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Mycobacterium tuberculosis Invades and Replicates within Type II Alveolar Cells

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Although *Mycobacterium tuberculosis* is assumed to infect primarily alveolar macrophages after being aspirated into the lung in aerosol form, it is plausible to hypothesize that *M. tuberculosis* can come in contact with alveolar epithelial cells upon arrival into the alveolar space. Therefore, as a first step toward investigation of the interaction between *M. tuberculosis* and alveolar epithelial cells, we examined the ability of *M. tuberculosis* to bind to and invade alveolar epithelial cells in vitro. The H37Rv and H37Ra strains of *M. tuberculosis* were cultured to mid-log phase and used in both adherence and invasion assays. The A549 human type II alveolar cell line was cultured to confluence in RPMI 1640 supplemented with 5% fetal bovine serum, L-glutamine, and nonessential amino acids. H37Rv was more efficient in entering A549 cells than H37Ra, *Mycobacterium avium*, and *Escherichia coli* HB101, a nonpiliated strain (4.7% ± 1.0% of the initial inoculum in 2 h compared with 3.1% ± 0.8%, 2.1% ± 0.9%, and 0.03% ± 0.0%, respectively). The invasion was more efficient at 37°C than at 30°C (4.7% ± 1.0% compared with 2.3% ± 0.8%). H37Rv and H37Ra were both capable of multiplying intracellularly at a similar ratio over 4 days. Binding was inhibited up to 55.7% by anti-CD51 antibody (antivitronectin receptor), up to 55% with anti-CD29 antibody (β_1 integrin), and 79% with both antibodies used together. Uptake of *M. tuberculosis* H37Rv was microtubule and microfilament dependent. It was inhibited by 61.4% in the presence of 10 μM colchicine and by 72.3% in the presence of 3 μM cytochalasin D, suggesting two separate pathways for uptake. Our results show that *M. tuberculosis* is capable of invading type II alveolar epithelial cells and raise the possibility that invasion of alveolar epithelial cells is associated with the pathogenesis of lung infection.

Mycobacterium tuberculosis is an important pathogen by medical and epidemiological standards. It has been estimated that it infects approximately one-third of the world's population (3). *M. tuberculosis* establishes infection after inhalation of the bacilli into the alveolar space in the lungs (23, 24). It is largely accepted that during this process, *M. tuberculosis* is ingested or enters alveolar macrophages (6, 27). Once within macrophages, *M. tuberculosis* has the ability to avoid the phagocytic cells' bactericidal mechanisms and replicate. Although it is assumed that *M. tuberculosis* infection occurs primarily as described above, it is plausible to hypothesize that the aerosolized bacterium can bind to and invade epithelial cells upon arrival into the alveolar space.

The ability of mycobacteria to bind to and invade epithelial cells was initially demonstrated in studies by Shepard with HeLa cells (22) and more recently by Mapother and Sanger (14) and by ourselves (1). Invasion of alveolar epithelial cells by *M. tuberculosis* would create a niche for the bacterium to replicate and establish the infection, avoiding the potentially hostile environment of the macrophage. Therefore, we sought to examine the ability of *M. tuberculosis* strains H37Rv (virulent) and H37Ra (attenuated) to invade A549 human type II alveolar cells.

MATERIALS AND METHODS

Type II alveolar cells. A549 cells, a human type II alveolar cell line, were used in the reported studies. They were purchased from the American Type Culture Collection (ATCC, Rockville, Md.) and maintained in RPMI 1640 (Gibco Laboratories, Detroit, Mich.) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, Mo.), 2 mM L-glutamine, and 1% nonessential amino acids (Sigma). For the assays, A549 cells were seeded (10^5 cells per well) in a 24-well tissue culture plate (Costar, Cambridge, Mass.) and allowed to grow to semiconfluence (approximately 4 to 5 days). The cell line was used between passages 45 and 55 in all the reported assays.

Bacteria. *M. tuberculosis* strains H37Rv and H37Ra were purchased from the ATCC, cultured in Middlebrook 7H10 agar for 20 days, harvested, and resuspended in Middlebrook 7H9 broth for 7 days. *Mycobacterium avium* strains 101 (serovar 1) and 109 (serovar 4) were cultured in Middlebrook 7H10 for 10 days, and transparent colonies were transferred to 7H9 broth for 5 days. *Escherichia coli* HB101, a nonpiliated strain, was cultured in Luria-Bertoni (LB) broth overnight. Bacteria were spun down, washed, and resuspended in Hanks' buffered salt solution (HBSS) before the assays. Because of the tendency of mycobacteria to form clumps, the mycobacterial suspension in HBSS was briefly sonicated (20 W for 5 s), vortex agitated for 1 min, and then placed in a 10-ml plastic tube for 10 min. The top 3 ml were recovered, and the concentration was adjusted to 10^7 bacteria per ml by using a McFarland turbimetric standard. The bacterial suspension was stained with Ziehl-Nielsen stain and observed under light microscopy. Only disperse suspensions were used in the experiments.

The viability of the bacterial suspensions was determined before the assays to be in a range of 40 to 60% by using the live-dead BAC-light (Molecular Probes, Eugene, Oreg.). In addition, an aliquot of the bacterial suspension was plated in Middlebrook 7H10 agar (mycobacteria) or LB agar (*E. coli*) to establish the exact number of bacteria in the inoculum.

