

## Infection by *Mycobacterium tuberculosis* Promotes Human Alveolar Macrophage Apoptosis

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**The effect of *Mycobacterium tuberculosis* infection on the viability of healthy (control) human alveolar macrophages was evaluated by staining with ethidium homodimer and calcein to discriminate live from dead cells. Infection with *M. tuberculosis* H37Ra or H37Rv increased macrophage mortality at 6 days from the control level of 3.8% ± 0.7% to 28.7% ± 6.9% or 12.6% ± 3.1%, respectively ( $P < 0.001$  for comparisons of all conditions). A role for tumor necrosis factor alpha (TNF- $\alpha$ ) in the *M. tuberculosis*-induced cytolysis of alveolar macrophages was demonstrated by increased cytotoxicity following the addition of exogenous TNF- $\alpha$  to the cultures and by enhancement of macrophage survival when *M. tuberculosis*-infected alveolar macrophages were treated with pentoxifylline or anti-TNF- $\alpha$  antibody. The cytolytic mechanism was determined to be apoptosis by the demonstration of a characteristic internucleosomal ladder of genomic DNA by agarose gel electrophoresis, by finding nuclear fragmentation and condensation by electron microscopy, and by in situ terminal transferase-mediated nick end labeling of fragmented DNA in alveolar macrophages infected with *M. tuberculosis* in vitro. The latter technique was employed to reveal extensive apoptosis within caseating granulomas from lung tissue samples from clinical tuberculosis cases. The induction of apoptosis in alveolar macrophages by *M. tuberculosis* may play a role in the macrophage-pathogen interaction of tuberculosis in vivo.**

The alveolar macrophage is the first line of defense in the lung against infection by *Mycobacterium tuberculosis*. While resident unactivated alveolar macrophages phagocytose *M. tuberculosis* and generate signals for the induction of the cell-mediated immune response, they may be less effective at killing internalized microorganisms than macrophages which have been preactivated by lymphokines. After ingestion by macrophages, mycobacteria may persist and replicate as intracellular parasites, in part by modulating the phagosomal compartment to prevent incorporation of the vesicular proton-ATPase and subsequent acidification (1, 21). Failure to eradicate an initial tuberculosis challenge may lead to primary disease, or the process may be contained by the cell-mediated immune response after limited dissemination has occurred, leaving the host susceptible to future reactivation and disease. There is growing awareness that apoptosis can be induced or inhibited in certain host cell-pathogen interactions (22), including infection of human monocyte-derived macrophages with *Mycobacterium avium* (9). Mechanisms which would reduce the potential for macrophages to serve as protected sanctuaries for tuberculosis might be of significant benefit to the host. Conversely, efficient killing of alveolar macrophages could favor the mycobacteria by reducing the numbers of phagocytes and possibly interfering with the induction of cell-mediated immunity.

Tuberculosis remains an important cause of morbidity and mortality worldwide. Host factors play a key role in susceptibility to the development of tuberculosis disease following infection. A better understanding of the host response to *M. tuberculosis* may contribute to improvements in the treatment

and prevention of tuberculosis. To this end, we evaluated the effect of *M. tuberculosis* infection on the viability of healthy (control) human primary alveolar macrophages in vitro, finding evidence for the induction of apoptosis by a mechanism involving tumor necrosis factor alpha (TNF- $\alpha$ ).

### MATERIALS AND METHODS

**Alveolar macrophages.** Alveolar macrophages were obtained from bronchoalveolar lavage fluid samples from healthy nonsmoking volunteers with their informed consent under a protocol approved by the Institutional Review Board of Boston University Medical Center. Lavage fluids and cells were filtered through a single layer of sterile gauze and centrifuged (450 × *g*, 10 min), and the cell pellet was suspended in RPMI 1640 medium (Gibco BRL, Gaithersburg, Md.) with 10% fetal calf serum and cefotaxime (50 µg/ml). Cells were plated on petri dishes or glass slides as described below, and nonadherent cells were removed by washing at 24 h. Differential counts were performed on cytocentrifuged preparations by using the Leuko Stat Stain Kit (Fisher, Pittsburgh, Pa.) according to the manufacturer's protocol. Viability of adherent alveolar macrophages was examined in representative samples by trypan blue dye exclusion.

