Comparison of In Vitro Models for the Study of *Mycobacterium tuberculosis* Invasion and Intracellular Replication

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We recently evaluated several tissue culture model systems for the study of invasion and intracellular multiplication of *Mycobacterium tuberculosis*. These model systems include a human alveolar pneumocyte epithelial cell line, a murine macrophage cell line (J774), and fresh human peripheral blood-derived macrophages. Our data indicated that the initial level of association of *M. tuberculosis* with human alveolar pneumocyte cells (2%) was less than that observed with fresh human peripheral blood macrophages (9%) or J774 murine macrophages (13%) within 6 h of the addition of the bacteria. *M. tuberculosis* replicated in association with the pneumocyte cells by more than 55-fold by day 7 postinfection. In contrast, total bacterial growth in the J774 cells and human macrophages was considerably less, with increases of only fourfold and threefold, respectively, over the same 7-day period. Amikacin, an aminoglycoside antimicrobial agent, was added to inhibit the growth of extracellular bacteria after the initial 6-h infection period. Decreases in viable counts were observed in all three cell cultures within the first 3 days after infection. However, unlike the case with either macrophage culture, intracellular bacterial CFU obtained from the infected pneumocytes increased by fourfold by day 7 after the addition of amikacin. These data indicate that *M. tuberculosis* infects and multiplies intracellularly in human lung epithelial cells and that these cells may be an alternative in vitro model for the study of intracellular multiplication of *M. tuberculosis* in the human lung.

During the past decade, the resurgence of tuberculosis has been documented in both developed and developing countries (25, 32, 33, 43). The causative agent, *Mycobacterium tuberculosis*, is a facultative intracellular bacterial pathogen that establishes infection after inhalation of bacilli into the alveoli of the lower lungs (6). It is suspected that during the initial stages of this process, the bacilli reside principally intracellularly, surviving within the host's phagocytic cells and primarily in the alveolar macrophages (12, 38). These macrophages are localized on the alveolar surface and in the local capillary beds (12) and with blood monocytes may move freely between the epithelial and endothelial cells. Though the alveolar macrophages are suspected to play a role during the disease process, the mechanism(s) by which *M. tuberculosis* is able to translocate from the alveolar spaces through lung epithelium and endothelium and ultimately into the bloodstream and lymphatic system has not been demonstrated. However, it is well accepted that *M. tuberculosis* bacilli survive, multiply, and are transported through the body via the bloodstream as intracellular parasites of monocytes and macrophages (13, 15, 20).

The alveolar epithelium consists of two cell types: type I and type II pneumocytes. The large flattened type I cells cover a majority of the alveolar surface area, while only a small portion is covered by rounded type II cells (5). Type I cells are respon-

sible for creating a thin gaseous barrier, while type II cells secrete surfactants, thus preventing alveolar collapse during expiration; type I cells are formed from the differentiation of type II cells (31). Several species of pathogenic mycobacteria, including *M. avium* and *M. marinum*, have been shown to invade and replicate within a variety of human nonprofessional phagocytes, such as fibroblasts and human epithelial cells (2, 3, 28, 36, 40).

During this study, we examined the association of the bacteria with lung pneumocytes and compared these data with those obtained with the more traditional in vitro systems, including cultured J774 murine macrophage cells and fresh peripheral human blood macrophages. Our ultimate goal is to understand the interaction between *M. tuberculosis* and the cells composing the lung alveoli (epithelium, endothelium, and alveolar macrophages) (5, 13) and how these interactions contribute to pathogenesis.

MATERIALS AND METHODS

Bacterial culture and growth conditions. *M. tuberculosis* H37Rv (TMC102) was stored at -70°C and, when needed, was maintained both on Löwenstein-Jensen medium and Middlebrook 7H9 broth with ADC (5% [wt/vol] albumin, 2% [wt/vol] glucose, and 0.003% [wt/vol] catalase) (Carr-Scarborough, Atlanta, Ga.) at 37°C in 5% CO_2 . Bacteria were grown in Middlebrook 7H9 broth with ADC and occasional shaking for 6 to 7 days. Immediately before the infection was begun, the bacterial culture was transferred to a fresh tube and vortexed vigorously for several minutes. This produced a culture with small amounts of bacterial clumping and a mid-logarithmic-phase viable count of approximately 3×10^7 to 5×10^7 CFU/ml. Viable count assays were performed on Middlebrook 7H10 medium (Carr-Scarborough).