Invasion assay. The invasion assay was carried out by the method of Isberg and Falkow (11) as modified by us (1). Briefly, prior to the assay, the culture medium of the monolayers was removed and replenished by warm culture medium. Bacterial samples (10^6 bacteria per well) were added as described above. Invasion assays were incubated at 37°C in a 5% CO_2 atmosphere for 2 h. Assays were terminated by replacing the overlying medium with 1 ml of tissue culture medium supplemented with either 100 μg (*M. avium*), 20 μg (*M. tuberculosis*), or 2 μg (*E. coli*) of amikacin per ml. Amikacin, an aminoglycoside antibiotic, at the concentration used (approximately 10 times the MIC), kills extracellular *M. avium* (1), both strains of *M. tuberculosis*, and *E. coli* HB101 (8, 11) in 2 h, while intracellular bacteria remain viable. After the incubation with amikacin for 2 h at 37°C in a 5% CO_2 atmosphere, the monolayers were washed twice with HBSS, and the viable intracellular bacteria were released by incubation with 0.5 ml of 0.1%

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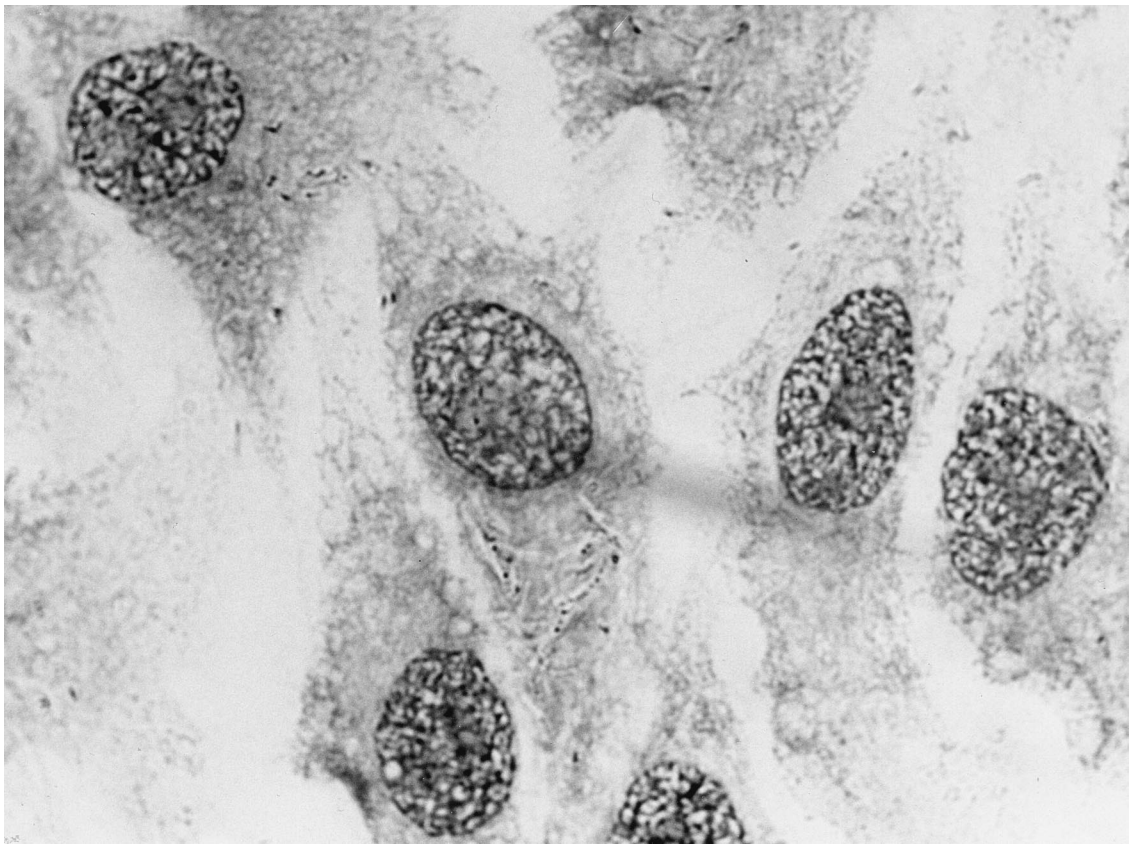


FIG. 1. A549 type II alveolar cell monolayer exposed to strain H37Rv of *M. tuberculosis* for 2 h and then washed and stained for acid-fast bacilli (MOI, 10:1).

Triton X-100 in sterile water for 10 min. Controls containing bacteria (but no cells) and treated with Triton X-100 were run in parallel to ensure viability of the bacteria. Samples were vigorously mixed with 0.5 ml of 7H9 broth or LB broth and harvested. The suspension was then vortex agitated for 2 min, and viable bacteria were quantitated by plating for CFU onto 7H10 agar or LB agar.

Effect of temperature on invasion. To examine whether the host temperature has any influence on the ability of *M. tuberculosis* to invade A549 cells, bacteria were grown for 7 days in Middlebrook 7H9 broth and then separated into two groups: one to grow at 37°C and another to grow at 30°C for 24 h. After the incubation, the bacteria were washed, prepared according to the method described above to adjust the inoculum, and used in the invasion assay.

