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Effects of *Mycobacterium tuberculosis* on the Bioelectric Properties of the Alveolar Epithelium

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To investigate the hypothesis that *Mycobacterium tuberculosis* penetrates the alveolar epithelium by downregulating its barrier properties, we evaluated the interactions between *M. tuberculosis* and rat alveolar epithelial cell monolayers that are believed to share electrophysiologic properties of the human alveolar epithelium. Nonproteinaceous components of *M. tuberculosis* caused marked declines in electrical resistance and equivalent short-circuit current of the alveolar epithelial cell monolayers, indicating a reduction in the capacity to maintain tight intercellular junctions and to actively reabsorb sodium. *M. tuberculosis* elicited production of TNF-α mRNA and protein by alveolar epithelial cells, and the effects of recombinant TNF-α on the bioelectric properties of the alveolar epithelial paralleled those of *M. tuberculosis*. Furthermore, the effects of *M. tuberculosis* on alveolar epithelial resistance were abrogated by neutralizing anti-TNF-α antibodies. These results indicate that *M. tuberculosis* elicits production of TNF-α, which in turn reduces the bioelectric barrier properties of the alveolar epithelium. These findings provide insight into potential mechanisms by which *M. tuberculosis* establishes infection and disease in the lung.

Despite the availability of effective antituberculosis agents for decades, tuberculosis continues to be the leading cause of death from infectious pathogens, claiming an estimated 3 million lives annually worldwide (Tuberculosis Surveillance Update, World Health Organization, 1992). Development of novel strategies to prevent tuberculosis is hampered by our inadequate understanding of the pathogenetic mechanisms by which *Mycobacterium tuberculosis* establishes infection in the lung.

Infection with *M. tuberculosis* occurs when organisms in droplet nuclei are inhaled into the alveoli (24). Passage of the organisms through the alveolar epithelium and into the lymphatics is a prerequisite to the establishment of infection and development of disease. However, little is known about the mechanisms by which *M. tuberculosis* crosses the alveolar epithelium, which constitutes a physiologic barrier that resists invasion by microbial pathogens.

Two features of alveolar epithelium that make it an effective barrier are tight intercellular junctions and the capacity to actively reabsorb sodium from the alveolar space, with water following passively. A recently developed monolayer model of rat alveolar epithelium exhibits junctional and active transport properties that are believed to reflect those of alveolar epithelium in vivo (11, 13, 21, 29). The integrity of tight junctions can be monitored by measurement of electrical resistance, and the capacity for active ion reabsorption through the epithelium can be measured by short-circuit current through the epithelium (18). We hypothesized that *M. tuberculosis* penetrates the alveolar epithelium by downregulating its barrier properties. As a first step to evaluate this hypothesis, we investigated the interactions between *M. tuberculosis* and rat alveolar epithelial cell monolayers.

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MATERIALS AND METHODS

Measurement of bioelectric properties of rat alveolar epithelial cell monolayers. We established confluent monolayers of rat pneumocytes in primary culture, as previously described (13, 19). Briefly, lungs of male, specific-pathogen-free, Sprague-Dawley rats (Harlan Bioproducts for Science, Indianapolis, Ind.) were perfused and lavaged, and then type II pneumocytes were isolated by treatment with intratracheal elastase, followed by panning with rat immunoglobulin G. The cell culture medium used was a 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F12 (DME/F12; Sigma Chemical, St. Louis, Mo.) supplemented with 10% newborn bovine serum (GIBCO, Grand Island, N.Y.) and 100 U of penicillin (GIBCO) per ml. Type II pneumocytes (>90% purity) were plated onto tissue culture-treated polycarbonate filter inserts (0.33 cm^2; Transwell; Costar, Pleasanton, Calif.) in 12-well plates at 1.5×10^6 cells/cm^2. After 2 days, fresh culture medium was added to both sides. After approximately 3 days in culture, the alveolar epithelial cells form confluent monolayers, exhibit morphological features of type I pneumocytes, and express epitopes reacting with a rat type I cell-specific monoclonal antibody (11, 13). The monolayer generates spontaneous transepithelial electrical potential difference and resistance. The equivalent short-circuit current, hereafter abbreviated as "current," is calculated by dividing the potential difference by the resistance and is an index of active sodium reabsorption (18). On day 4, the alveolar pneumocyte monolayers were washed twice with DME/F12 containing 0.125% bovine serum albumin (BSA) (Sigma) on both sides and allowed to stabilize for 24 h, with 250 μl of apical bathing fluid and 1 ml of basolateral fluid. The transepithelial electrical potential difference and monolayer resistance were monitored daily with a MilliCell ERS device (Millipore, Marlborough, Mass.).

