Intracellular Fate of *Mycobacterium leprae* in Normal and Activated Mouse Macrophages

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*Mycobacterium leprae* replicates within mononuclear phagocytes, reaching enormous numbers in the macrophage-rich granulomas of lepromatous leprosy. To examine the capability of macrophages to digest *M. leprae*, we studied the intracellular fate of *M. leprae* organisms in normal and activated mouse macrophages by using the electron-dense secondary lysosome tracer Thoria Sol. Intracellular *M. leprae* organisms, surrounded by a characteristic electron-transparent zone, were contained within phagosomal vacuoles of macrophages cultured in vitro for 1 to 6 days. In normal macrophages, a majority of phagosomes containing freshly isolated live *M. leprae* cells resisted fusion with Thoria Sol-labeled lysosomes. The extent of fusion was not significantly affected by pretreatment of *M. leprae* with human patient serum high in specific immunoglobulin G and M antibodies. In contrast, a majority of phagosomes containing gamma-irradiated *M. leprae* cells underwent lysosome fusion in normal macrophages. In addition, increased phagolysosome fusion was observed with live *M. leprae*-containing phagosomes in macrophages activated with gamma interferon. Increased fusion was associated with an increase in the number of fragmented and damaged bacilli, suggesting that increased digestion followed fusion. This study indicates that activated macrophages may have an increased capacity for clearance of normally resistant *M. leprae*.

The enormous number of intracellular *Mycobacterium leprae* organisms within granuloma macrophages of lepromatous leprosy patients (30, 31) and infected nude mice (4) indicates that *M. leprae* survives the microbicidal capacity of normal macrophages. Indirect evidence suggests that activation of macrophages for nonspecific microbicidal activity leads to killing and clearance of *M. leprae* in mice (22) and in human patients (5, 6, 29). Among the microorganisms which survive in mononuclear phagocytes by interfering with digestive processing, *Mycobacterium tuberculosis* (1), *Legionella pneumophila* (16), and *Toxoplasma gondii* (20) all reside in modified phagosomes that resist fusion with host cell lysosomes. However, in activated macrophages, enhanced microbicidal capacity leads to increased rates of lysosome fusion and digestion of these microorganisms (17, 37, 38).

Although numerous ultrastructural observations of *M. leprae*-infected tissues have been reported (2, 9, 10, 18, 21, 26, 31, 33), the intracellular processing of the bacilli has not been systematically examined with relation to host cell lysosomal contents. In the present study, we characterize the intracellular compartments occupied by *M. leprae* by using Thoria Sol as a specific marker for secondary lysosomes of normal and activated mouse macrophages.

**MATERIALS AND METHODS**

**Cell culture.** Resident peritoneal macrophages were harvested from Swiss Webster mice (Simonsen Laboratories, Gilroy, Calif.) in Hanks balanced salt solution (HBSS; Gibco Laboratories, Grand Island, N.Y.) containing 10 U of heparin (Sigma Chemical Co., St. Louis, Mo.) per ml. Peritoneal cells were plated in two-chambered Lab-Tek slides (Miles Scientific, Naperville, Ill.) with RPMI 1640 containing 20% heat-inactivated fetal calf serum (FCS; Hyclone, Sterile Systems, Inc., Ogden, Utah), 25 mM 2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, and 100 U of penicillin (GIBCO) per ml. After 4 h, nonadherent cells were removed by rinsing, and the monolayers of confluent adherent cells were recultured in RPMI–20% FCS. Activated peritoneal macrophages, obtained from Swiss Webster mice harboring chronic C-56 strain *T. gondii* infection (23), were cultured as above with the addition of 250 U of recombinant murine gamma interferon supplied by H. Michael Shepard (Genentech, Inc., San Francisco, Calif.), per ml and 2 ng of endotoxin (*Escherichia coli* O111:B4; Sigma) per ml. Activated macrophages were also obtained by stimulation of normal resident peritoneal macrophages with either concanavalin A-stimulated spleen cell lymphokines as described previously (35) or with recombinant murine gamma interferon as above.

**Thoria Sol labeling.** Macrophage monolayers were incubated for 4 h at 37°C with electron-dense colloidal Thoria Sol (Polysciences, Warrington, Pa.) diluted 1:80 in RPMI–20% FCS. After repeated washing in HBSS, monolayers were recultured in RPMI–20% FCS for 16 h before infection.