Eukaryotic cell lines. The mouse macrophage cell line J774A.1 (American Type Culture Collection no. TIB67) and a human type II alveolar pneumocyte cell line, A549 (American Type Culture Collection no. CCL185), were obtained

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and maintained at the Centers for Disease Control and Prevention Tissue Culture Facility. These tissue culture lines were maintained in antibiotic-free Dulbecco's modified Eagle medium (DMEM) with high-level glucose (Gibco/BRL, Grand Island, N.Y.) and supplemented with 584 mg of glutamine (Gibco) per liter and 10% fetal bovine serum (FBS) (Gibco).

Isolation of human blood monocyte-derived macrophages. Heparinized venous blood was obtained from tuberculin-negative, healthy individuals; diluted 1:1 with sterile phosphate-buffered saline (PBS) (Gibco); and layered onto a Ficoll-Hypaque gradient. Samples were centrifuged at $400 \times g$ for 20 min. Cells in the buffy coat were removed and washed twice with Hanks' buffered salt solution (without calcium and magnesium) and once in RPMI 1640 tissue culture medium (Gibco). The mononuclear cells were resuspended in RPMI 1640 medium containing 2 mM glutamine, 1% unheated autologous human serum, and 20 μ g of gentamicin per ml. Cells were seeded and maintained in 24-well microtiter plates at a density of 2.5×10^6 cells per well and incubated at 37°C in 5% CO_2 . After 2 h, the wells were washed twice with warm medium to remove nonadherent cells. It was found that at least 10% of the added mononuclear cells adhere as monocytes and convert into monocyte-derived macrophages when cultured at 37°C in 5% CO_2 for 6 to 7 days. By esterase staining, it was observed that >96% of the monolayer consisted of monocytes. The viability of macrophage culture was assessed by trypan blue exclusion, and more than 98% of the macrophages were found to be viable. One day before infection, the monolayers were washed and fresh medium without gentamicin, but supplemented with 5% unheated autologous human serum, was added.

Invasion and intracellular growth. Pneumocytes and J774 cells were seeded onto six-well microculture plates at a density of 3×10^5 cells per well 3 to 4 days before use. Before infection, the medium was replaced with DMEM supplemented with 5% FBS. Viability of the eukaryotic cells was determined by trypan blue vital stain and direct hemocytometer counts.

Bacteria ($5 \times 10^7/\text{ml}$) were harvested by centrifugation, washed twice with DMEM, and resuspended in DMEM supplemented with 5% FBS. For human macrophages, RPMI 1640 was substituted for DMEM and 5% autologous human serum was substituted for FBS. Enough bacteria (10^6 to 10^7) were added to the wells to achieve multiplicities of infection (MOIs) of approximately 1 to 5 (one to five bacteria per tissue culture cell) for the J774 murine and human macrophages and approximately 1 to 11 for the human pneumocyte cells. This translated to approximately 100 μ l of the bacterial suspension per well for the human macrophage cultures and approximately 300 μ l per well for the J774 macrophage and human pneumocyte cultures. After inoculation, the microculture plates were incubated for 6 h at 37°C in 5% CO_2 . Subsequently, the monolayers were washed twice with warmed DMEM or RPMI 1640 and returned to the incubator in warmed medium supplemented with 1% FBS or autologous human serum.

Infected tissue culture cells were lysed with 1 ml of 0.1% Triton X-100 for 15 min. Serial 10-fold dilutions of the lysate were prepared in Middlebrook 7H9 broth and plated on Middlebrook 7H10 medium. After about 3 weeks of incubation in sealed plastic bags at 37°C with 5% CO_2 , colonies were counted. Controls designed to elucidate background attachment to the plastic dish demonstrated that a negligible amount (<0.0001%) of the inoculum was sticking nonspecifically. This background was automatically subtracted from each assay point.

In some experiments, amikacin, an aminoglycoside antimicrobial agent, was used to inhibit the growth of extracellular bacteria (36). After incubation of the J774 murine macrophage, human macrophage, and human pneumocyte cell cultures for 6 h at 37°C , the monolayers were washed and subsequently incubated in DMEM or RPMI 1640 (supplemented with 1% FBS or autologous human serum) containing 200 μ g of amikacin (Sigma Chemicals, St. Louis, Mo.) per ml for 2 h at 37°C . The infected cell cultures then were washed twice with serum-free warm medium, and one monolayer was assessed for bacterial invasion by lysis as detailed above. The remainder of the monolayers were reincubated at 37°C with 5% CO_2 in tissue culture medium (supplemented with 1% FBS or autologous human serum) containing 20 μ g of amikacin per ml. On alternate days during the 7-day assay, media were replaced in both amikacin-supplemented and unsupplemented experiments. At several time points after the infections, the media were removed, the monolayers were washed twice and lysed as described above, and lysates were diluted and plated for viable count analysis.