***M. tuberculosis.*** Stock cultures of *M. tuberculosis* H37Ra and H37Rv (ATCC 25177 and ATCC 25618; American Type Culture Collection, Rockville, Md.) were prepared and stored frozen in 1-ml aliquots. Prior to inoculation of alveolar macrophages, the mycobacteria were dispersed by aspiration through a 25-gauge needle five times, vortexed, and then sonicated (15 s, 500 W) in a bath sonicator (Laboratory Supplies, Inc., Hicksville, N.Y.). Following sonication, the suspension of bacilli was allowed to stand (10 min) and the upper 500 µl was removed for use in experiments. Dilutions of these dispersed mycobacterial preparations were made to yield the desired multiplicity of infection for specific experiments. For each experiment, the adequacy of dispersion and the multiplicity of infection were checked by infecting macrophages and performing an acid-fast stain of washed cells at 3 h. The rate of internalization was also confirmed subsequently in selected cases by electron microscopy (EM).

**Analysis of macrophage viability.** Alveolar macrophages were cultured in two-well chamber slides (Nunc, Inc., Naperville, Ill.) at 400,000 cells per well in 1 ml of medium and incubated overnight (37°C, 5% CO<sub>2</sub>). Culture medium was replenished at 24 h, and at 72 h cells were infected with H37Ra or H37Rv to give an infection ratio of approximately 5 to 10 bacilli per macrophage. In selected experiments, recombinant TNF- $\alpha$  (100 U/ml) (R&D Systems, Miles, Calif.), monoclonal anti-TNF- $\alpha$  neutralizing antibody (5 µg/ml) (gift of M. Duerr, Miles Inc., Berkeley, Calif.), or pentoxifylline (100 µg/ml) (Sigma Chemical Company, St. Louis, Mo.) was added. After 5 days, the wells were removed and the cells were incubated (25°C, 45 min) in RPMI 1640 with 150 µl of a mixture of 4 µM

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ethidium homodimer and 0.2  $\mu$ M calcein (Oncor, Gaithersburg, Md.). Slides were washed with phosphate-buffered saline (PBS), and a coverslip was applied to the slide for examination by epifluorescence microscopy. Viable cells process calcein and display green fluorescence. Dead cells do not retain calcein and display red fluorescence because their nuclear membranes fail to exclude ethidium. One thousand cells on each slide were counted and scored as live or dead.

**Measurement of TNF- $\alpha$  release.** Alveolar macrophages were cultured and challenged with *M. tuberculosis* strains as described above, in duplicate. Supernatants were harvested at 4 and 24 h, and the level of immunoreactive TNF- $\alpha$  was determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (R&D Systems) in accordance with the manufacturer's specifications.

**Analysis of DNA fragmentation.** DNA was prepared by a modification of the method of Sellins and Cohen (20). Four million alveolar macrophages per condition were infected with H37Ra (1 to 5 bacilli per macrophage) or cultured in control medium alone for 3 or 6 days. Cells were then washed and lysed with 0.2% Triton X-100 in 10 mM Tris-1 mM EDTA buffer (pH 7.5). Following 10 min of incubation on ice, the lysate was centrifuged (14,000  $\times$  g, 10 min), the supernatant was then extracted with phenol-chloroform, and large DNA fragments were removed by sodium acetate precipitation and centrifugation (8,000  $\times$  g, 4 min, 25°C). The resulting supernatant was precipitated in ethanol, and DNA was resolved in a 1.5% agarose gel with 5  $\mu$ g of RNase per ml added to the loading buffer. DNA was visualized by ethidium bromide staining.

**EM.** Two hundred thousand alveolar macrophages were cultured on round glass coverslips in a 24-well plate. Cells were infected with H37Ra or cultured in control medium alone and washed after 4 h. At 48 h, a pellet of cells which had become nonadherent was fixed in 2% glutaraldehyde and cells which remained adherent were similarly fixed on the coverslips. Cell pellets and coverslips were postfixed in OsO<sub>4</sub> (4°C, 6 h), stained with 0.25% uranyl acetate, dehydrated in increments with alcohol, and embedded in Epon blocks. Sections were stained with uranyl acetate and lead citrate and then examined with a JEOL 100CX transmission electron microscope. Photographs were taken on Kodak electron imaging film.