**Purification of *M. leprae*.** *M. leprae* was harvested from footpads of HSD nu/nu mice (Harlan Sprague Dawley, Indianapolis, Ind.) injected 12 months previously with 10⁷ *M. leprae* organisms as previously described (3). Footpad tissue was minced in HBSS and homogenized with a ground-glass homogenizer (Wheaton Scientific, Milville, N.J.). Homogenized tissue was incubated at 37°C for 2 h in HBSS containing 15 U of collagenase (Cappel-Worthington Biochemicals, Malvern, Pa.) per ml and 25 μg of DNase (Calbiochem, San Diego, Calif.) per ml. A 1-ml sample of the enzyme-digested suspension was layered onto gradients consisting of 8 ml of 50% Percoll (Pharmacia, Inc., Piscataway, N.J.) in RPMI above a 2-ml cushion of 100% Percoll. *M. leprae* cells were purified from the less dense mouse tissue debris by sedimentation at 2,700 × g for 90 min. Density marker beads (Pharmacia) were run in separate
In vitro infection. For some experiments M. leprae were preincubated for 30 min in a 1:10 dilution of human patient serum (Gillis W. Long Hansen's Disease Center Serum Bank) containing high levels of immunoglobulin G (IgG), IgM, and IgA antibodies to M. leprae as determined by enzyme-linked immunosorbent assay for anti-phenolic glycolipid-1 antibody (titers of 1:932, 1:1,294, and 1:736, respectively) (36). Gamma-irradiated (25 megarads) armadillo-derived M. leprae cells purified by the method of Draper (P. Draper. Report of the Fifth Meeting of the Scientific Working Group on the Immunology of Leprosy, Geneva, Switzerland, 1980) were obtained from Patrick Brennan, Colorado State University, Fort Collins, Colo., and stored at 4°C in HBSS until used. Macrophage monolayers were infected with M. leprae by incubation at 37°C for 15 min at a challenge ratio of 10 bacilli per adherent cell. Extracellular M. leprae organisms were removed by rinsing, and monolayers were cultured in RPMI-20% FCS.

Electron microscopy (EM). Monolayers were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 2 h at 4°C and postfixed with 1% osmium tetroxide containing 0.5% potassium ferricyanide for 1 h at room temperature. Monolayers were stained for 30 min with 1% uranyl acetate, dehydrated in ethanol, and embedded in LR white resin (London Resin Co., Ltd., Hampshire, England). Sections ranging in thickness from 60 nm to 300 nm were stained with lead citrate and examined using a Phillips 410 electron microscope operated at 60 or 100 kV accelerating voltage.

RESULTS

Separation of M. leprae organisms by Percoll gradient centrifugation resulted in concentration of bacilli into two bands which sedimented with a buoyant density of 1.09 to 1.10 g/ml. The majority of tissue debris remained at the surface of the gradient. Analysis of ATP content indicated that Percoll purification substantially enriches for ATP-rich bacilli. The mean (± standard deviation) concentrations of ATP were as follows: for the crude homogenate, 351.07 ± 35.86, pg/10⁶ cells; for the surface layer, 143.7 ± 8.5 pg/10⁶ cells; for the lower bands, 696.48 ± 138.40 pg/10⁶ cells (n, 3 to 5). No difference in ATP content between the lower two bands was found; therefore these bands were pooled for further use. Morphologically, bacilli in these two lower bands were substantially free of host cell debris as judged by acid-fast stained preparations counterstained with soluble blue and by direct EM examination of negatively stained material on carbon-Formvar-coated grids (data not shown).

Purification was essential because our initial attempts in feeding crude homogenates to macrophages led to an intracellular concentration of large masses of bacilli, which was observed with the purified preparations used for the EM studies reported here.

The intracellular location of M. leprae was examined by conventional thin-section EM of macrophages fixed at 1 h, 14 h, and 6 days postinfection. There was no apparent morphologic difference between intracellular live and gamma-irradiated M. leprae cells. Both live and gamma-irradiated M. leprae cells were contained within membrane-bound vacuoles in the cytoplasm of infected macrophages and were surrounded by a prominent electron-transparent zone (ETZ) (Fig. 1). The appearance and size of this ETZ was similar in both normal and activated macrophages at 1 and 6 days postinfection. No evidence of intracellular replication of M. leprae was observed.