Invasion assay in the presence of biochemical inhibitors. Invasion was carried out in the presence of individual inhibitors added to the A549 cell monolayers 1 h prior to the addition of bacteria for inhibition of the mammalian cell process of internalization. Inhibitors were maintained throughout the 2-h invasion period. The concentration of each inhibitor was tested for maximum effect on internalization without affecting the viability of epithelial cells, as determined by both trypan blue dye exclusion and acridine orange staining. None of the inhibitors, at the concentrations used, had an effect on bacterial viability (data not shown). Colchicine, cytochalasin D, nocodozole, and caffeine were purchased from Sigma.

Role of epithelial cell receptors in the process of internalization. In an attempt to determine the putative receptors for *M. tuberculosis* in A549 cells, the invasion assay was performed in the presence of mouse anti-human CD51 (vitronectin receptor; Biosource, Camarillo, Calif.), mouse anti-human CD29 (β_1 subunit of integrins; Biosource), mouse anti-human VLA4 (Upstate Biochemicals, Lake Placid, N.Y.), or mouse anti-human CD71 (transferin receptor; Biosource) antibodies (all immunoglobulin G1 [IgG1]). A mouse anti-*Pseudomonas aeruginosa* lipopolysaccharide (which does not cross-react with mycobacteria) IgG1 antibody was used as a nonsense antibody control. A549 cells were incubated with increasing concentrations of antibodies at 4°C for 30 min, and then medium was substituted for warmed (37°C) RPMI 1640, bacteria were added, and subsequently the monolayer was incubated at 37°C. At the concentrations used, the antibodies had no effect on A549 cell viability (data not shown).

Intracellular duplication of *M. tuberculosis*. Monolayers of A549 cells were inoculated with either H37Rv or H37Ra (10^6 bacteria) in 1 ml of RPMI 1640 medium. After 2 h at 37°C and 5% CO_2, extracellular bacteria were killed with amikacin. Following exposure to amikacin, the medium was changed, and intracellular bacteria growth without antibiotic was examined for 4 days. Cell viability was monitored by acridine orange viability staining. Approximately 8% ± 2% of the infected and uninfected monolayers were not viable after 4 days. *M. tuberculosis* did not grow extracellularly in RPMI 1640 (data not shown).

Microscopy techniques. (i) Light microscopy. A549 monolayers infected with *M. tuberculosis* strain H37Rv were fixed with methanol at −20°C and subsequently stained for acid-fast bacilli by the method of Ziehl-Nielsen.

(ii) Electron microscopy. Cultured cells were infected with strain H37Rv for different periods of time. Then, monolayers were detached from the plastic with 0.2% trypsin for 5 min at 37°C and subsequently washed twice with HBSS. The pellet was then resuspended in ice-cold 1% glutaraldehyde in phosphate buffer for 1 h. The cells were centrifuged and transferred to small conical plastic capsules. Melted agar (0.7%) was added, mixed with the cells, and quickly centrifuged. The solid agar tip was cut off, and small pieces of agar containing cells were dehydrated through 50 and 80% ethyl alcohol at 4°C.

Statistics. All experiments were done in triplicate and repeated three times. Statistical analysis between results at the same time point was done with analysis of variance. $P < 0.05$ was considered significant.

RESULTS

Internalization of *M. tuberculosis* H37Rv by type II alveolar epithelial cells. Light microscopy and electron microscopy were used to localize *M. tuberculosis* within type II alveolar epithelial cells.

Figure 1 shows both an isolated bacillus and clusters of bacilli within vacuoles.

Figure 2 shows electron micrographs of A549 cells infected with *M. tuberculosis* H37Rv. Figure 2A shows an infected A549 cell after 2 h of incubation with H37Rv at a multiplicity of infection (MOI) of 10:1. Intracellular bacteria can be observed, with more than one bacterium occupying one vacuole. Figure 2B shows an A549 cell from a monolayer infected with H37Rv at an MOI of 100:1. Large numbers of intracellular bacteria