Mycobacteria and their components. Avirulent H37Ra and virulent H37Rv *M. tuberculosis* strains were used. In some experiments, *M. smegmatis* and nonpathogenic *Escherichia coli* (strain AR120; American Type Culture Collection, Rockville, Md.) were used instead of *M. tuberculosis*. Single-cell suspensions of *M. tuberculosis* were obtained by standard methods. Briefly, aliquots of frozen *M. tuberculosis* were cultured in 7H9 medium for 7 days, pelleted at $4,500 \times g$ for 15 min, and resuspended in RPMI (GIBCO). After clumps of mycobacteria were dispersed with an ultrasonic cell disrupter (Virtis, Gardiner, N.Y.), the sample was centrifuged at $400 \times g$ to pellet residual clumps, and the upper bacterial suspension was used in all experiments. Bacteria were counted in a Petroff-Hauser chamber. Using this technique, 90 to 95% of the organisms were single bacilli, with the remaining 5 to 10% in small aggregates of up to five organisms. Mycobacterial viability, as assessed by culture, was approximately 50%.

Mycobacterial components were isolated from mid-logarithmic-phase cultures of *M. tuberculosis* H37Rv grown in glycerol alanine salts media. Culture filtrate

protein was prepared as described previously (14). After removal of culture supernatant, the mycobacterial cells were inactivated by autoclaving at 80°C for 60 min, washed with phosphate-buffered saline (PBS), and suspended at a final concentration of 2 g of cells per ml of PBS with 0.005% Tween 80. Cells were lysed by shearing in a Bead Beater (Bio-spec Products, Bartlesville, Okla.) with 0.1-mm zirconium beads at 4°C. The lysate was centrifuged at 3,000 × *g* for 10 min at 4°C to remove unbroken cells, and the supernatant was termed *M. tuberculosis* lysate. Lipoarabinomannan was purified from *M. tuberculosis* H37Rv and from a rapidly growing nonpathogenic *Mycobacterium* species, as described previously (9).

Effects of *M. tuberculosis* on epithelial bioelectric properties. Rat alveolar epithelial cell monolayers were incubated in DME/F12 containing 0.125% BSA and 100 U of penicillin per ml as controls. To the apical fluid of some monolayers, live or heat-killed *M. tuberculosis* H37Ra or H37Rv was added. In other experiments, 10 μg of *M. tuberculosis* lysates, culture filtrate protein, heavily mannosylated lipoarabinomannan from *M. tuberculosis* H37Rv, or arabinofuranosyl-terminated lipoarabinomannan from a nonpathogenic mycobacterium per ml was added to the apical fluid. In some instances, *M. tuberculosis* lysates (1 mg/ml) were treated with 100 μg of proteinase K (Sigma) per ml at 55°C for 24 h, and proteinase K was inactivated by being boiled for 2 min and then cooled to room temperature, prior to addition to the alveolar cell monolayers. In separate experiments, graded concentrations of recombinant murine tumor necrosis factor alpha (TNF-α) (Genzyme, Cambridge, Mass.) were added to the basolateral or apical fluid bathing the monolayer. In other cases, polyclonal neutralizing rabbit anti-mouse TNF-α antibodies (Genzyme) were added to the apical or basolateral fluid. These antibodies are strongly cross-reactive with rat TNF-α.

Because the baseline resistance and current were variable from monolayer to monolayer and both values declined by approximately 15% over 4 days in the presence of media alone, we adjusted for these factors by expressing the data as "relative resistance" and "relative current." For addition of *M. tuberculosis*, the relative resistance at day *x* = (resistance in well containing *M. tuberculosis* at day *x*/resistance in well containing *M. tuberculosis* at day 0)/(resistance in well containing medium alone at day *x*/resistance in well containing medium alone at day 0). By substituting current for resistance in the formula above, relative current was calculated.

Semi-quantitative evaluation of TNF mRNA expression. Alveolar epithelial cell monolayers were cultured for various periods, in the presence or absence of apically added 2×10^6 *M. tuberculosis* H37Ra organisms, and then harvested, lysed with 4 M guanidinium isothiocyanate, and stored at −20°C prior to isolation of RNA and synthesis of cDNA as described previously (2). We normalized all samples for β-actin cDNA content by using 26 cycles of amplification with primers specific for rat β-actin cDNA (2). The 5′ and 3′ primer sequences were TTCTACATTGAGCTGCGTGTGG and TATGGGTCCTTCCTTCCGACCT TC, respectively. PCR product (10 μl) was subjected to electrophoresis on 1.5% agarose gels, visualized by staining with ethidium bromide, and quantified by a gel documentation system, as previously described (36). After normalization for β-actin cDNA content, PCR amplification for 35 cycles was performed with primers specific for rat TNF cDNA. The 5′ and 3′ primer sequences were CCACGTCGTAGCAAACCACCAAG and GATCCACTCAGGCATCGACA TTCG, respectively (28). Upon PCR amplification of serial twofold dilutions of sample cDNAs, a concomitant decrease in sample PCR product was observed, indicating that PCR conditions were not within the plateau phase of amplification. Negative controls containing no cDNA were employed in each set of reactions.

