1994; Beron *et al.*,

1995; Gruenberg and Maxfield, 1995); therefore, understanding the influence that *Mycobacterium* species exert over this process requires examination of many factors. In this current study, we present data demonstrating that *M. avium*-containing vacuoles retain cathepsin D in an immature form, suggesting that it has been derived relatively recently from the cell's synthetic pathway. Moreover, *M. avium* vacuoles remain accessible to the transferrin recycling pathway of the macrophage. These results, coupled with our earlier observations regarding the paucity

Fig. 6. Immunoblots demonstrating the kinetics of transit of digoxigenin-transferrin through mycobacterial vacuoles. The gels were run with macrophage homogenates (A) and isolated *M. avium*-containing vacuoles (B and C). Infected macrophages were incubated with 10 µg/ml digoxigenin-transferrin on ice, then washed and transferred to medium at 37°C for the time period indicated. (A) and (B) were probed with anti-digoxigenin, demonstrating the rate of loss of transferrin from the whole cell (A) versus the increase, from 0 to 5 min, and decrease, from 5 to 15 min, of transferrin in the mycobacterial vacuoles (B). (C) The mycobacterial vacuole lanes probed with ID4B, anti-LAMP 1 to demonstrate equivalent loading of material from the different time points.

of proton-ATPases and the ready delivery of glycosphingolipid from the cell surface to *M. avium* and *M. tuberculosis* vacuoles (Russell *et al.*, 1996), indicate that mycobacteria arrest the normal maturation of their phagosomes prior to fusion with acidic endosomes or lysosomes. These data, together with their IgG-bead controls, have implications for both survival of *Mycobacterium* and the process of phagosome maturation.

Current models of phagosome maturation invoke multiple fusion events with ready-formed endocytic compartments facilitating acquisition of endosomal constituents prior to fusion with lysosomal compartments (Mellman, 1992; Beron *et al.*, 1995; Desjardins, 1995; Gruenberg and Maxfield, 1995). The intracellular routing of certain endosomal constituents prior to their addition to the endosomal network, most notably the proton-ATPases responsible for acidification of intracellular vacuoles, remains to be defined. Data presented here suggest that LAMP 1 and cathepsin D precede proton-ATPases into maturing phagosomes. Early phagosomes may acquire LAMP 1 and cathepsins through fusion with an early or 'pre'-endosomal vesicle, or directly from vesicles derived from the *trans*-Golgi network, as indicated in a recent study on intracellular targeting of LAMP 1 (Rohrer *et al.*, 1996), but in either case our results indicate that such a delivery vesicle lacks significant numbers of proton-ATPases. This suggestion is consistent with previous studies on the trafficking of cathepsin D in mammalian cells which report that the conversion of the 51–55 kDa proenzyme through the 48 kDa immature enzyme is initiated prior to delivery to early, or 'light', endosomal compartments (Ludwig *et al.*, 1991; Rijnboutt *et al.*, 1992; Delbrueck *et al.*, 1994), and the appearance of the mature, two chain enzyme is restricted to late endosomes or lysosomes (Rijnboutt *et al.*, 1992). This

Fig. 7. Immunoblot demonstrating that the acquisition of labeled transferrin by IgG-bead phagosomes was a transient phenomenon, peaking at 5 min following internalization. IgG-bead phagosomes isolated at different time points following internalization by macrophages incubated to steady-state (45 min) with 10 µg/ml digoxigenin-transferrin prior to and during phagocytosis. The samples were normalized for protein content prior to electrophoresis.

latter processing requires an acid pH thought to induce a structural change in the protein; however, autocleavage of procathepsin D at low pH does not generate the forms of the enzyme found in nature, indicating that other hydrolases, possibly cathepsins B and L, are involved (Richo and Connor, 1991; Larsen *et al.*, 1993). The sequence of acquisition of endosomal/lysosomal constituents, and the form of cathepsin D found in the bacterial vacuoles, suggests that *Mycobacterium* restricts transition of its vacuole to a proton-ATPase-rich compartment. The rate of accumulation of proton-ATPases in IgG-bead phagosomes mirrors closely the accumulation of proton-ATPase reported in *Staphylococcus aureus*-containing phagosomes in J774 cells (Pitt *et al.*, 1992).