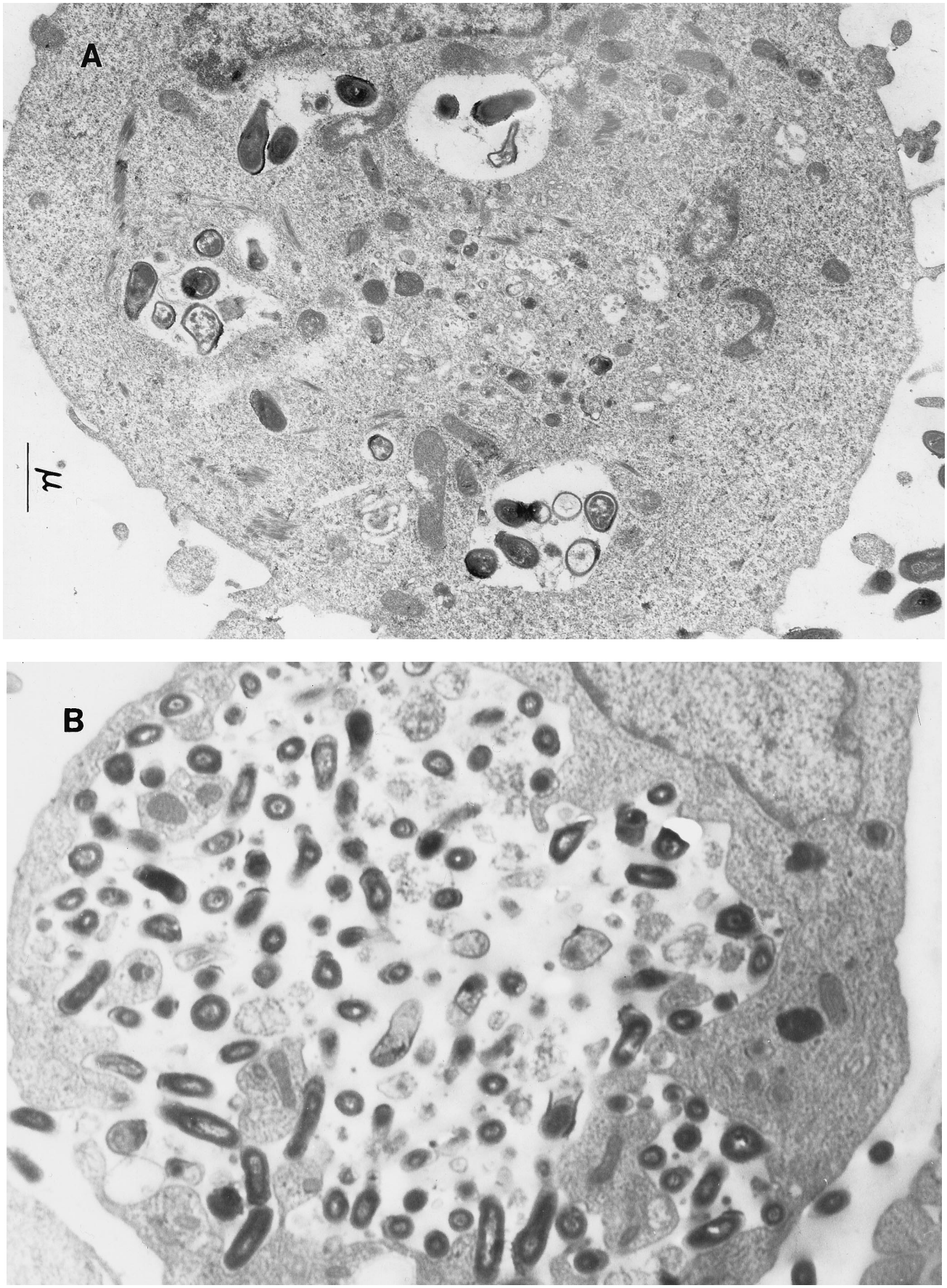
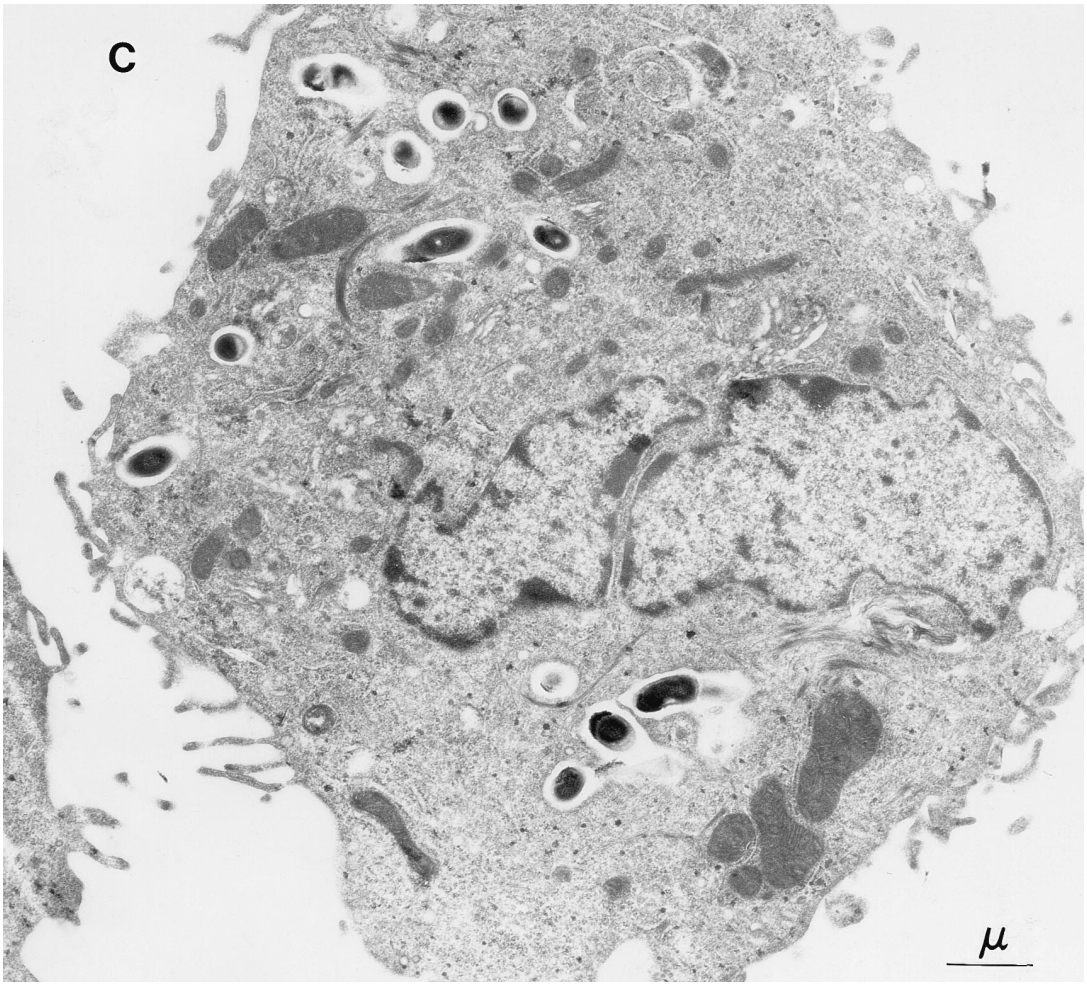