Measurement of TNF-α bioactivity. Supernatants were harvested from the apical and basolateral fluids bathing the alveolar epithelial cell monolayers, which had been cultured in the presence or absence of *M. tuberculosis*. The presence of bioactive TNF-α in the supernatants was measured by the WEHI 164 clone 13 assay, as previously described (3). This clone is extremely sensitive to the cytotoxic effects of TNF-α. Recombinant murine TNF-α was used as a standard. If a supernatant was cytotoxic for the WEHI cells, the assay was repeated after incubation of the supernatant with polyclonal neutralizing rabbit anti-mouse TNF-α antibodies.

Statistical analysis. Data are presented as means ± standard errors of the means and were analyzed by one-way or two-way (for unequal cell sizes) analyses of variance. Modified Newman-Keuls procedures were used to contrast group means within the data matrices. $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of live *M. tuberculosis* on bioelectric properties of the alveolar epithelium. For 84 monolayers, representing seven culture preparations, the mean baseline resistance of the alveolar epithelial cell monolayers was 2,890 ± 80 ohm/cm^2, the mean potential difference (apical side negative) was 10.1 ± 0.3 mV, and the mean current was 3.5 ± 0.1 μA/cm^2, comparable to previously reported results (19). These values indicate that the rat alveolar epithelial cell monolayer maintains a physiologic barrier with tight junctions and has the capacity for active ion absorption (18).

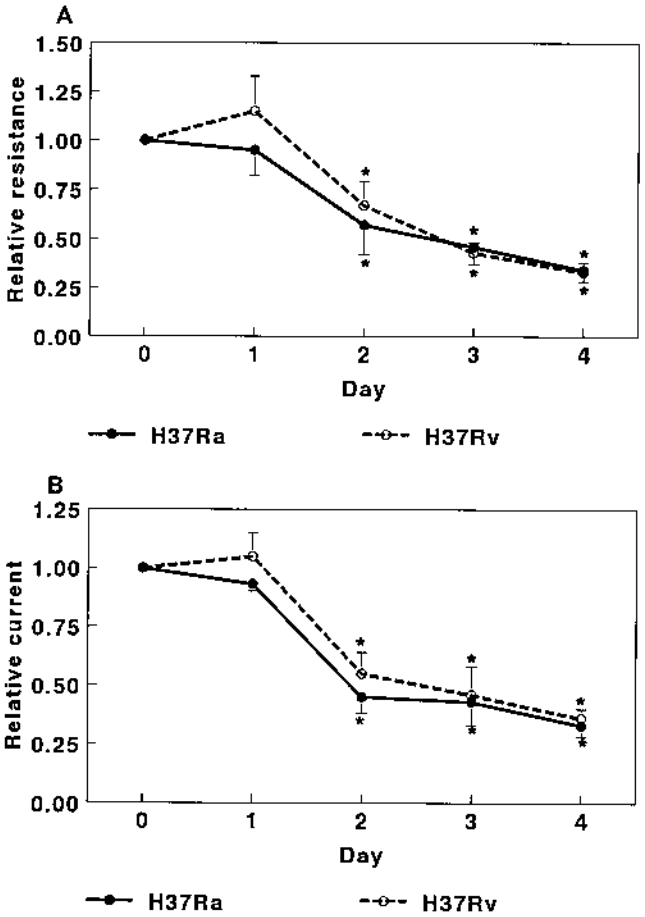

FIG. 1. Effects of *M. tuberculosis* H37Ra and H37Rv on the transepithelial resistance (A) and current (B) of alveolar epithelial cell monolayers. *M. tuberculosis* H37Ra or H37Rv (2×10^6 cells) was added to the apical fluid of the alveolar epithelial cell monolayers, and resistance and current were measured daily. Values shown are the mean (± standard error) relative resistance and relative current for 14 experiments. Asterisks denote values that are significantly different from the control value of 1.0.

Live *M. tuberculosis* H37Ra was added in 10-fold dilutions to the apical surface of the epithelial cell monolayer, and the resistance and potential difference across the monolayer were measured after 24 to 96 h. In eight experiments, addition of up to 2×10^5 *M. tuberculosis* organisms had no consistent effect (data not shown). However, addition of 2×10^6 organisms, which approximates four bacilli per alveolar epithelial cell in the monolayer, caused a significant decline in relative resistance across the monolayer (Fig. 1A). The mean relative resistance at 48, 72, and 96 h fell significantly below the control value of 1.0 ($P = 0.0001$, 0.0002, and 0.0001, respectively). Similar reductions in relative current were also observed over 96 h (Fig. 1B). These results indicate that *M. tuberculosis* diminishes the capacity of the alveolar epithelium to maintain tight intercellular junctions and actively reabsorb sodium, therefore reducing the epithelial barrier properties. The effects of virulent *M. tuberculosis* H37Rv and avirulent H37Ra on the transepithelial resistance and current were indistinguishable (Fig. 1). In five experiments, addition of 5×10^6 to 10×10^6 *M. smegmatis* cells did not affect relative resistance (0.87 ± 0.03, $P > 0.05$) or relative current (0.99 ± 0.07, $P > 0.05$). Non-

pathogenic *E. coli* and latex beads (3-μm diameter; Sigma) also had no effect on relative resistance and current (data not shown).

