Cytotoxicity for Lung Epithelial Cells Is a Virulence-Associated Phenotype of *Mycobacterium tuberculosis*

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Dissemination of viable tubercle bacilli from the lung is a critical event in the establishment of *Mycobacterium tuberculosis* infection. We examined the possibility that *M. tuberculosis* bacteria could infect and damage lung epithelial cells to determine whether direct penetration of the alveolar epithelium is a plausible route of *M. tuberculosis* infection. While both virulent H37Rv tubercle bacilli and the attenuated *Mycobacterium bovis* BCG vaccine strain were able to enter A549 human lung epithelial cells in culture, only the virulent tubercle bacilli were cytotoxic for both polarized and nonpolarized epithelial monolayers and macrophages. In addition, bacterial entry into epithelial cells, but not macrophages, was increased by intracellular passage through macrophages, suggesting enhancement of a bacterially mediated cell entry mechanism in bacteria grown within macrophages. These findings suggest that *M. tuberculosis* bacteria might have the ability to gain access to the host lymphatics and circulatory system by directly penetrating the alveolar epithelial lining of an infected lung.

Resolution of the current tuberculosis (TB) epidemic will require prevention of new TB infection as well as improved methods for treating existing disease. This point is emphasized by the risk of generating additional drug-resistant isolates during treatment of TB infection and the frequency with which *Mycobacterium tuberculosis* establishes a latent infection that can reactivate years or decades later (6). Design of such preventive measures would be facilitated by an understanding of how tubercle bacilli establish infection at the cellular and molecular levels.

TB infection occurs when droplet nuclei smaller than 0.5 μ m and containing one to three tubercle bacilli are inhaled deep into the alveoli (13, 34). Dissemination of viable tubercle bacilli from the lumen of the alveolus into the lymph or circulatory system is a critical stage in the establishment of *M. tuberculosis* infection because this event marks the transition from a localized clearance event to a potential systemic infection (8, 12, 33). There is also evidence that hematogenous spread is an important factor in the establishment of infectious pulmonary TB in the majority of cases in which initial implantation of bacilli does not occur in the more vulnerable apices of the lung (4, 5). The means by which this hematogenous seeding occurs is not known.

One route of particle clearance from the alveoli to the hilar lymph nodes is mediated by alveolar macrophages (21), and it is likely that *M. tuberculosis* bacteria can reach the lymph nodes, and eventually the blood stream, in this way. However, it has not been determined whether tubercle bacilli can also translocate across the alveolar epithelium on their own. The distinction between macrophage-mediated and bacterially mediated translocation mechanisms is significant with respect to the accessibility of bacteria to the mucosal immune system during transport and the sensitivity of the transport process to the host physiological state.

Bacterial pathogens have been shown to breach the intestinal epithelium by a variety of strategies, including specific access through M cells, cytotoxicity, transcytosis, and weakening of the junctions between adjacent epithelial cells (15, 16, 19, 31, 36, 37). To determine whether virulent tubercle bacilli can disrupt the integrity of an epithelial cell barrier, we have examined the interaction of tubercle bacilli with cultured human lung alveolar epithelial cells. We found that virulent H37Rv tubercle bacilli, but not attenuated *Mycobacterium bovis* BCG bacteria, are cytotoxic for cultured A549 human lung alveolar epithelial cells, including cells polarized by extended growth on porous filter membranes. These detrimental effects on polarized A549 cells were greatest when cells were infected in the absence of serum by virulent H37Rv bacteria that had been grown previously within macrophages.

MATERIALS AND METHODS

Bacterial growth conditions. *M. tuberculosis* H37Rv (ATCC 25618) and *M. bovis* BCG (Pasteur strain; Trudeau Institute) were grown as described previously (28). Briefly, frozen aliquots of bacteria were thawed, diluted into 7H9 liquid medium (Difco) supplemented with 0.5% glycerol, a 10% oleic acid-albumin-glucose complex (Difco), and 0.25% Tween 80, and grown at 37°C in tissue culture flasks (Falcon) or square medium bottles (PETG; Nalgene) without shaking. H37Rv cultures were not passaged further, whereas BCG cultures were passaged weekly at 1:100 for use up to the ninth pass. Bacteria were used after 4 to 7 days of growth, at a density of approximately 1×10^7 to 5×10^7 /ml. Bacteria grown in this way form a silt-like precipitate on the bottom of the flasks that resuspends to a uniform suspension of mostly small clumps containing 1 to 10 bacteria.