Electron microscopic analysis. Infected cells were prepared for electron microscopy by first removing the infected tissue culture medium, adding 2% glutaraldehyde fixative, and incubating the cells at 4°C for 1 h. The glutaraldehyde solution was then removed and ice-cold PBS was added, after which the tissue cultures were stored at 4°C for 6 to 24 h. The cells were fixed with 2% osmium tetroxide, dehydrated in ethanol, passed through propylene oxide, and embedded in Maraglas resin. One-micrometer-diameter sections were cut and stained with uranyl acetate and lead citrate (18). The sections were observed by transmission electron microscopy using a Philips (Amsterdam, The Netherlands) 410 electron microscope.

RESULTS

From our J774 infection studies, we determined that 10% FBS in the DMEM tissue culture medium, which is nearly

FIG. 1. Colony counts of *M. tuberculosis* at various times in association with pneumocytes (■), pneumocytes in the presence of amikacin (□), J774 cells (●), and J774 cells in the presence of amikacin (○) after incubation at 37°C for 7 days. The infections were performed in six-well dishes with MOIs of approximately 1 to 5 for the J774 cells and 1 to 11 for the pneumocytes. Five percent FBS was used for the initial 6 h of infection, and 1% FBS was used for the 7 subsequent days. The data are the means of results from three independent experiments, and standard errors of the means have been calculated for all datum points.

universally used to perform these types of studies (35, 37, 42), would result in detachment and lysis of the tissue culture cells after 5 days. Alternatively, with medium containing 5% FBS for the initial infection and DMEM with 1% FBS for maintenance of the monolayers during the subsequent 7- to 10-day incubation period, the rate of tissue culture growth and, thus, degeneration of the monolayers was significantly decreased (data not shown). Though the detachment and lysis of the uninfected control monolayers were not as rapid as the detachment and lysis observed with the infected monolayers, decreasing the concentration of serum in the maintenance medium of the uninfected monolayers also prolonged their integrity by several days. These conditions subsequently were used for the human macrophage and pneumocyte infection studies.

Results showed that *M. tuberculosis* could associate (attach and invade) in large numbers with the pneumocyte, mouse macrophage, and human macrophage cells within 6 h. The percentage of the inoculum which associated with the various monolayers by 6 h after addition of the bacteria was more pronounced for the J774 mouse macrophage cells (13%), followed by human macrophages (9%), than for the pneumocyte cells (2%). However, replication of the associated *M. tuberculosis* bacilli, over the long term, was considerably more efficient in association with the human pneumocyte cells; the bacterial counts increased by more than 55-fold by day 7 postinfection, while bacteria associated with J774 cells increased in number by 4-fold after 3 days (Fig. 1). Bacteria associated with human macrophages were found to increase in number by only threefold after 7 days (Fig. 2). Viability of the macrophage cultures did not change appreciably, decreasing from >98% to >90% over the 7-day incubation period. Infections with smaller bacterial inocula (MOIs of 0.35 and 1) also were performed with the J774 and human macrophage monolayers; similar four- and threefold increases, respectively, were observed with these smaller inocula, indicating that the limit of attachment, invasion, and intracellular as well as extracellular growth with the J774 and human macrophages is not related to initial association numbers.

To ascertain the true intracellular growth rate of *M. tuberculosis* in these cultured cell lines, amikacin was added to the

FIG. 2. Colony counts of *M. tuberculosis* at various times in association with human macrophages (■) and human macrophages in the presence of amikacin (●) after incubation at 37°C for 7 days. The infections were performed with an MOI of approximately 1 to 5. Five percent autologous human serum was used for the initial 6 h of infection, and 1% autologous human serum was used for the 7 subsequent days. The data are the means of results from three independent experiments, and standard errors of the means have been calculated for all datum points.

medium. We previously examined the inhibition of growth of *M. tuberculosis* by 10 to 600 µg of amikacin per ml and found that amikacin had a MIC of 20 µg/ml and an MBC of approximately 200 µg/ml in DMEM with 1% FBS (data not shown).