Proton-ATPases are present on the plasmalemma; however, they are usually found in low density except when recruited to the cell surface by a specific signal, such as activation of neutrophils (Grinstein *et al.*, 1992; Nanda *et al.*, 1992). Their point of addition to the early endocytic network has not been determined. Experiments by Fuchs and colleagues demonstrated that early endocytic vesicles did not exhibit ATP-dependent acidification (Fuchs *et al.*, 1994), whereas other investigators showed that the mildly acidic pH of the sorting/recycling machinery was sensitive to the V-ATPase inhibitor bafilomycin A (Johnson *et al.*, 1993). Intriguingly, weak bases and inhibitors of proton-ATPase activity, such as bafilomycin A, have profound effects on the retention of material within the sorting and recycling endosomal machinery, suppressing its delivery to lysosomes (Clague *et al.*, 1994; van Weert *et al.*, 1995). Furthermore, the treatment of cells with lysosomotropic weak bases can modify the fusion capacity of phagosomes markedly. Chloroquine enhances phagosome-lysosome fusion (Hart and Young, 1978), whilst ammonium chloride, which also affects cytosolic pH, suppresses phagosome-lysosome fusion but enhances phagosome-endosome fusion (Hart and Young, 1991). These data indicate an intimate relationship between acidification and the passage of material through to the late endosomal-lysosomal system. Our results suggest that it is the exploitation of this decision point encountered early in phagosome biogenesis that allows *Mycobacterium* to survive in macrophages by avoiding fusion with lysosomes. Rather than inhibit intracellular fusion, *Mycobacterium* appears to have evolved a mechanism to sustain the regulated, homotypic fusion of early endosomal vesicles, as demonstrated through their access to transferrin and GM1 (Russell *et al.*, 1996) from the plasmalemma. This may be a general feature of early endosomes which exhibit homotypic fusion in *in vitro* fusion assays (Gorvel *et al.*, 1991; Mayorga

et al., 1991) yet resist fusion with late endosomes or lysosomes. Therefore, rather than actively inhibit fusion with lysosomes, mycobacterial vacuoles may merely retain the fusion facilitators responsible for endosome–endosome fusion which appear to be non-permissive for endosome–lysosome fusion.

The maintenance of this vectorial fusion profile by mycobacterial vacuoles appears to correlate with the ability of *Mycobacterium* species to survive in macrophages. Whilst low pH is bacteriostatic to *M.bovis* (BCG) and *M.tuberculosis*, *M.avium* continues to grow at pH 5.0 (Oh and Straubinger, 1996; D.G.Russell, unpublished data). It is unlikely, therefore, that pH alone is responsible for the death of intracellular bacilli. Once macrophages are activated, there is a marked increase in the fusion of mycobacterial vacuoles with lysosomes (Sibley *et al.*, 1987; Orme *et al.*, 1993). Activation of murine macrophages by interferon- γ and tumor necrosis factor- α is accompanied by the up-regulation of superoxide production, and expression of the inducible nitric oxide synthase (iNOS) (Nathan and Hibbs, 1991). Inhibitors of iNOS prevent the clearance of *Mycobacterium* species from infected mice and murine macrophages. It is likely that the fusion of lysosomes with mycobacterial vacuoles leads to a drop in pH which is conducive to the cumulative microbicidal activities of nitric oxide, superoxide and lysosomal hydrolases. We currently are examining the alterations in mycobacterial vacuoles following macrophage activation and correlating these changes with the viability of the infecting bacilli.

Our data indicate that the ability of pathogenic *Mycobacterium* to manipulate the differentiation of their phagosomes provides invaluable insights, not only into the virulence strategies of an important group of pathogens, but also into the decision points active in normal phagosome biogenesis.