FIG. 2. *M. tuberculosis* H37Rv within A549 type II alveolar cells at (A) 2 h after infection, MOI of 1:10, (B) 2 h after infection, MOI of 100:1, (C) 24 h following infection, and (D) 6 days after infection. Bars, 1 μm.



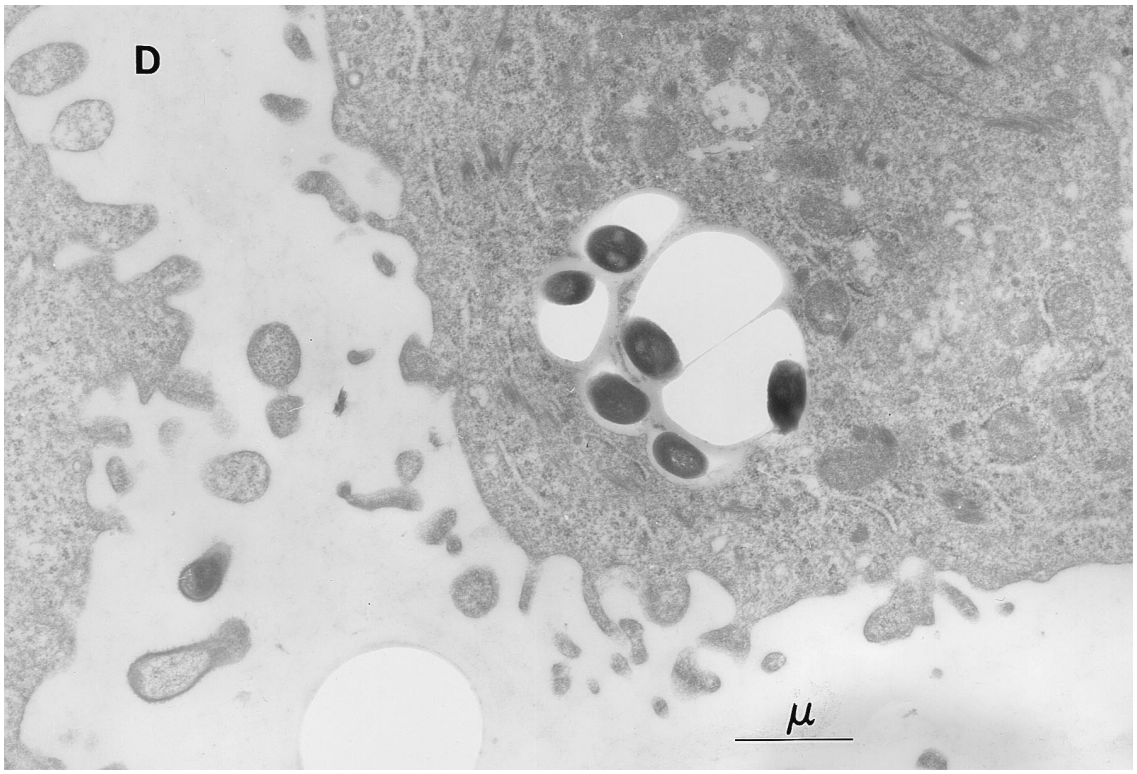


FIG. 2—*Continued.*

TABLE 1. Invasion of A549 cells by bacterial strains[a]

Strain	Relative invasion[b] (mean ± SD)			
	37°C		30°C	
	10:1	100:1	10:1	100:1
M. avium 101	2.1 ± 0.9 (C)*	3.1 ± 1.2 (C)*	0.8 ± 0.2 (B)*	1.3 ± 0.6 (B)*
M. avium 109	0.9 ± 0.2 (B)*	2.0 ± 0.9 (B)*	0.4 ± 0.1 (B)*	0.9 ± 0.4 (B)*
M. tuberculosis H37Rv	4.7 ± 1.0 (D)*†‡	5.7 ± 1.6 (D)*†‡	2.3 ± 0.8 (C)*†‡	3.1 ± 0.9 (C)*†‡
M. tuberculosis H37Ra	3.1 ± 0.8 (D)*	3.6 ± 0.8 (D)*	1.5 ± 0.5 (B)*	1.4 ± 0.9 (B)*
E. coli HB101	0.02 ± 0.00 (A)	0.03 ± 0.01 (A)	0.03 ± 0.01 (A)	0.03 ± 0.01 (A)

[a] Assays were performed at 37°C and 30°C at ratios of bacteria to cells of 100:1 and 10:1. The results are means for three experiments. The results represent the percentage of the initial inoculum that was intracellular. Monolayers were 99 to 100% viable during the assays.