Preparation of single-cell mycobacterial suspensions. Clumping of bacterial cultures was reduced further by applying three 5-s pulses at setting 4 to cultures in closed polypropylene tubes at 4°C by using a Virsonic 475 ultrasonic cell disrupter fitted with a cup horn attachment (VirTis Company, Gardiner, N.Y.). Cooling was achieved by continuously circulating 4°C water through the cup horn with a Lauda refrigerated recirculator. The viability of these cultures was assessed by staining the bacteria with 2 μ g of fluorescein diacetate (FDA; Sigma) (5a) and 8 μ g of ethidium (Et) homodimer (Molecular Probes, Eugene, Oreg.) per ml. The nonfluorescent FDA substrate is cleaved by esterases in metabolically active cells to produce fluorescein. With this method, viable bacteria stain yellow-green and nonviable bacteria fluoresce red when viewed with a long-pass fluorescein filter set. Single-cell suspensions containing from 65 to 85% viable bacteria were routinely obtained in this manner (Fig. 1). Bacterial viability was also determined for each experiment by plating in triplicate on 7H10 agar in conjunction with counting bacterial suspensions in a Petroff-Hausser chamber using phase-contrast microscopy.

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FIG. 1. Viability assessment of single-cell BCG cultures using the FDA-Et homodimer method. (A) Viable culture; (B) culture heat killed at 90°C for 30 min.

Tissue culture conditions. The human lung pneumocyte A549 cell line (CCL 185) was obtained from the American Type Culture Collection at passage 77 and used between passages 79 and 95. Cells were maintained without antibiotics in Ham's F12 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, Utah) with weekly passage and feedings every 3 to 4 days. A549 cells were seeded on glass coverslips (22 by 22 mm) in 6-well microtiter dishes or directly into plastic 24-well microtiter plates (Falcon) at a density of approximately 5×10^4 /ml. These cells were polarized by growing for 10 to 14 days after seeding at a density of approximately 5×10^5 /ml on 0.33-cm² porous filter membranes (3415; Costar, Cambridge, Mass.) in 24-well microtiter plates (Costar) containing media. Both apical and basolateral media were changed every 3 days. The mouse macrophage cell line J774.16 (11) was maintained as described previously (28). Briefly, cells were cultured in antibiotic-free Dulbecco's modified Eagle's medium (GIBCO) containing 20% FBS, 5% NCTC 109 (GIBCO), 1% nonessential amino acids (GIBCO), and 1% glutamine. J774.16 cells were seeded at approximately 10^5 /ml 18 to 24 h before use.

Preparation of IC and XC-passaged bacterial cultures. To obtain intracellularly (IC) passaged bacteria, J774.16 monolayers in 75-cm² tissue culture flasks were infected by diluting bacterial cultures at least 1:20 directly into fresh tissue culture medium to achieve a multiplicity of infection (MOI) of approximately 5 bacteria per cell. This inoculating medium was used to replace the maintenance medium on the cells before incubation at 37°C with 5% CO₂. At 16 to 40 h postinfection, the monolayers were washed two or three times for 5 min with 10 ml of Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) at pH 7.4. Cells were lysed by replacing the last wash with 1 ml of 0.5% Triton X-100–0.5% deoxycholate in Ca²⁺- and Mg²⁺-free PBS for 3 to 5 min before addition of 30 ml of serum-free medium (SFM) to dilute the detergent. Bacteria were harvested by centrifugation, resuspended in the final inoculation medium (usually 10 to 20 ml), and briefly (5 to 15 s) sonicated as described earlier. Extracellularly (XC) passaged bacteria were grown in tissue culture media without macrophages at 37°C with 5% CO₂, and, except for the initial washing of the infected monolayers, were treated identically to IC-passaged bacteria during the harvest and wash steps. As in other experiments, inoculation titers were determined by plating for CFU counting on 7H10 agar. In many experiments, microscopy counts were also used to estimate bacterial input on the day of the experiment.