M. tuberculosis is cytotoxic for a transformed human lung carcinoma cell line (22). To determine if the changes in bioelectric properties were due to lysis of alveolar epithelial cells, we used trypsin and EDTA to detach alveolar epithelial cells from the filter and then determined cell viability by Trypan blue exclusion and by cytofluorometric analysis after staining with propidium iodide. Detachment reduced cell viability, and both methods showed that the percentage of viable cells varied from 85 to 95% ($n = 4$) in monolayers cultured in medium alone. In monolayers to which *M. tuberculosis* had been added, the percentage of viable cells was similar (range, 87 to 94%, $n = 4$).

Effects of *M. tuberculosis* components on bioelectric properties of the alveolar epithelium. To determine if the reduction in resistance and current across the alveolar epithelial cell monolayer could be mediated exclusively by live *M. tuberculosis* organisms or by structural components that are common to live and dead bacilli, we first compared the effects of live and heat-killed organisms on the bioelectric properties of the monolayer. Because no differences were observed (data not shown), we tried to identify the *M. tuberculosis* components that mediate this effect. Compared to control cultures, *M. tuberculosis* lysates caused significant decreases in relative resistance and current after 2 to 4 days ($P < 0.0001$ for both resistance and current at days 2, 3, and 4; Fig. 2), similar to changes induced by live *M. tuberculosis*. Because 10 μg of *M. tuberculosis* lysates per ml caused consistent effects on relative resistance and current, we used this concentration for all the *M. tuberculosis* components. Lysates treated with proteinase K also caused parallel reductions in relative resistance ($P = 0.01$, 0.01, and 0.00002 for days 2, 3, and 4, respectively) and current ($P = 0.005$, 0.0002, and 0.00002 for days 2, 3, and 4, respectively). The effects of lysates treated with proteinase K were not statistically different from those of untreated lysates ($P > 0.1$). Culture filtrate proteins had no effect on monolayer resistance and current (Fig. 2), indicating that the reduction in bioelectric barrier properties is not mediated by a protein of *M. tuberculosis* but probably by carbohydrate or lipid components. A prominent lipoglycan of *M. tuberculosis* is lipoarabinomannan, which is a heteropolysaccharide that exhibits potent immunoregulatory effects and is a putative virulence factor (4, 8, 12, 23, 30, 31). Lipoarabinomannan derived from *M. tuberculosis* H37Rv had no effect on the transepithelial resistance and current (Fig. 2), indicating that these changes were caused by lipids or carbohydrates other than lipoarabinomannan.

TNF-α production by alveolar epithelial cells exposed to *M. tuberculosis*. TNF-α enhances transepithelial permeability in a *Corynebacterium parvum* model of pulmonary infection (20). In addition, TNF-α is present in the alveolar epithelium of patients with pulmonary fibrosis (26) and is produced by human macrophages in response to *M. tuberculosis* (25, 27). We therefore hypothesized that *M. tuberculosis* reduced the resistance and current across the alveolar epithelial cell monolayer by inducing TNF-α production by cultured alveolar epithelial cells. We harvested apical and basolateral supernatants of the monolayer after incubation with medium alone or after addition of *M. tuberculosis* H37Ra to the apical fluid for 1 to 4 days. Supernatants were added to WEHI cells, and cell lysis was used as a measure of TNF-α bioactivity. Twenty-four hours after addition of *M. tuberculosis*, the mean concentration of TNF-α in supernatants harvested from the apical fluid was 3,441 ± 2,235 pg/ml ($n = 4$) and that in supernatants harvested from the basolateral fluid was 18 ± 5 pg/ml ($n = 3$). Apical