[b] The values shown represent the percentage of initial inoculum. Letters in parentheses indicate the percentage of infected cells: (A), 0 to 1%; (B), 2 to 20%; (C), 21 to 40%; (D) 41 to 90%. *, $P < 0.05$ compared with *E. coli* HB101; †, $P < 0.05$ compared with *M. avium* strains; ‡, $P < 0.05$ compared with H37Ra.

can be observed in a vacuole. Figure 2C shows a cell that has been infected for 24 h. Most of the bacteria are in isolated compartments. Figure 2D, obtained 6 days after infection, shows H37Rv bacteria individually in vacuoles. Except on rare occasions, no intracellular organelle could be seen in the proximity of *M. tuberculosis*-containing vacuoles.

Invasion of A549 cells by *M. tuberculosis* strains. To determine the percentage of the bacterial inoculum that would invade A549 alveolar epithelial cells, monolayers of A549 cells were incubated with strain H37Rv or H37Ra for 2 h, and invasion was compared with that by *M. avium* and *E. coli* HB101. Subsequently, the monolayers were treated with amikacin at a concentration that kills extracellular *M. tuberculosis* in 2 h (reduction to an undetectable number of bacilli).

Aminoglycosides are known to concentrate in cells at 5% of the extracellular concentration. Therefore, at the concentration used, amikacin would at most achieve a subinhibitory concentration for the intracellular bacteria (MIC for *M. avium* 101 and 109, 12 μg; MIC for H37Rv and H37Ra, 2 μg; MIC for *E. coli* HB101, 0.4 μg). As shown in Table 1, strain H37Rv invaded A549 cells with greater efficiency than *M. avium*, strain H37Ra, and *E. coli* HB101 (negative control). The percent invasion was dependent on the inoculum used and on the temperature at which the assay was carried out. At 37°C, approximately 83% ± 4% of the cells were infected at an MOI of 100:1, 62% ± 6% were infected at an MOI of 10:1, and 41% ± 4% were infected when the MOI was 1:1. Significantly greater invasion was observed when the bacteria used in the assay were grown at 37°C (host temperature) than at 30°C, suggesting that expression of virulence determinants occurs at the latter temperature. As a control to rule out amikacin-dependent inhibition of intracellular bacteria, uninfected A549 monolayers were treated with a similar concentration of amikacin (20 μg/ml for *M. tuberculosis*) for 2 h, washed, and then lysed by freezing and thawing. The lysate was incubated with both *E. coli* HB101 (MIC, 0.4 μg/ml) and H37Rv (MIC, 2 μg/ml), and inhibition of growth was monitored for 24 h by comparison with bacteria not exposed to amikacin. No effect of the lysate was observed on H37Rv, but inhibition or killing was seen with *E. coli* HB101 (data not shown).

Replication of *M. tuberculosis* in monolayers of alveolar epithelial cells. To examine the ability of *M. tuberculosis* to replicate within A549 cells, the monolayers were infected with a 10:1 ratio of *M. tuberculosis* to A549 cells for 2 h and exposed to amikacin for an additional 2 h. The monolayers were washed and monitored for 96 h. Control monolayers infected with *M. tuberculosis* H37Rv were observed daily for cell lysis, which would release intracellular bacteria that would possibly multiply in the extracellular environment. No cytotoxicity was observed in monolayers infected at the 10:1 ratio (bacteria to cell). As shown in Table 2, both strains H37Rv and H37Ra replicated within A549 cells.

Effect of inhibitors of invasion mechanisms. To examine the role of microfilaments and microtubules on the uptake of *M. tuberculosis* H37Rv by A549 alveolar epithelial cells, we used cytochalasin D, which has been shown to cause microfilament depolymerization (26), and colchicine and nocodazole, which are inhibitors of microtubule polymerization (25). In addition, caffeine, an inhibitor of pinocytosis at the concentration used, was used to determine whether pinocytosis was involved as a mechanism of internalization of *M. tuberculosis* by A549 cells (18). As shown in Fig. 3, cytochalasin D at 3 μM inhibited 73% of H37Rv uptake by A549 cells. A concentration-dependent effect was observed when cytochalasin D was used; 0.5 μM inhibited uptake 33% ± 4%, 1 μM inhibited uptake 49% ± 11%, and 2 μM inhibited uptake 56% ± 6%.

Colchicine at 10 μM and nocodazole at 20 μM had similar effects on the uptake of strain H37Rv by A549 cells. As shown in Fig. 3, both nocodazole and colchicine inhibited *M. tuberculosis* uptake by approximately 60%. Concentrations of colchicine and nocadazole lower than those shown in Fig. 3 had a less significant effect on uptake (data not shown). No toxic effect on the A549 cell monolayers was observed.

To examine whether pinocytosis had any role in H37Rv invasion of A549 cells, we used caffeine in increasing concentrations up to 200 μM to inhibit pinocytosis. However, as shown in Fig. 3, 200 μM caffeine had no significant influence on the ability of strain H37Rv to invade A549 epithelial cells.