Infection of monolayers. J774.16 or A549 cells were infected with IC- or XC-passaged bacterial cultures at MOIs ranging from 1 to 200 bacteria per cell, depending on the experiment. After an initial incubation period of 4 to 16 h, the inoculating medium was removed, the infected monolayers were washed twice with PBS at pH 7.4, and fresh tissue culture medium was added to the cells. Thereafter, fresh medium was provided to the cells every 2 days for the duration of the experiment. Unless specified otherwise, infections were carried out in tissue culture medium containing 10% (A549) or 20% (J774.16) FBS. When infections were performed in SFM, the inoculating medium was replaced with fresh medium containing FBS within 4 to 6 h. No detrimental effects on the cells were observed during these short periods of serum deprivation.

Cell detachment assays. At various times postinfection, cells were washed

three times with PBS, fixed for 1 h at room temperature in methanol, and stained overnight with 4 μ M ethidium bromide (EtBr; Sigma) in PBS. The resulting fluorescence signal was read by using an EX 485(20)-EM 645(40) filter combination in a Cytofluor 2350 multiwell fluorescence plate reader (Millipore). All fluorescence values were derived from summing 12 overlapping readings encompassing the entire surface area of each well to avoid discrepancies that may arise from uneven distribution of cells throughout a well. For each experiment, the mean of replicate uninfected control monolayers was assigned a value of 100%, and the percent monolayer remaining under each experimental condition was calculated as mean sample fluorescence/mean fluorescence of uninfected wells $\times$ 100. Following these EtBr measurements, plates were rinsed and restained for 30 min with Giemsa so that the degree of monolayer detachment in each well could be assessed visually. Periodically throughout an experiment, the infected monolayers were examined with a Zeiss axiovert inverted microscope using Hoffman modulation optics to monitor the condition of the cells.

Microscopy. Cell-associated mycobacteria were stained with auramine O (Sigma) by the method of Smithwick and David (42) following methanol fixation. Samples were viewed with a Zeiss filter set 10 on an Axioskop fluorescence microscope, and photographs were taken with Kodak Tmax 400 film.

Electron microscopy (EM) samples were prepared as described previously (28). Briefly, infected monolayers and Costar filter membranes were fixed for 45 min on ice in a solution of 2% glutaraldehyde and 2% osmium tetroxide (1:1) in 0.1 M cacodylate buffer (pH 7.3). Fixed samples were washed and then left standing overnight in 4% paraformaldehyde with 1% glutaraldehyde, dehydrated in ascending ethanol, and embedded in Spurr's resin (Polysciences, Warrington, Pa.) (43). Thin sections were stained in 3% uranyl acetate for 20 min and lead citrate for 2 min before they were examined in either a Siemens 102 or a JEOL 100S electron microscope. One-micrometer sections were stained with toluidine blue before light-microscopic examination with a Zeiss Axioskop.

For EM-level cytotoxicity assessment, from 100 to 200 cells from at least eight thin sections cut from different areas of the monolayer were counted. The number of bacteria per cell and the condition of each cell were recorded. Degraded cells were scored as dead, as shown in Fig. 2.

RESULTS

Previous reports on the ability of tubercle bacilli to exert a cytotoxic effect in the absence of host factors have varied, and it has been postulated that tumor necrosis factor alpha produced by local macrophages may be responsible for the tissue necrosis observed during TB disease (17, 18). To determine whether tubercle bacilli are cytotoxic to host cells in the absence of host immune cells, we infected cultured lung epithelial cells with single-cell suspensions (Fig. 1) of virulent H37Rv or

FIG. 2. Representative electron micrographs showing A549 lung epithelial cells infected with H37Rv bacteria for 7 days scored for mortality. D, degraded cells that would have been scored as dead in Fig. 6; n, nucleus. Bars, 1 μ m. (a) Well-preserved, healthy-looking lung cell infected with numerous bacilli (arrows). (b) Degraded cell adjacent to a cell that is beginning to show signs of toxicity (center). Flanking cells appear normal. Note the darker staining, numerous vacuoles (v), swollen mitochondria (short arrows), distorted rough endoplasmic reticulum (long arrows), and disrupted microvilli (m) of the morbid cell (this cell would not have been scored as dead in Fig. 6). (c) Example of a heavily infected cell that could have been lysed because of bacterial overload. The nucleus appears nearly degraded, and very little is left of the cytoplasm. This lysed cell is adjacent to a normal-looking cell. (d) Severely degraded cell between two healthy infected cells. Note the intact apical membrane (arrows) and the relatively small number of bacilli in this cell compared with the overloaded lysed cell shown in panel c. (e) Lower magnification showing cells in a variety of stages including healthy, infected, and degraded.