The extent of lysosome fusion with M. leprae-containing phagosomes was evaluated using both thin (60-nm) and thick (200- to 300-nm) sections. M. leprae-containing vacuoles which failed to fuse with lysosomes remained in membrane-bound phagosomes that were segregated from Thoria Sol-labeled lysosomes. Amorphous, electron-dense material was occasionally observed within these phagosomes but surrounding the ETZ (Fig. 2). Fusion of M. leprae-containing vacuoles with lysosomes resulted in Thoria Sol occupying the same compartment with M. leprae cells (Fig. 3). Thoria Sol was excluded from the ETZ surrounding the bacterial cell. On closer examination, it was evident that the ETZ is often bordered by an electron-dense coat. However, this outer edge of the ETZ does not have a unit membrane profile characteristic of the phagolysosome membrane (Fig. 4).

Thick sections produced suitable images when examined at an accelerating voltage of 100 kV. Thick sections were more useful for evaluating the presence or absence of Thoria Sol within M. leprae-containing compartments due to the increased depth of the specimen and reduced tearing of the section associated with thin sectioning of M. leprae cells. Thus, it was possible to accurately compare the extent of fusion and morphological appearance of the bacilli under a variety of experimental conditions. Whereas little fusion occurred in the first hour after infection, the extent of lysosome fusion reached a plateau by 14 h and remained unchanged for 6 days in culture. The majority of live M. leprae-containing phagosomes remained segregated from Thoria Sol-labeled lysosomes in normal macrophages (Fig. 5). In counts based on 100 or more infected normal macrophages, 27.2% of live M. leprae cells were observed in fused phagolysosomes; 38.8% of M. leprae cells precoated with human sera were observed in fused phagolysosomes. Whereas preincubation of M. leprae with specific antibodies to surface determinants had little effect on the extent of fusion, gamma-irradiated M. leprae-containing vacuoles underwent extensive fusion in normal macrophages (66.2%). In activated macrophages, live M. leprae-containing vacuoles readily underwent lysosome fusion (Fig. 6; 66.7 and 68.6% fusion for Toxoplasma- and gamma interferon-activated macrophages, respectively). M. leprae in fused phagolysosomes of activated macrophages often appeared morphologically disrupted (Fig. 6).

DISCUSSION

M. leprae is generally regarded as an obligate intracellular microorganism capable of prolific growth in macrophages. The immune-mediated activation of macrophages and their capability to effectively kill and digest M. leprae is of central importance to understanding the spectrum of host resistance to this pathogen (29). In the present study, we have used in vitro-cultured mouse peritoneal macrophages with well-defined microbicidal capacity to examine the intracellular processing of live and killed M. leprae cells by a uniform population of macrophages. Viable M. leprae organisms

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FIG. 1 to 4. 1. *M. leprae* cell in the phagosome vacuole of a mouse macrophage surrounded by an ETZ. Bilaminar membranes surround the phagosome vacuole and a nearby mitochondrion (arrowheads) (60-nm section at 60 kV). 2. Amorphous electron-dense material surrounding the ETZ within macrophage phagosome (open arrow) is contained within the phagosome membrane (closed arrow). Thoria Sol is located in nearby vacuoles (60-nm section at 60 kV). 3. Fusion with Thoria Sol-labeled lysosomes evident, although Thoria Sol remains excluded by the ETZ (60-nm section at 60 kV). 4. Prominent electron-dense border of ETZ (open arrow) lacks the unit membrane profile of the phagolysosome membrane (closed arrow) (60-nm section at 60 kV).
were freshly harvested by a process which selected for a suspension of ATP-rich organisms. The ATP content of M. leprae is correlated with viability as demonstrated by titration in mouse footpads (7). In comparison, gamma-irradiated M. leprae cells lacking ATP activity served as a killed preparation.

Phagocytosed M. leprae were observed in membrane-bound cytoplasmic vacuoles in cultured macrophages. Numerous other reports substantiate that M. leprae resides in membrane-bound vacuoles in vivo (2, 9, 10, 26, 33). This phagosomal compartment often contains additional amorphous material that has been interpreted to represent lysosomal contents (2, 10). However, this amorphous material is excluded from the prominent ETZ which characteristically surrounds mycobacteria (8). The appearance of an electron-dense rim at the edge of the ETZ often resembles a membrane at low magnification. However, this border fails to show the characteristic unit membrane profile at higher magnification and thus cannot be considered a true membrane.