Effect of antibodies on the ability of H37Rv to invade A549 cells. Integrins and other receptors have been shown to be the receptors on the cell membrane for a number of bacteria, such as *M. tuberculosis* (21), *M. avium* (2), and *Mycobacterium leprae*

TABLE 2. Intracellular growth of *M. tuberculosis* strains H37Rv and H37Ra[a]

Time of infection (h)	Mean no. of intracellular bacteria ± SD	
	H37Rv	H37Ra
2	$3.4 (\pm 0.4) \times 10^5$	$2.1 (\pm 0.4) \times 10^5$
24	$6.8 (\pm 0.5) \times 10^5$*	$3.8 (\pm 0.5) \times 10^5$
48	$9.6 (\pm 0.4) \times 10^5$*	$5.1 (\pm 0.4) \times 10^5$
72	$1.0 (\pm 0.3) \times 10^6$*	$7.6 (\pm 0.3) \times 10^5$
96	$2.1 (\pm 0.4) \times 10^6$*	$7.8 (\pm 0.4) \times 10^5$

[a] The ratio of infection was 10 bacteria per cell. The results are the means of three experiments. Approximately 8 to 10% of the cells were lost during the period of infection. No difference was seen between H37Rv and H37Ra. *, $P < 0.05$ compared with H37Ra.

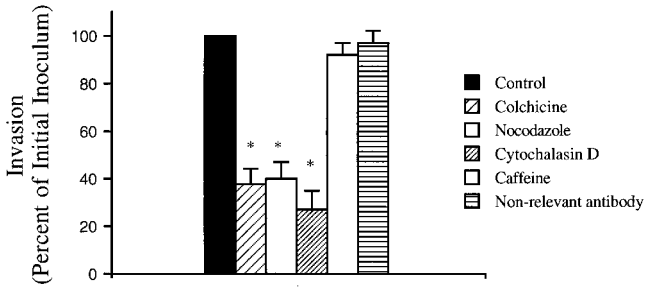


FIG. 3. Effects of various biochemical inhibitors of *M. tuberculosis* strain H37Rv uptake into A549 alveolar epithelial cells. Monolayers were incubated with either no inhibitor (control) or 10 μM colchicine, 3 μM cytochalasin D, 20 μM nocodazole, or 200 μM caffeine for 1 h prior to the assay and during the 2 h of the assay. Percentage of initial inoculum was calculated by subtracting invasion in the presence of inhibitors from invasion in the absence of inhibitors. The results are means of three experiments ± SD. *, $P < 0.05$ compared with control group.

(4). Recently, Ratliff and colleagues (19) identified a fibronectin receptor on the outer structure of mycobacteria that was associated with the binding of bacillus Calmette-Guerin to bladder epithelial cells (20). To examine whether integrins and transferrin receptors were linked to strain H37Rv entry into A549 cells, monolayers were incubated with antivitronectin receptor (CD51), anti-β_1 integrin (CD29), antitransferrin receptor (CD71), or anti-VLA-4 antibodies at different concentrations at 4°C for 30 min. Then, *M. tuberculosis* H37Rv was added to the monolayer and incubated at 37°C for 2 h to allow invasion. As shown in Table 3, both the anti-CD51 and anti-CD29 antibodies inhibited H37Rv invasion in a dose-dependent manner, but no effect was observed with anti-CD71 or anti-VLA-4. When used in combination, an additive effect of anti-CD51 and anti-CD29 in inhibiting uptake of H37Rv was observed. A mouse anti-*P. aeruginosa lipopolysaccharide* was used as a control and could not inhibit *M. tuberculosis* uptake.

DISCUSSION

Infection with *M. tuberculosis* is assumed to occur primarily by inhalation of small drops of proteinaceous pulmonary secretion containing live bacteria (3, 23). Once in the respiratory tree, the droplet nuclei, ranging between 5 and 10 μm, will deposit in the alveoli, where *M. tuberculosis* can then invade local macrophages. However, it is plausible to assume that an interaction between the bacillus and alveolar epithelial cells occurs during the infectious process. Type II alveolar cells are cuboid epithelial cells that cover approximately 5 to 8% of the alveolar surface (5). They specialize in the synthesis of alveolar lining material surfactant. In addition, type II cells also differentiate into type I cells to reconstitute the epithelial surface when it is lost as a result of normal turnover or acute injury (13).

In the present study, we have made the observation that *M. tuberculosis* strain H37Rv can invade type II alveolar cells with significant efficiency in vitro, in the absence of serum. The observation that *M. tuberculosis* can invade epithelial cells was first reported by Shepard for HeLa cells (5). In his original studies, it was also determined that the uptake of *M. tuberculosis* was more efficient in the presence of serum than in its absence.

Invasion ability was observed to be dependent upon the MOI and on the temperature. A temperature of 37°C was associated with augmented uptake of all microorganisms tested except for *E. coli HB101* compared with that at 30°C. This result suggests that at the host temperature (37°C), *M. tuberculosis* expresses an invasive phenotype. In a previous study, invasion of both HEp-2 and HT-29 cells by *M. avium* was also shown to be significantly more efficient when the bacteria were cultured at 37°C (1). In addition, the *inv* gene of *Yersinia pseudotuberculosis*, a gene associated with the ability to invade intestinal cells, has been demonstrated to be expressed at 37°C and not at lower temperatures (12).

The experiments reported here demonstrate that both *M. tuberculosis* strains H37Rv and H37Ra were capable of replicating within A549 type II alveolar cells. Gut pathogens such as *Salmonella* spp., enteropathogenic *E. coli*, and *Yersinia* spp. as well as respiratory pathogens such as *Haemophilus influenzae* adhere to and enter epithelial cells but do not require epithelial cells for replication. These bacteria presumably invade epithelial cells to facilitate migration to the subepithelial space. While *M. leprae* invades host cells to replicate (5), it is currently unknown if *M. tuberculosis* enters epithelial cells either to replicate or to avoid the mechanisms of host defense.