EFFECT OF INTRACELLULAR PASSAGE ON Mtb CYTOTOXICITY

FIG. 4. Effect of MOI on cytotoxicity for cultured lung epithelial cells (A549) (A and B) and macrophages (J774.16) (C and D). At 1 (solid bars), 4 (hatched open bars), and 8 (hatched solid bars) days postinfection, cells were washed, fixed, and stained with EtBr, as described in Materials and Methods. Approximate MOIs: BCG, 65 (A), 125 (B), 25 (C), and 50 (D); *M. tuberculosis* (Mtb), 10 (A and D), 20 (B), and 5 (C). Values exceeding 100% were read as 100%.

avirulent *M. bovis* BCG bacteria. Cell cytotoxicity was assessed microscopically and by using two versions of a monolayer detachment assay at various times postinfection. Because it is possible that some of the tubercle bacilli that encounter the alveolar epithelium during natural infection will have previously resided within a host macrophage, we used bacteria that had been grown IC within J774.16 macrophages in addition to XC-grown bacilli in all of these experiments.

At an MOI of approximately 20 bacteria per cell, avirulent BCG bacteria were not toxic to cultured lung epithelial cells at 1 day postinfection and had only slightly deleterious effects at 4 days postinfection (see Fig. 3). In contrast, virulent H37Rv bacteria that had been passaged for 2 days intracellularly within J774.16 murine macrophages had destroyed more than 75% of the epithelial cell monolayer by 4 days postinfection. Changes in the cell monolayers which had been infected with XC-grown *M. tuberculosis* were less dramatic but still obvious at 4 days postinfection (Fig. 3B and C).

The degree of cytotoxicity, its apparent enhancement with IC passage, and the length of time before these effects can be detected are affected by several factors. One variable we investigated was MOI. A549 and J774.16 cells were infected at various MOIs with IC- and XC-passaged H37Rv and BCG bacteria for 16 h before removal of the inoculation media. At 1, 4, and 8 days postinfection, cells were washed, fixed, and stained with EtBr, as described in Materials and Methods. The percent intact monolayer was calculated as described before, except that, owing to the large number of samples in this experiment, it was not possible to produce a replicate for each condition.

As shown in Fig. 4, BCG bacteria were not toxic to either A549 epithelial cells or J774.16 macrophages, even at 8 days postinfection, with MOIs exceeding 100 bacteria per cell. In contrast, virulent *M. tuberculosis* bacteria were toxic to macrophages at 8 days postinfection at an MOI of 5 bacteria per cell. When the MOI was increased to greater than 10 bacilli per cell,

effects were apparent as early as 1 day postinfection. A549 cells were less sensitive than J774.16 macrophages to *M. tuberculosis* cytotoxic effects, although by 8 days postinfection much of the A549 monolayers were destroyed when MOIs of at least 10 bacilli per cell were used for infection.

Cytotoxic effects can be exerted on a cell from either the XC milieu or the IC milieu (20, 23, 24, 32). Several observations suggest that this *M. tuberculosis*-mediated toxicity is a localized event resulting from IC rather than from XC bacteria. As shown in Fig. 3C and assessed by trypan blue exclusion (not shown), cells that remained adherent to the glass or plastic substrate often appeared healthy despite high levels of monolayer detachment. When examined at the EM level, the cytotoxic effect was observed primarily in infected cells with apparently healthy cells immediately adjacent to degraded cells. The lysed cells contained from 3 to 50 H37Rv bacteria per cell. In addition, many healthy-appearing cells in close contact with degraded cells were also infected with different numbers of bacilli (Fig. 2).