The unit membrane which surrounds the ETZ and enclosed bacillus is relatively light staining and in this respect resembles plasma membranes and other phagosomal membranes which are often poorly preserved by standard EM staining. The use of potassium ferricyanide during osmium staining (11) and staining of preembedded specimens with uranyl acetate greatly enhances membrane staining, making phagosomal membranes more apparent. In the present report we have used this enhanced staining and found no evidence for bacilli free in the cytoplasm of macrophages cultured up to 6 days, such as that reported for virulent M. tuberculosis (27) or previously suggested for M. leprae (9). It is conceivable that in cells which are less specialized for phagocytosis and phagosome digestive processing, M. leprae cells could be found free in the cytoplasm of the host cell (18).

To examine the nature of the intracellular compartment occupied by intracellular M. leprae we used the electron-dense secondary lysosome marker Thoria Sol. In normal macrophages a majority of phagosomes containing freshly isolated viable M. leprae organisms resisted fusion with secondary lysosomes. However, this trend was reversed with gamma-irradiated M. leprae cells, indicating that viable M. leprae resist lysosome fusion in normal macrophages. Thus, M. leprae is similar to M. tuberculosis, M. bovis, M. microti, and M. avium, which previous EM studies have shown resist lysosome fusion (12, 14, 25). In contrast to the observation that coating M. tuberculosis cells with specific antibody results in increased lysosome fusion (1), we did not observe any appreciable increase in fusion when live M. leprae cells were precoated with human serum rich in specific antibodies to the surface phenolic glycolipid.

Macrophages activated by gamma interferon or by chronic protozoan infection have a heightened capacity to nonspecifically kill a variety of obligate intracellular microorganisms (15, 23, 28, 32). Therefore, we examined the intracellular fate of M. leprae in activated macrophages from mice with chronic Toxoplasma infection and in macrophages activated in vitro with spleen cell lymphokines or recombinant murine gamma interferon. In both groups of activated macrophages,

FIG. 5 and 6. 5. Majority of intact M. leprae cells in live inoculum remain segregated from Thoria Sol-containing lysosomes in normal macrophages (300-nm section at 100 kV). 6. Extensive fusion of M. leprae cells with Thoria Sol-containing lysosomes associated with morphological disruption (arrows) of bacilli in activated macrophages (300-nm section at 100 kV).
phagosomes containing viable *M. leprae* cells underwent extensive fusion with secondary lysosomes. Bacilli that underwent fusion were often morphologically damaged, suggesting enhanced digestion by activated macrophages. Interestingly, gamma-irradiated *M. leprae* cells did not appear to be digested by normal macrophages.

Despite the report that secondary lysosomes are not required for macrophage killing and digestion of microorganisms (13), the present report indicates that secondary lysosome fusion is correlated with viability of the *M. leprae* inclusion, the morphological appearance of the bacilli, and the activation state of the macrophage. In several previous studies, Thoria Sol labeling has provided a useful morphological correlate of the intracellular fate of *T. gondii* (20) and of *L. pneumophila* (16). Like *M. leprae*, both of these microorganisms survive in normal macrophages. Although we have not examined the role of primary lysosomes, previous studies have shown a close relationship between fusion of primary and secondary lysosomes with phagosomes occupied by intracellular microorganisms (16, 20). Several previous reports have indicated that *M. leprae*-infected macrophages contain abundant acid phosphatase (19, 21, 26) and that *M. leprae* can be observed in compartments containing this lysosomal enzyme. However, these previous reports involved biopsied tissue specimens which necessarily contain macrophages of an unknown state of activation and bacilli of uncertain viability. In the present report we have shown that both these parameters affect the intracellular fate of *M. leprae*, making the interpretation of biopsy sections difficult.

The present study demonstrates enhanced lysosome fusion in activated macrophages challenged with *M. leprae* and is consistent with involvement of activated macrophages in bacterial clearance (5, 6, 22, 29). The suspension of freshly harvested *M. leprae* cells used in the present study was enriched for viability by Percoll gradient purification as determined by ATP content. The purification of *M. leprae* on Percoll gradients also eliminated most of the cellular debris associated with *M. leprae*-infected tissue homogenates. Thus it was possible to assess accurately the fate of *M. leprae* cells ingested by macrophages without the contribut- ing influence of host cell components. Despite these improvements, uncertainty still remains about the viability of individual *M. leprae* organisms observed in the present study and prevents us from concluding whether the observed increase in digestion of bacteria is accompanied by heightened killing of *M. leprae* by activated macrophages. Corroborating data have recently been obtained showing that activated macrophages markedly inhibit ATP content and phenolic glycolipid-1 synthesis by *M. leprae* (N. Ramasesh, S. Franzblau, L. D. Sibley and J. Krahenbuhl, manuscript in preparation).

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