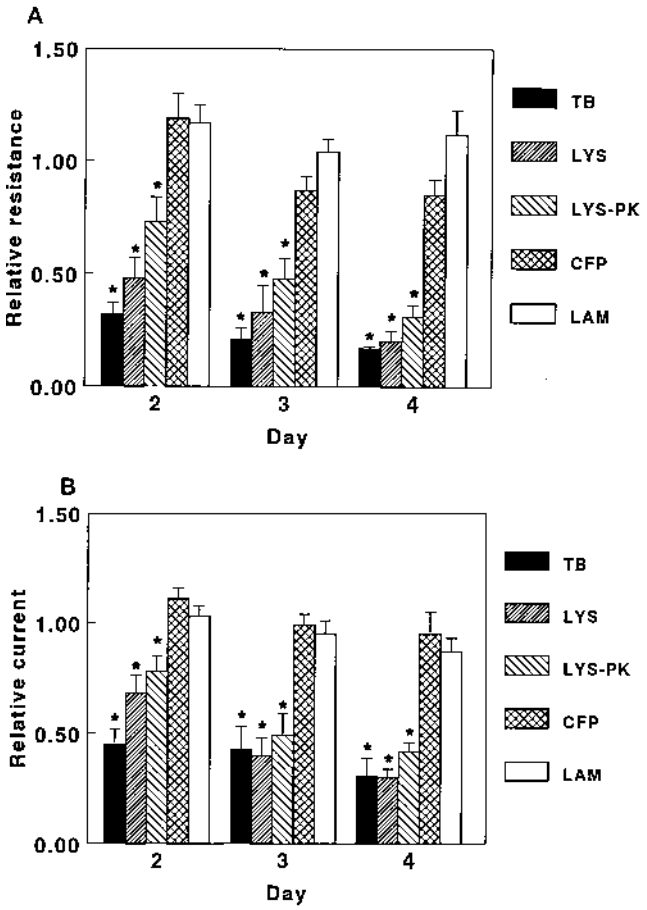

FIG. 2. Effects of *M. tuberculosis* components on the resistance (A) and current (B) of alveolar epithelial cell monolayers. Lysates of *M. tuberculosis* (LYS), proteinase K-treated lysates (PK-LYS), culture filtrate proteins (CFP) and lipoarabinomannan from H37Rv (LAM) were added to the apical fluid of alveolar epithelial cell monolayers, and resistance and current measured daily for 4 days. At day 1, the mean relative resistance and current for all preparations were not different from control values (data not shown). Values shown are the mean (± standard error) relative resistance and relative current for 12 experiments. Asterisks denote values that are significantly different from control values of 1.0.

fluid TNF-α concentrations were significantly higher than those in apical fluid from control monolayers cultured in medium alone (10 ± 5 pg/ml, $P = 0.01$). Concentrations of TNF-α ranged from 1,000 to 2,800 pg/ml in the apical supernatants 2 to 4 days after addition of *M. tuberculosis*. To confirm that lysis of WEHI cells was mediated by TNF-α, we used the WEHI bioassay to measure TNF-α concentrations in the supernatants after addition of neutralizing anti-TNF-α antibodies. Addition of anti-TNF-α completely abrogated the bioactivity of the apical supernatants of *M. tuberculosis*-treated alveolar epithelial cells, demonstrating that bioactivity was mediated by TNF-α. Figure 3 shows a representative time course of TNF-α concentrations measured in one of four experiments. *M. tuberculosis* lysates also elicited production of TNF-α (2,726 pg/ml), whereas lipoarabinomannan from H37Rv or from a nonpathogenic mycobacterium did not (22 pg/ml).

To determine if *M. tuberculosis* induced TNF-α production through upregulation of TNF mRNA levels, we used reverse transcription (RT)-PCR to detect TNF mRNA. TNF mRNA was present in alveolar epithelial cells exposed to *M. tuberculosis* after 3 h and persisted for 72 h, whereas TNF mRNA was

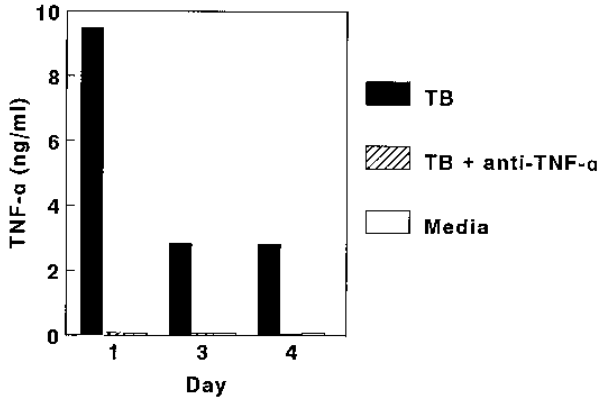

FIG. 3. Concentrations of bioactive TNF-α in apical supernatants of alveolar epithelial cell monolayers cultured in the presence and absence of *M. tuberculosis*. Results are shown for a representative experiment in which 2×10^6 *M. tuberculosis* H37Ra cells (TB) were added to the apical fluid of alveolar epithelial cell monolayers and supernatants were harvested for measurement of TNF-α concentrations after 24, 72, and 96 h. For supernatants from *M. tuberculosis*-exposed monolayers, measurement of TNF-α bioactivity was repeated after addition of neutralizing anti-TNF-α antibodies (TB + anti-TNF-α). TNF-α concentrations were also measured in apical supernatants of alveolar epithelial cell monolayers cultured in media alone (Media).

barely detectable in alveolar epithelial cells cultured in medium alone (Fig. 4).