To determine whether BCG was less cytotoxic than H37Rv because of a reduced capacity to enter host cells and whether IC passage affected the ability of these bacteria to associate with host cells, we examined the degree of bacterial association with lung epithelial cells at 1 day postinfection (Fig. 5). A549 lung epithelial cells were infected for 16 h with IC- or XC-passaged H37Rv and BCG bacteria. After methanol fixation, samples were stained with auramine O and viewed with a Zeiss Axioskop fluorescence microscope. As shown in Fig. 5, both BCG and H37Rv showed higher levels of association with the epithelial cells after having been pregrown in macrophages, although the patterns of association differed. In these experiments, H37Rv bacteria appeared to be more invasive than adherent, whereas the reverse was true with BCG.

To allow more precise differentiation between adherent and internalized bacteria, infected monolayers were embedded in Spurr's resin and examined in ultrathin sections at the EM level and in 1- μ m-thick sections by light microscopy. In this experiment, IC bacteria were counted at the light level to facilitate counting a large number of cells (Table 1). J774.16 macrophages were included in these experiments for comparison as a functional control. We reasoned that bacterial uptake by macrophages would be generally nonspecific because of the multiplicity of phagocytic routes in these cells (14, 38, 39) and should provide a relative index of the number of bacilli available for phagocytosis. In contrast, bacterial entry into nonphagocytic epithelial cells is likely to be mediated by specific bacterial factors.

We found that the infectivity index in A549 lung epithelial cells in the IC-passaged samples was higher than that in the XC-passaged samples of both BCG and *M. tuberculosis*, although the increase was greater with *M. tuberculosis* (4.5-fold) than with BCG (2-fold) (Table 1). In contrast, the number of bacteria in the macrophage samples was not affected by passage conditions, suggesting that the results observed with epithelial cells reflect a true enhancement of a bacterially mediated cell entry mechanism. In both cases, the increase in the number of IC bacteria resulted more from additional cells becoming infected than from an increase in the number of bacteria per infected cell. Forty-five percent more epithelial cells were infected with IC-passaged BCG than with XC-passaged BCG, and nearly 200% more cells became infected with IC- than with XC-passaged *M. tuberculosis*.

Previously, tubercle bacilli have been shown to enter a variety of nonphagocytic cell types (17, 18, 40, 41), and recently a DNA fragment from an attenuated H37Ra *M. tuberculosis* strain was shown to confer an invasive phenotype on *Esche-*

FIG. 5. Effect of IC passage on bacterium-host cell association. A549 lung epithelial cells were infected with XC *M. tuberculosis* (A), IC *M. tuberculosis* (B), XC BCG (C), or IC BCG (D) bacteria (BCG MOIs were 10-fold higher than those for *M. tuberculosis*).

TABLE 1. Effects of IC passage and XC passage on infectivity

Sample	Total no. of cells	Total no. of bacteria	No. of cells infected (%)	No. of bacteria/infected cell	Infectivity index ^a
J774.16					
BCG					
IC	822	2,391	613 (74.6)	3.9	291
XC	637	1,665	483 (75.8)	3.5	261
<i>M. tuberculosis</i>					
IC	543	276	157 (28.9)	1.8	51
XC	688	380	231 (33.6)	1.7	55
A549					
BCG					
IC	201	1,393	183 (91.0)	7.6	693
XC	163	568	102 (62.6)	5.6	348
<i>M. tuberculosis</i>					
IC	247	545	160 (64.8)	3.4	220
XC	211	104	46 (21.8)	2.3	49

^a Number of bacteria per 100 cells.

richia coli bacilli (3). However, a role for bacterial entry into these nonphagocytic cell types during natural infection has yet to be assigned. To penetrate the alveolar epithelium, tubercle bacilli must be able to enter through the apical side of a polarized epithelial cell monolayer.

To determine whether virulent tubercle bacilli can enter polarized lung epithelial cells, we infected A549 cells that had been grown for 10 days on polycarbonate filters suspended in tissue culture media. Because the serum conditions at the time of infection have been shown to influence the fate of internalized tubercle bacilli (2), we performed these infections in both the presence and the absence of serum. Polarized A549 cells were infected with XC- or IC-passaged H37Rv bacteria in the presence of either heat-inactivated FBS, fresh normal human serum (NHS) from a BCG-negative donor, or serum-free medium (SFM). Samples were washed at 4 to 6 h postinfection and maintained in tissue culture medium. At 7 days postinfection, samples were fixed, processed, and examined at both the EM level and the light microscopic level. Bacterial uptake and epithelial cell mortality were scored at the EM level (Fig. 2 and 6).