Materials and methods

Cell and bacterial culture

Experiments were performed with bone marrow-derived macrophages isolated from BALB/c mice and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 5% horse serum, and 20% L-cell conditioned medium. Macrophages were cultured in bacteriological Petri dishes for 7 days, then transferred to tissue culture-treated T75 flasks at a density of $5\text{--}8 \times 10^6$ cells, and left for 24 h to establish a monolayer. All bone marrow macrophages were used between 1 and 3 weeks in culture.

Mycobacterium avium 101 is a highly mouse-virulent, clinical isolate that was used for all the phagosome isolation procedures. The bacteria were maintained in Middlebrook broth (Difco) supplemented with OADC. Initial colonies were obtained from the spleens of infected mice, expanded and frozen in aliquots. Since loss of virulence has been associated with continuous passage of mycobacteria in liquid culture, freshly thawed aliquots were passaged no more than once before infection of macrophages. Fresh aliquots have a >85% smooth colony morphology, the phenotype associated with virulence, and were >75% viable before infection, as determined by plating and fluorescence viability assay (McDonough and Kress, 1995).

Phagosome isolation techniques

IgG-bead phagosomes. Tosyl-activated magnetic beads (Dynal) were covalently linked to human IgG to facilitate phagocytosis via the macrophage's Fc receptors. In routine preparations, beads were added to a monolayer of bone marrow-derived macrophages at 4°C for 10 min to allow binding to the cell surface. The cells were placed at 37°C to allow phagocytosis to proceed for 15 min, after which the macrophage monolayer was washed to remove excess beads. Warm medium was

added and phagocytosis was allowed to continue at 37°C for the designated amount of time. Cells were scraped into homogenization buffer (250 mM sucrose, 20 mM HEPES pH 7.0, 0.5 mM EGTA, 0.1% gelatin plus proteinase inhibitors) and IgG-bead phagosomes were isolated from cells disrupted by multiple passage through a tuberculin syringe with a 25 gauge needle. The cell lysate was layered onto 15% high molecular weight Ficoll in homogenization buffer and centrifuged at 900 g for 10 min at 4°C. Following removal of the supernatant, the pellet was gently resuspended and layered over 300 μ l of 30% sucrose in a microfuge tube. The tube was placed on the Magnetic Particle Concentrator (Dynal) with the tip towards the magnet to allow the bead phagosomes to run through the sucrose cushion to the base of the tube. This procedure was repeated 3–4 times.

For the time windows shown in Figure 3, IgG-beads were bound to macrophages on ice for 10 min, washed gently and placed in medium at 37°C on a hot block for the period stated, prior to scraping and isolation.

***M.avium*-containing vacuoles.** Bone marrow macrophages were infected with late log-phase *M.avium* at 20 bacteria/macrophage in DMEM with 10% FCS, 5% horse serum and no antibiotic for 30 min, washed, and left to complete internalization at 37°C in fresh medium for the period stated. The infected cells were scraped into homogenization buffer (see above) and lysed by passage through a tuberculin syringe. Following centrifugation at 200 g for 5 min, the supernatant was run, under gravity, through a 47 mm 5 μ m pore Nucleopore filter, followed by 5 ml of homogenization buffer. The flow-through was loaded onto a step gradient consisting of 50 and 12% sucrose and centrifuged at 800 g for 45 min at 4°C. The 12–50% interface was removed, loaded onto a 15% low molecular weight Ficoll cushion, and centrifuged at 1300 g for 45 min at 4°C to concentrate the *M.avium*-containing vacuoles at the base of the tube. Examination of the isolated vacuoles by immunofluorescence revealed that >90% of the bacilli were LAMP 1 positive, confirming that the bacteria were retained inside phagosomes.

Following isolation, aliquots of vacuoles were quantitated using a bacterial counting chamber. The length of a mycobacteria phagosome is 1 μ m while that of a bead is 3 μ m; however, the mycobacteria vacuole has a greater luminal volume than that of a bead. We found that a ratio of two mycobacterial vacuoles to one bead vacuole contained roughly equivalent quantities of protein when compared with material from macrophages labeled with [35 S]methionine. This was confirmed by SDS-PAGE and immunoblotting of the phagosomes with an antibody against LAMP 1. Both phagosomes acquire LAMP 1, although the membrane density of this marker is lower in the mycobacterial phagosomes.