Bacterial invasion is a complex process that involves bacterial as well as host cell determinants (9). The epithelial cell endocytosis processes triggered by most invasive bacterial species are heavily microfilament dependent, and they are inhibited by cytochalasins, which are microfilament depolymerizers. Previous studies in our laboratory (1) and a study by Mapother and Sanger (14) have demonstrated that invasion of epithelial cells by *M. avium* is inhibited by cytochalasin. In the present study, we observed that the entry of *M. tuberculosis* into A549 epithelial cells was microfilament dependent and was inhibited up to approximately 70% by 3 μM cytochalasin D. However, entry was also microtubule dependent. Microtubule depolymeralization following treatment with colchicine or nocodazole resulted in inhibition of *M. tuberculosis* uptake.

Entry was not inhibited by caffeine, an inhibitor of pinocytosis (18), potentially ruling out a pathway that has recently been suggested for *Salmonella* invasion of macrophages. Why *M. tuberculosis* invasion of type II alveolar cell line appears to be dependent on both microfilaments and microtubules is not known. It seems plausible to hypothesize that *M. tuberculosis* expresses at least two different mechanisms of invasion, one

TABLE 3. Effect of antibodies against membrane receptors on uptake of *M. tuberculosis* H37Rv by A549 alveolar epithelial cells[a]

Antibody	Concn (μg/ml)	Mean no. of intracellular bacteria ± SD[b]	% Inhibition of uptake[c]
None (control)		$1.4 (\pm 0.5) \times 10^5$	
Anti-CD51	5	$9.5 (\pm 0.4) \times 10^4$	32.1
	10	$7.4 (\pm 0.4) \times 10^4$	47.1*
	30	$6.2 (\pm 0.5) \times 10^4$	55.7*
Anti-CD29	5	$8.9 (\pm 0.4) \times 10^4$	36.4
	10	$7.1 (\pm 0.4) \times 10^4$	49.2*
	30	$6.3 (\pm 0.5) \times 10^5$	55.0*
Anti-CD71	5	$1.4 (\pm 0.5) \times 10^5$	0
	10	$1.5 (\pm 0.3) \times 10^5$	0
	30	$1.1 (\pm 0.5) \times 10^5$	0
Anti-VLA-4	5	$1.6 (\pm 0.3) \times 10^5$	0
	10	$1.5 (\pm 0.3) \times 10^5$	0
	30	$1.2 (\pm 0.4) \times 10^5$	0
Anti-CD51 + anti-CD29	5 + 5	$4.3 (\pm 0.4) \times 10^4$	69*
	10 + 10	$2.9 (\pm 0.3) \times 10^4$	79*

[a] The initial inoculum was $3.0 (\pm 0.6) \times 10^5$.
[b] The results are means for three experiments.
[c] *, $P < 0.05$ compared with the control.

that is microfilament dependent and another that is microtubule dependent. Expression of one or more bacterial uptake pathways may depend on the presence of different cell membrane receptors according to different environmental conditions as well as the influence of other, surrounding cells. More than one mechanism of entry has been described for *Yersinia* spp. as well as *Salmonella typhimurium* (10, 16). Why both pathways were present on the same cell at the same time is presently difficult to explain. Recent reports have shown that *Candida albicans* invasion of endothelial cells is also microtubule and microfilament dependent (7). In addition, a microtubule-dependent endocytosis mechanism has been reported to occur in the invasion of epithelial cells by *Campylobacter jejuni* and *Citrobacter freundii* (17).

Bacteria utilize a number of different cell receptors to be internalized by the cells. It is assumed that pathogenic bacteria bind to receptors linked to intracellular signal pathways before triggering interaction with the cytoskeleton, and a number of pathogenic organisms have been shown to use integrins as the receptor. Integrins are connected to the intracellular trafficking machinery, and the association with an integrin on the cell membrane may trigger a chain of intracellular events (2, 4, 21). For example, *M. leprae* binds to β_1 integrin to interact with nasal epithelial cells (4), and BCG has been shown to use the fibronectin receptor to be ingested by bladder epithelial cells (19, 20). We have demonstrated that blocking of the β_1 integrin and the vitronectin receptor was associated with significant inhibition of *M. tuberculosis* uptake. Furthermore, blocking of both the vitronectin receptor and β_1 integrin resulted in up to 79% inhibition of *M. tuberculosis* binding. The presence of β_1 integrin and the vitronectin receptor ($\alpha v\beta 3$) in A549 alveolar epithelial cells in vivo has been demonstrated (15).

There are a number of possible reasons why *M. tuberculosis* has not been shown to attach to and invade alveolar epithelial cells in vivo. Specimens from patients are often obtained at autopsy, and examination the late events in the pathogenesis of early infection of alveolar epithelial cells would not be possible. In addition, animal studies may not have focused on involvement of alveolar epithelial cells, or epithelial cells may not be important host cells for *M. tuberculosis* in vivo. Our observation that *M. tuberculosis* can enter alveolar epithelial cells in vitro is certainly the first step in the investigation of the role of alveolar epithelial cells and their possible interaction with macrophages in the pathogenesis of tuberculosis.

In summary, *M. tuberculosis* can enter an alveolar epithelial cell line by both microfilament- and microtubule-dependent pathways by using integrins as receptors. Future studies will address the importance of the current observation in vivo.

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