Effects of recombinant TNF-α on bioelectric properties of the alveolar epithelium. To determine if *M. tuberculosis*-induced TNF-α could reduce the resistance and current across the alveolar epithelium, we evaluated the effects of recombinant TNF-α on epithelial barrier properties. Addition of TNF-α to the apical fluid caused small reductions in transepithelial resistance and current only at very high concentrations, whereas lower concentrations of TNF-α caused more marked effects when added to the basolateral fluid (Fig. 5).

Neutralization of the effects of *M. tuberculosis* on bioelectric properties of the alveolar epithelium. To confirm that some of the effects of *M. tuberculosis* on the transepithelial resistance and current were mediated through a soluble factor(s) in the

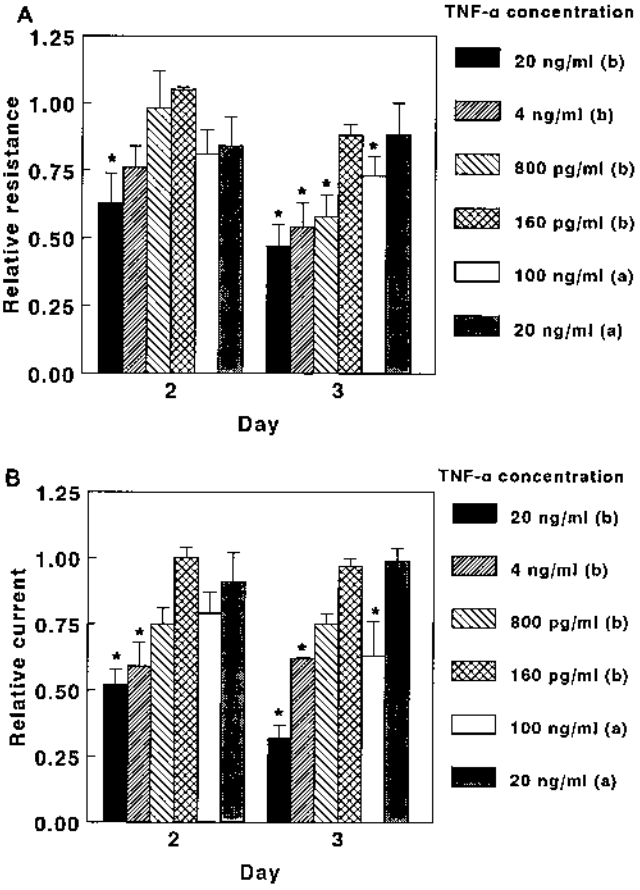

FIG. 5. Effects of recombinant TNF-α on resistance (A) and current (B) of alveolar epithelial cell monolayers. Recombinant murine TNF-α was added to the apical (a) or basolateral (b) fluid of alveolar epithelial cell monolayers, and resistance and current were measured daily for three days. At day 1, the mean relative resistance and relative current for all TNF-α concentrations were approximately 1 (data not shown). Values shown are the mean (± standard error) relative resistance and relative current for eight experiments. Asterisks denote values that are significantly different from control values of 1.0.

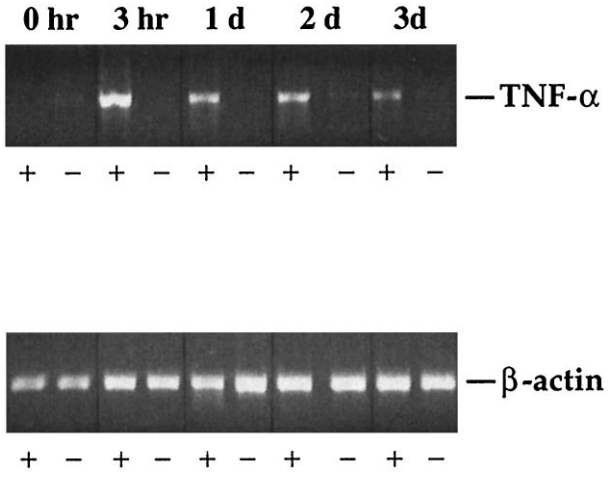

FIG. 4. TNF-α mRNA expression in alveolar epithelial cell monolayers cultured in the presence and absence of *M. tuberculosis*. Alveolar epithelial cell monolayers were cultured with media alone (lanes labeled −) or in the presence of 2×10^6 *M. tuberculosis* H37Ra cells added to the apical fluid (lanes labeled +). Cells were harvested at various time points, and mRNA for TNF-α and β-actin were detected by semi-quantitative RT-PCR.

bathing fluids, we took supernatants from the apical and basolateral fluids of alveolar epithelial cell monolayers after exposure to *M. tuberculosis* for 24 h, centrifuged the supernatants, and discarded the pellets containing *M. tuberculosis*. When these supernatants were added to their respective sides of a fresh alveolar epithelial cell monolayer, the transepithelial relative resistance decreased by 64% ± 9% over 1 to 2 days, similar to findings when *M. tuberculosis* was added. The relative current declined only slightly by 15% ± 6%, indicating that the decrease in relative current required the continued presence of *M. tuberculosis*. This finding suggests that resistance is more sensitive than current to soluble factors produced by *M. tuberculosis*-exposed monolayers.