Assaying the purity of isolated phagosomes

The level of contamination within the phagosome preparations was assayed by the following procedures. To evaluate the overall purity of isolated vacuoles, four T75 flasks of macrophages were prepared, two of which were metabolically labeled with [35 S]methionine overnight. Particles were added to one labeled and one unlabeled flask. The cells from all four flasks were scraped and combined for processing as follows. The labeled cells with particles were combined with unlabeled cells with no particles, and the unlabeled cells with particles were combined with the labeled cells with no particles. After the isolation procedures were completed, an equivalent aliquot from the two samples was measured for radioactivity. The percentage contamination was calculated as c.p.m. from particles in unlabeled cells/c.p.m. from particles in labeled cell + c.p.m. from particles in unlabeled cells $\times 100$. In the protocols given, the levels of contamination varied between 3 and 5% for IgG-bead phagosomes and between 6 and 10% for *M.avium* vacuoles. Two-dimensional PAGE analysis of labeled proteins co-purified with phagosomes from unlabeled cells indicated that there was no selection or enrichment of specific contaminating proteins.

To evaluate the level of contamination with specific proteins, such as the digoxigenin-labeled transferrin employed in the trafficking experiments, particles were added to flasks on macrophages that were scraped and combined with an equivalent number of macrophages incubated with labeled transferrin as described for individual experiments. Equivalent levels of protein were loaded onto gels that were immunoblotted for detection of digoxigenin.

SDS-PAGE and immunoblotting

An equivalent amount of sample was loaded onto each lane of a 12% polyacrylamide gel. The electrophoresed proteins were transferred to nitrocellulose and blocked at 4°C overnight in PBS with 5% non-fat milk with 5% goat serum.

The blots were washed in wash buffer (PBS, 0.05% Tween-20,

0.05% Triton-114) and incubated in primary and horseradish peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Labs). The blots were developed with Luminol chemiluminescence reagent (Pierce). Although each gel lane was loaded with the same amount of protein and can be compared with other lanes on the gel blotted with the same antibody, this approach does not allow quantitative comparison between different gels.

Rabbit anti-procathepsin D antibody was raised against ovalbumin coupled to a synthetic peptide corresponding to the first 20 amino acids of the murine cathepsin D proenzyme sequence (IRIPLRKFT-SIRRTMTEVG) (Diedrich *et al.*, 1990; Grusby *et al.*, 1990). Other antibodies used were the monoclonal antibodies ID4B (LAMP 1) (Chen *et al.*, 1988), E11 (E subunit proton-ATPase), N2 (110 kDa proton-ATPase accessory protein) (Sturgill-Koszycki *et al.*, 1994) and 17C (digoxigenin) (Boehringer Mannheim) and affinity-purified polyclonal rabbit anti-cathepsin D and rabbit antiserum against cathepsins B and L.

Cysteine proteinase assays

Isolated phagosomes were suspended at comparable protein concentrations in 100 µl of 50 mM phosphate buffer pH 6.2 containing 1.2 mM EDTA, 4 mM cysteamine, 350 µM cbz-Phe-Arg-methyl-β-naphthylamide and 1% Triton X-100 and incubated at 37°C for 1 h in the presence and absence of the cysteine proteinase inhibitors E.64 and cbz-Phe-Tyr(Obut)-CHN₂ at 10 µM. The reaction was terminated by addition of 1 ml of 200 mM bicarbonate buffer pH 9.0, and fluorescence was measured at 340 nm excitation/406 nm emission in a Perkin Elmer fluorimeter.

Immunoelectron microscopy

Macrophages were processed for immunoelectron microscopy as described previously (Russell, 1994). In brief, cells were washed in medium and then fixed in 4% formaldehyde and 0.5% glutaraldehyde in HEPES saline pH 7.0. Fixed cells were scraped, pelleted, embedded in gelatin and infiltrated with 2.3 M sucrose/20% polyvinyl pyrrolidone in PBS. The blocks were trimmed, frozen and sectioned with an RMC MT7/CR21 cryoultramicrotome, and probed with antibodies.