Under such growth conditions in the presence of amikacin, bacterial numbers in cultured pneumocytes declined by about fourfold during the first 3 days after the addition of amikacin and this decline was followed by a fourfold increase in bacterial numbers by day 7 postinfection (Fig. 1). In contrast, bacterial numbers in J774 cells did not increase but viable counts actually decreased throughout the infection period (Fig. 1). The decreases were even more pronounced with human macrophages (Fig. 2).

Electron microscopic preparations of infected tissue culture cells showed bacteria within vacuoles of J774 macrophages and human pneumocytes (Fig. 3 and 4A and B). Interestingly, bacteria also were observed in the cytoplasm of the pneumocytes (Fig. 4B and C).

DISCUSSION

Once inhaled into the alveoli of the lung, *M. tuberculosis* likely associates with and ultimately passes through the epithelial and endothelial cells lining the capillary beds before entering the blood. Macrophages in the alveolar spaces, between the epithelial and endothelial cells, and in the blood play a significant role in the survival and transmission of the tubercle bacilli through the body of an infected host (11, 15). Additionally, days or weeks after the initial infection, CD4⁺ and CD8⁺ T-cell types likely are important in the response of lung cells to mycobacterial infection. Recent research suggests that, in particular, CD8⁺ cells are important in the lung immune response as cytolytic cells that lyse macrophages and other nonprofes-

FIG. 3. Transmission electron photomicrograph of *M. tuberculosis*-infected J774 mouse macrophages (MOI = 1 to 5) after incubation at 37°C for 7 days. Arrowheads indicate vacuolar membranes surrounding bacteria.

FIG. 4. Transmission electron photomicrographs of *M. tuberculosis*-infected human lung pneumocytes (MOI = 1 to 11) after incubation at 37°C show the presence of multiple bacteria in vacuoles (A), in a vacuole and free in the host cell cytoplasm (B), and free in the host cell cytoplasm (C). Arrowheads indicate the presence of vacuolar membranes surrounding bacteria, while arrows indicate bacteria apparently free in the cytoplasm.

FIG. 4—Continued.

sional phagocytic cells (perhaps pneumocytes) that are damaged by the invading mycobacteria (19, 24, 34). However, the macrophage is the only one of these cell types that has been thoroughly examined to study invasion and replication of *M. tuberculosis*. The present study was initiated as a comparative analysis of invasion and intracellular multiplication of *M. tuberculosis* in macrophages and in pneumocytes, a nonprofessional phagocytic cell type found in lung alveoli.

One of the standard models for examining the interaction of *M. tuberculosis* with macrophages is J774 mouse macrophages (35, 37, 42). Our results with J774 cells revealed that the concentration of FBS in the tissue culture medium was important because 10% FBS resulted in the detachment and lysis of J774 cells, likely because of their enhanced rate of multiplication in culture. As suggested by Rastogi et al. (37), we decreased the concentration of FBS in the assay system to slow the growth of the host cells during the long incubation period; 5% FBS was optimal for initial infection and 1% FBS was adequate for subsequent monolayer maintenance.

Our results concerning the survival and multiplication of *M. tuberculosis* H37Rv in association with J774 and human macrophages differ considerably from those of some previously reported studies, wherein 10- to 15-fold increases in the number of bacteria were observed during a 7-day period (9, 12, 14, 37). In our studies, the level of bacterial association was high in J774 cells: by 6 h after infection approximately 13% of the inoculum was cell associated. However, over the 5- to 7-day course of the experiment, only a small increase in bacterial numbers was observed. When amikacin was included and maintained throughout the experiment, a decrease in viable counts was observed. With the use of fresh human blood-

derived macrophages in these assays, initial bacterial association was similar to that in the J774 model. The addition of amikacin to these infected cells caused an actual decrease in viable counts: after 3 days of incubation with the amikacin, a viable count equalling only 0.02% of the original inoculum was detected. Alternatively, fewer bacteria were found associated initially with the pneumocyte cell line than with either the mouse or the human macrophages. However, in the presence of amikacin, the level of intracellular growth increased by fourfold in this cell line from day 3 to day 7 postinfection.

We have observed similar amikacin-associated intracellular growth differences while studying variants of *Mycobacterium haemophilum* in human epithelial cells (18). *M. haemophilum* bacteria grew in number by more than 100-fold at 37°C in Hec1B cell cultures without amikacin, but no growth was observed in the presence of the aminoglycoside amikacin. However, at 33°C, substantial intracellular growth was observed both in the presence and in the absence of amikacin.