As shown in Fig. 6A to D, the presence of NHS at the time of infection increased the uptake of XC bacteria relative to the other conditions and eliminated the enhancement of IC bacterial uptake observed with either heat-inactivated serum or no serum. In contrast to the other conditions, the increased uptake of XC bacteria with NHS did not correlate with an increase in cytotoxicity for lung epithelial cells, as determined at the EM level. As seen microscopically in Fig. 6a to h, H37Rv entry into polarized lung epithelial cells resulted in selective lysis of infected areas of the monolayers. Monolayer destruction was greatest when infection with IC-passaged bacteria was carried out in the absence of serum, resulting in clear gaps in the cell monolayer.

DISCUSSION

Tissue destruction is believed to play a role in at least two stages of *M. tuberculosis* pathogenesis: liquefaction of granulomas preceding reactivation of latent infection and lung cavitation associated with advanced disease (12, 13). There is evidence that host factors are involved in these processes (17, 18, 35). However, it is not clear whether any of the observed

effects are bacterially mediated. In this report, we show that virulent H37Rv *M. tuberculosis*, but not avirulent BCG, bacteria are cytotoxic for human lung epithelial cells and murine macrophages in culture, and we propose a model for TB infection via direct penetration of the alveolar epithelium. In addition, effects of IC passage within macrophages on the ability of these bacilli to enter and kill lung epithelial cells are discussed.

Cytotoxicity is a virulence-associated phenotype. Cytotoxicity was consistently observed with virulent *M. tuberculosis* bacteria but not avirulent BCG. The length of time necessary to see significant destruction of *M. tuberculosis*-infected A549 monolayers in different experiments varied (Fig. 3 and 4) but was often dependent on the initial MOI and the length of time before the initial wash following infection. Because no antibiotics were used to kill XC bacilli in these studies, bacteria released from lysed cells were also available to infect new cells for periods of up to 48 h between changes of the media. This could result in accelerated destruction in heavily infected monolayers. Unfortunately, it was not possible to use antibiotics to prevent reinfection in these experiments because all antibiotics tested also killed IC bacilli when present in the media for extended periods.

Higher levels of cytotoxicity were observed with IC-passaged *M. tuberculosis* than with XC-passaged bacteria in just more than one half the experiments performed. The reasons for this variability are not clear, although it seems likely that some regulatory components are involved. It is possible that subtle differences in the environmental conditions such as pH and/or oxygen tension influence expression of the cytotoxic phenotype. Stimulatory conditions found routinely in an IC environment may be duplicated under XC conditions depending upon batch-to-batch variation in tissue culture medium, bacterial concentration, and possibly even positional effects within the CO₂ incubator. Both *Yersinia pestis* (26, 27) and *Salmonella typhimurium* (1) express new proteins in response to the acidic pH within phagolysosomes, and oxygen tension has been shown to influence the uptake of *Salmonella* organisms by mammalian cells (25a) and the growth rate of tubercle bacilli within macrophages (30).

The precise mechanism of the observed cytotoxicity cannot be determined from the present study, although it appears that these effects are caused by IC rather than XC bacteria (Fig. 3c). The higher bacterial loads found in the IC-passaged *M. tuberculosis*-infected epithelial cell samples may contribute to an increase in cytotoxicity in epithelial cells, but they cannot fully account for increased cytotoxicity in macrophages that ingested similar numbers of IC- and XC-passaged bacilli. In addition, when cells were infected in the presence of NHS, similar infection levels were achieved after both IC passage and XC passage, but the cytotoxicity associated with the IC-passaged bacteria was six times greater than that seen with the XC-grown bacilli (12 versus 2%) (Fig. 6). It is possible that the bacterial route into the cell influences the outcome of infection, as has been observed with *Toxoplasma gondii* (22) and that serum-mediated bacterial uptake routes may result in less toxicity to the cells.