All labeling experiments were conducted, in parallel, with controls omitting the primary antibody, and in the presence of 'non-immune' IgG or irrelevant monoclonal antibodies. These controls were consistently negative (<5%) at the concentrations of gold-conjugated second antibodies used in these studies.

The antibodies used were ID4B, a rat monoclonal antibody against LAMP 1 (from the Developmental Hybridoma Bank, NICHHD); and 17C, a mouse monoclonal antibody against digoxigenin (Boehringer Mannheim, IN). The gold-conjugated second antibodies, goat anti-rat IgG (5 nm gold) (Amersham Life Science, IL) and goat anti-mouse IgG (18 nm gold) were checked for species cross-reactivity in all double-labeling experiments. Each of the immunoelectron microscopy experiments described was repeated at least three times.

Quantitation of the distribution of label was scored by counting the number of gold particles in 50 mycobacterial vacuoles and 50 IgG-bead phagosomes in two independent experiments. No more than five vacuoles were scored in an individual cell. The final values are displayed as a distribution bar graph indicating the percentage of vacuoles containing the relative number of gold particles and as the relative densities of gold particles/µm.

Transferrin trafficking experiments

Iron-loaded human transferrin was labeled with a 10-fold molar excess of *n*-hydroxysuccinimide digoxigenin (Boehringer Mannheim) in PBS, pH 7.6, on ice for 20 min. Labeled transferrin was separated from unreacted label by gel filtration. Because the level of substitution can affect the trafficking of transferrin inside cells (Russell *et al.*, 1992; Russell, 1994), we confirmed that uptake was specific by co-localization of digoxigenin-transferrin with the macrophage transferrin receptor by immunofluorescence, and by outcompeting uptake by addition of a 10-fold excess of unlabeled transferrin.

Macrophages were incubated with IgG beads or mycobacteria as described, and left for 60 min, or infected with mycobacteria and left for 9 days. Macrophages were depleted of transferrin in DMEM containing 1% serum for 30 min, and then incubated for 45 min with 10 µg/ml digoxigenin holo-transferrin. Matched sets of macrophages were incubated with a 10-fold excess of unlabeled holo-transferrin to prevent the receptor-mediated uptake of the digoxigenin-transferrin. The macrophages were washed in PBS, the respective phagosomes isolated and normalized for protein content, and SDS-PAGE and immunoblotting with an anti-digoxigenin antibody performed.

For the pulsed-labeling experiments of mycobacteria phagosomes,

macrophages were infected for 2 h with mycobacteria, washed and left for an additional 14 h. The monolayer was incubated for 30 min in DMEM with 1% serum at 37°C, then with 10 µg/ml digoxigenin transferrin for 10 min at 4°C to promote binding of the ligand to its receptor. The macrophages were warmed to 37°C for 5 min to allow for internalization of the ligand, the cells washed extensively and internalization allowed to continue in the presence of 10% FCS. Phagosomes were isolated immediately following binding, 0 min, and at 5, 15 or 60 min following internalization. Samples were prepared for SDS-PAGE and immunoblotting with anti-digoxigenin antibody.

In the experiments designed to determine access of IgG-bead phagosomes to transferrin-containing compartments, macrophages were incubated with digoxigenin-labeled holo-transferrin for 45 min prior to and following binding and internalization of the beads. The IgG-beads were bound on ice, washed and allowed to internalize in medium at 37°C for the times stated.

Acknowledgements

The authors are extremely grateful to Drs S.Kornfeld, D.Buttler and M.Gottesman for their generous gifts of anti-cathepsin antibodies, and to Drs P.Schlesinger, D.Sibley and D.Goldberg for their critical comments during the course of this study. This work was supported by USPHS grants AI 33348 and HL 55936 to D.G.R. D.G.R. is a recipient of the Burroughs Wellcome Scholar Award in Molecular Parasitology. U.E.S. was a recipient of a Deutsche Krebsforschungszentrum AIDS-Stipendium fellowship.

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Received on July 25, 1996; revised on September 6, 1996