Few of the previously reported studies of *M. tuberculosis* association with human or murine macrophages or other cell types incorporated aminoglycoside agents to eliminate extracellular bacterial growth (8, 9, 14). The substantial macrophage-associated growth reported to have occurred in these other studies, therefore, may represent extracellular growth due to lysed host cell components, differences in tissue culture media used, or some other nonspecific growth conditions not present in our study. More substantial sloughing of the infected monolayers with the subsequent washing and removal of the associated bacteria could account for some of the decreased viable counts observed in our assays. However, the most likely reason for differences in intracellular growth may be virulence differences

among the various laboratory strains of *M. tuberculosis* used in these studies. Unpublished data from our laboratory indicate that *M. tuberculosis* Erdman replicated three- to fourfold more efficiently in J774 cells and human macrophages than did strain H37Rv.

Additionally, high intracellular levels of amikacin, representing perhaps as much as 10% of the extracellular concentration in the pneumocytes and perhaps far higher percentages in the macrophages, may accumulate and thus may slow or prevent intracellular bacterial growth. Several decades ago Mackaness and Smith (27), and later Crowle et al. (10), observed that streptomycin, another aminoglycoside, could penetrate into human macrophages and inactivate intracellular tubercle bacilli. This observation could explain the tremendous decreases in viability, particularly in the human macrophage assays. Nonetheless, even under these circumstances, the in vitro pneumocyte model appears to be an excellent alternative to the macrophage model for the study of intracellular growth.

Invasion and intracellular replication by other intracellular bacterial pathogens, such as *Salmonella typhimurium*, *Yersinia pseudotuberculosis*, *Listeria monocytogenes*, *Shigella flexneri*, and *Legionella pneumophila*, in nonprofessional phagocytic cells such as fibroblasts, epithelial cells, and endothelial cells have been well documented (16, 17, 21, 26, 30). With *M. tuberculosis*, Shepard (40), and more recently Arruda et al. (1), reported that the bacteria were able to invade HeLa epithelial cells. On the basis of these and other data, Shepard (41) suggested that the ability of *M. tuberculosis* to invade HeLa cells and its ability to cause disease in humans and experimental animals were related. Electron microscopic studies conducted by Kanai et al. (23) on the lungs of mice infected intravenously with virulent *Mycobacterium bovis* Ravenel revealed marked alterations in the alveolar epithelial cells, and particularly type II cells, and the development of interstitial and intra-alveolar granulomas, perhaps indicating the direct involvement of pneumocytes in mycobacterial pathogenesis or containment.

The decreased uptake of bacteria by human pneumocyte cells compared with macrophages suggests the existence of different mechanisms for the entry of mycobacteria into nonprofessional phagocytes and may involve pathways and receptors more diverse than those used by professional macrophages (39). The other possibility for the observed decrease in uptake by the pneumocytes could be simply a lower rate of bacterial phagocytosis by these epithelial cells compared with macrophages. Multiple entry mechanisms exist for other intracellular organisms, such as *Toxoplasma gondii* (22), and the mode of entry has been shown to impact intracellular survival. For the mycobacteria, *M. avium* has been shown to invade epithelial cell lines such as HT-29 and HEp-2, suggesting that these bacteria possess the ability to interact with and penetrate nonprofessional phagocytic cells before translocating into the lamina propria of the intestine (3). *L. pneumophila* also has been shown to invade and replicate significantly within alveolar epithelial cells (7, 31). A similar type of interaction between *M. tuberculosis* and pneumocytes may occur in the lung alveoli before translocation into the alveolar capillaries. Such a mode of infection via nonprofessional phagocytic cells may enable the bacteria to escape traditional host immune responses and hence help to maintain the chronic bacteremia associated with some late-stage, acute mycobacterial diseases (2).

In summary, this study demonstrated that *M. tuberculosis* readily associated with macrophages and became intracellular. However, in the presence of amikacin, intracellular replication occurred to a lesser extent than did that observed with a human alveolar pneumocyte cell line. This cell line may be an appropriate alternative for the study of intracellular survival and

replication by *M. tuberculosis*. Currently, our laboratories are engaged in the development of a tissue culture bilayer model containing type II pneumocytes, human macrophages, and human lung endothelial cells sandwiched together into a structure resembling a human lung alveolus (29). We have already used a similar system to examine attachment and transport of *Neisseria meningitidis* (4). We are beginning to study transport and intracellular growth of *M. tuberculosis* within a more complex and relevant in vitro system.

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