To determine if the effects of the supernatants were mediated through TNF-α, apical and basolateral supernatants from *M. tuberculosis*-exposed monolayers were collected and incubated at 37°C for 1 h, with or without neutralizing antibodies to TNF-α. These treated supernatants were then added to their respective sides of a fresh unexposed monolayer. The effect of anti-TNF-α antibodies was estimated by the percent neutralization of the decrease in relative resistance after addition of anti-TNF-α antibodies. Percent neutralization = 100% × (relative resistance with addition of supernatants incubated with

anti-TNF-α − relative resistance with addition of supernatants alone)/(1 − relative resistance with addition of supernatants alone). In six experiments, anti-TNF-α antibodies neutralized 57% ± 9% of the decrease in relative resistance, indicating that TNF-α plays a significant role in mediating the reduced transepithelial resistance induced by *M. tuberculosis*. Because anti-TNF-α did not completely neutralize the effects of the supernatants, soluble factors other than TNF-α may also contribute to reduction of the transepithelial resistance.

DISCUSSION

Important barrier properties of the alveolar epithelium include its capacity to maintain tight intercellular junctions and to actively reabsorb sodium from the alveolar surface, with water following passively. Failure to maintain tight junctions may facilitate microbial invasion, and decreased active sodium reabsorption may allow fluid accumulation within the alveolar space. The data presented in this report provide insight into potential mechanisms by which *M. tuberculosis* penetrates the alveolar epithelium by altering its barrier properties. Nonproteinaceous *M. tuberculosis* components decreased the capacity of the alveolar epithelium to maintain tight intercellular junctions, as measured by the transepithelial resistance. In addition, *M. tuberculosis* reduced the alveolar epithelial capacity for active sodium reabsorption, measured by the transepithelial current. *M. tuberculosis* stimulated alveolar epithelial cells to express TNF-α mRNA and to secrete TNF-α into the apical fluid. Recombinant TNF-α in the basolateral fluid mimicked the bioelectric changes induced by *M. tuberculosis*. Furthermore, the capacity of supernatants from *M. tuberculosis*-exposed alveolar epithelial cell monolayers to reduce transepithelial resistance was abrogated by anti-TNF-α antibodies. These results suggest that, during the first 24 h of exposure to *M. tuberculosis*, alveolar epithelial cells secrete TNF-α apically. TNF-α then probably crosses the monolayer and binds to receptors on the basolateral cell surface, causing progressive reduction in epithelial barrier properties 48 to 96 h after infection. These changes are likely to favor establishment of tuberculosis infection in the lung.

Penetration of the alveolar epithelial barrier is of central importance in the pathogenesis of tuberculosis. For infection to progress to disease, mycobacteria must leave the alveolar space and enter the lymphatics. Furthermore, fluid and inflammatory infiltrate accumulate in the alveolar space in tuberculosis, indicating loss of integrity of the alveolar epithelium. There are several potential mechanisms by which *M. tuberculosis* may penetrate the alveolar epithelial barrier. The organisms may damage the epithelium by direct cytotoxicity, as virulent *M. tuberculosis* passaged through macrophages are cytotoxic for the human alveolar cell line A549 (22). Alternatively, *M. tuberculosis* may penetrate and proliferate in alveolar epithelial cells, as they can do so in A549 cells (5). A third possibility is that *M. tuberculosis* may cross the alveolar barrier in alveolar macrophages. We demonstrate a fourth potential mechanism by which *M. tuberculosis* may penetrate the alveolar epithelium through reduction of tight junctional integrity without cell lysis. To cause these bioelectric changes, *M. tuberculosis* may enter the alveolar epithelial cell directly or may exert its effects without entering the cell and then penetrate the alveolar barrier by paracytosis through the weakened intercellular junctions, as in the case of *Haemophilus influenzae* (34). Light and electron microscopy studies in the current model showed that *M. tuberculosis* organisms were present in alveolar epithelial cells (1a), but it remains uncertain if cell entry is necessary to mediate the electrophysiologic effects observed.

Although the current and prior studies demonstrate that *M. tuberculosis* induces morphologic and physiologic changes in alveolar epithelial cells and their barrier properties, they do not measure passage of *M. tuberculosis* through the alveolar epithelium. In our model, bacterial penetration of the epithelial barrier cannot be directly measured because few bacilli pass through the pores of the monolayer polycarbonate supports, which are only 0.4 μm in diameter (35a). Establishment of experimental systems which obviate this problem is critical to improve our understanding of the pathogenesis of tuberculosis.