IC passage increased bacterium-cell association. IC passage consistently enhanced the ability of both H37Rv bacteria and BCG bacteria to associate with epithelial cells, although their patterns of cell association were not identical (Fig. 5). The difference between *M. tuberculosis* and BCG in the enhancement of their internalization by lung epithelial cells following IC passage suggests that these bacteria respond differently to the macrophage environment, as we have shown previously (26). These findings also suggest that specific bacterial factors

FIG. 6. Effects of growth and serum conditions on cell entry and cytotoxicity for polarized lung epithelial cells. (A to D) A549 cells were infected with XC (solid bars) or IC (hatched bars) H37Rv bacteria in the presence of heat-inactivated FBS (HIS), fresh NHS, or SFM. Percent mortality, number of cells scored as dead per 100 cells; infectivity index, number of bacteria per 100 cells; number of bacteria per infected cell, total number of bacteria/number of infected cells. (a to h) Toluidine blue-stained cells in 1- μ m sections on a Zeiss Axioskop using bright-field illumination. (a and b) Enlargements of boxed areas in panels c and d, respectively. Bars, 1 μ m. c, XC, heat-inactivated FBS; d, IC, heat-inactivated FBS; e, XC, NHS; f, IC, NHS; g, XC, SFM; h, IC, SFM. Bars, 10 μ m. Arrowheads, heavily infected lysed cells; arrows, areas in which the monolayers have been fully breached.

that are influenced by the IC milieu mediate the process of bacterial internalization by epithelial cells (Table 1). Possible mechanisms include de novo protein synthesis by IC bacilli and/or bacterial association with macrophage-derived factors.

Others have reported both specific induction of bacterial genes within macrophages (7) and enhanced uptake of bacteria by epithelial cells following association of bacteria with eucaryotic cells (9, 10, 25, 37). We have observed a reduction in the number of H37Rv bacilli residing within fused phagolysosomes immediately after infection when bacilli are pregrown within macrophages. This effect is not seen with BCG and is eliminated when virulent *M. tuberculosis* bacteria are IC passaged in

the presence of chloramphenicol to block de novo protein synthesis (28a). Current investigations are directed towards determining whether expression of new bacterial uptake ligands is induced within macrophages.

An alternative explanation for the increased invasion levels seen with IC-passaged bacteria is that IC passage either selects for highly invasive tubercle bacilli or favors recovery of viable bacteria that are better able than dead bacilli to enter epithelial cells. However, we believe that this is unlikely. Genetic selection of invasive bacteria within macrophages is improbable because the length of passage required for enhanced cell uptake (<18 h) is less than the doubling time of intracellular

M. tuberculosis (20 to 24 h). In addition, bacterial viability was not increased following IC passage as determined by the FDA-Et homodimer method, and all MOIs were based on viable plate counts rather than microscopic counts.

Although one must use caution when interpreting in vitro results, these data raise the important possibility that virulent tubercle bacilli may facilitate their own transit from the lumen of the alveolus into the interstitial spaces or circulatory systems of the host. We believe that this model is tenable for the following reasons: (i) the enhanced monolayer disruption observed when infection occurs under serum-free conditions is consistent with the low serum levels found within the lumen in the alveoli, (ii) the local concentration of bacteria at a given focus of infection following multiplication within macrophages could be expected to reach levels similar to those used in these studies, and (iii) only transient disruption of the alveolar lining would be required for bacterial penetration.

An additional implication of this model is that host physiological factors that affect lung epithelial permeability may increase the efficiency with which inhaled tubercle bacilli are able to establish progressive infection. Such conditions have been reported for human immunodeficiency virus-infected individuals (29) and could contribute to an increased susceptibility to *M. tuberculosis* infection and disease in human immunodeficiency virus-positive individuals.

In conclusion, we provide evidence that virulent tubercle bacilli, but not the avirulent BCG vaccine strain, are cytotoxic to both lung epithelial cells and macrophages in the absence of additional host immune cells. We have also demonstrated that virulent tubercle bacilli are capable of entering and destroying polarized lung epithelial cells in culture and that bacterial invasion of epithelial monolayers and, possibly, cell cytotoxicity are enhanced by IC passage within macrophages. Further study is necessary to determine the relative contribution of each of these factors to the establishment of TB infection and progression to disease.

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