Our findings highlight a novel interaction between the cytokine network and the barrier properties of the alveolar epithelium, demonstrating that a human pathogen induces alveolar epithelial cells to produce TNF-α, which in turn reduces epithelial barrier properties and may facilitate pulmonary tissue invasion. It is intriguing to speculate that bacterium-induced TNF-α production is a general mechanism by which bacterial pathogens traverse the alveolar epithelial barrier, as several bacteria and bacterial lipopolysaccharide elicit TNF-α production by pulmonary and nonpulmonary epithelial cells (16, 17, 20), and *C. parvum* infection increases alveolar epithelial permeability through the effects of TNF-α (20). In addition, intestinal epithelial cells produce TNF-α in response to invasive microbial pathogens but not noninvasive ones (16).

Our data indicate that *M. tuberculosis* can elicit TNF-α production by alveolar epithelial cells. The high apical TNF-α concentrations produced by alveolar epithelial cell monolayers in response to *M. tuberculosis* are unlikely to have been due to TNF-α production by contaminating macrophages. First, morphological studies have shown that these monolayers contain virtually no macrophages (17a). Second, TNF-α was not produced when alveolar epithelial cell monolayers were cocultured with arabinofuranosyl-terminated lipoarabinomannan, which is a potent stimulus for TNF-α production by macrophages (10, 12).

Addition of exogenous TNF-α reduced the transepithelial resistance and current across the alveolar epithelium more markedly when added to the fluid bathing the basolateral rather than the apical surface of the alveolar epithelial cell monolayer, suggesting that functional TNF-α receptors are present predominantly on the basolateral cell membranes. These findings parallel those in intestinal epithelial cells, in which gamma interferon receptors that mediate reduction in transepithelial resistance are present predominantly on the basolateral cell membranes (1). When epithelial cell monolayers were exposed to *M. tuberculosis*, high TNF-α concentrations were measured in the apical but not in the basolateral bathing fluids. One possible explanation for this finding is that TNF-α is secreted predominantly through the apical surface, diffuses through the alveolar epithelium to the basolateral surface, and is rapidly bound to basolateral TNF-α receptors, where it is internalized or degraded. Alternatively, TNF-α may be secreted through both apical and basolateral cellular surfaces but is predominantly bound to basolateral TNF-α receptors. In our system, *M. tuberculosis* induced greater changes in transepithelial resistance and current than did exogenous TNF-α at concentrations comparable to those elicited by *M. tuberculosis*. This suggests that other cytokines or factors induced by *M. tuberculosis* may have contributed to these bioelectric changes. Further studies are needed to clarify these issues.

The relationship between bacterial virulence and the capacity to penetrate the alveolar epithelium remains uncertain. One study demonstrated that highly virulent *Pseudomonas aeruginosa* strains cause greater injury to the alveolar epithelial

barrier than do strains of lesser virulence (35). We found no difference between the bioelectric effects of virulent *M. tuberculosis* H37Rv and avirulent H37Ra strains, indicating that the difference in virulence between these strains may not be related to their capacity to penetrate the alveolar epithelium. This may be because both strains are similar in their capacity to establish pulmonary infection in animals but differ in their potential to cause progressive disease (11a). In support of the relevance of bioelectric effects of *M. tuberculosis* to pulmonary invasion, nonpathogenic *M. smegmatis* and *E. coli* did not reduce the relative resistance and relative current of the alveolar epithelial cell barrier.

TNF-α can contribute both to protection against tuberculosis and to immunopathology. In murine models, neutralization of TNF-α or deletion of the gene for the 55-kDa TNF receptor markedly increases mortality from tuberculosis (15), indicating that TNF-α is essential for protective immune responses. On the other hand, TNF-α can cause adverse effects, such as weight loss and tissue necrosis (6), and monocytes from patients with severe tuberculosis produce higher TNF-α concentrations than do monocytes from healthy tuberculin reactors (7, 25), indicating that this cytokine may contribute to immunopathology. Our findings add to the spectrum of physiologic effects mediated by TNF-α, which can facilitate establishment of pulmonary infection by altering the bioelectric properties of the alveolar epithelium.

Although the histopathologic and immunologic responses to tuberculosis differ in rats and humans (32), our study focused on the barrier properties of the alveolar epithelium. The rat alveolar epithelial cell monolayer mimics the morphologic (13) and physiologic (19, 21, 29) properties of human alveolar epithelium. Unfortunately, primary cultures of human alveolar epithelial cells are difficult to obtain, and transformed human alveolar cell lines such as A549 cells do not yield confluent monolayers with measurable electrical resistance and potential difference (33), indicating that they are unlikely to mimic the barrier properties of alveolar epithelium.

In summary, we found that nonproteinaceous components of *M. tuberculosis* diminished the capacity of the alveolar epithelium to maintain tight intercellular junctions and to actively transport sodium. These changes were mediated in part through TNF-α produced by alveolar epithelial cells in response to *M. tuberculosis*. Our findings suggest that *M. tuberculosis* may facilitate establishment of infection in the lung by adversely affecting the electrophysiologic barrier properties of the alveolar epithelium.

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