**In situ apoptosis analysis.** Individual cell apoptosis was demonstrated in alveolar macrophage cultures by an in situ terminal transferase-mediated nick end labeling (TUNEL) technique of DNA strand breaks (ApopTag; Oncor). *M. tuberculosis*-infected macrophages adherent to glass slides were rinsed with PBS and fixed in 4% neutral buffered formalin. Slides were washed with PBS and then covered in equilibration buffer. Terminal deoxynucleotidyl transferase (TdT) in reaction buffer with digoxigenin-dUTP was then added to the cells and incubated (37°C, 1 h) in a humidified chamber. The reaction was stopped by immersion in stop-wash buffer (37°C, 30 min). After the cells were washed, anti-digoxigenin-fluorescein antibody was added, and following incubation (25°C, 30 min), the cells were washed three more times in PBS and counterstained with a propidium iodide-antifade mix. A glass coverslip was applied to the slide for epifluorescence microscopy using standard fluorescein excitation. Apoptotic cells were identified by yellow-orange and green fluorescence. Rat genomic DNA, partially digested with DNase, was used as a positive control. The negative control consisted of *M. tuberculosis*-infected alveolar macrophage cultures treated by the Apoptag protocol but without the addition of TdT.

For quantitative studies of apoptosis, alveolar macrophages adherent to glass coverslips were infected with *M. tuberculosis* with approximately 1 to 5 bacilli per cell, then harvested at various time points, fixed with 4% formaldehyde in PBS, rinsed in double-distilled water, immersed in reaction buffer containing 0.5 U of TdT per  $\mu$ l and 1 mM biotin-16-dUTP (Boehringer Mannheim, Indianapolis, Ind.) and incubated in a humidified chamber (37°C, 60 min). The reaction was terminated by transferring the coverslips to a solution of 300 mM NaCl and 30 mM sodium citrate, pH 7.2, for 15 min at room temperature. Coverslips were rinsed and immersed in PBS (5 min) first and then in avidin peroxidase (1:10) (Boehringer Mannheim) for 30 min at 37°C. Cells were then stained with 1% 3-amino 9-ethyl carbazole and dimethylformamide (Sigma) diluted 1:20 with 50 mM acetate buffer, pH 4.5, and 0.018% hydrogen peroxide (37°C, 20 min). Macrophages from three different donors were tested in duplicate. Apoptotic cells were identified by brown staining in the nucleus. On each slide, three fields of approximately 200 cells were counted. The number of TUNEL-positive cells was subtracted from the background numbers for TdT-negative controls.

Paraffin-embedded archival tuberculosis tissue sections were first deparaffinized by washing the sections in two changes of xylene and then in two changes of ethanol. They were then washed sequentially in 95% ethanol, 70% ethanol, and PBS and then subjected to proteinase K digestion (25°C, 15 min). DNA strand breaks were identified by TUNEL assay using the Apoptag kit (Oncor) following the manufacturer's protocol as described above except that anti-digoxigenin-peroxidase and diaminobenzidine substrate were used. Hydrogen peroxide was used to quench endogenous peroxidase activity, and the cells were counterstained with methyl green. Negative controls were processed identically except that TdT was not added. Lung tissue sections from C57BL/6 mice treated by intratracheal instillation of an Fas-activating antibody were used as positive controls. Each lung section used for TUNEL was compared to an adjacent specimen stained with hematoxylin and eosin.

**Statistical analysis.** Cytotoxicity data were compared by analysis of variance using StatView software (Abacus Concepts, Berkeley, Calif.).

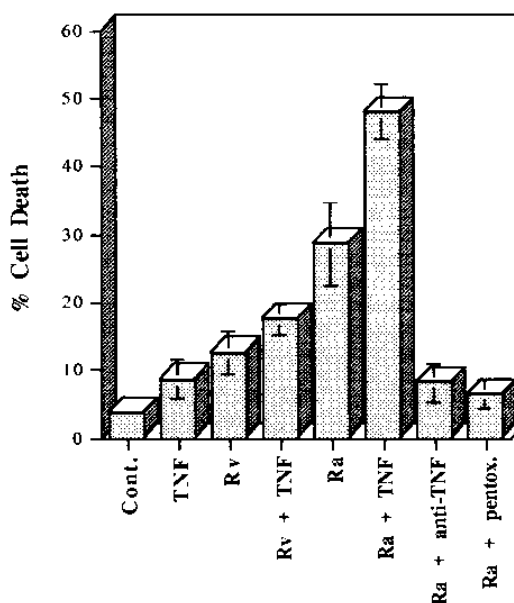


FIG. 1. *M. tuberculosis* H37Ra demonstrates greater cytopathic effect for alveolar macrophages than strain H37Rv, and the cytopathogenicities of both strains are enhanced by TNF- $\alpha$ . Alveolar macrophages were cultured on microscopy chamber slides in medium alone (Cont.) or medium containing TNF- $\alpha$  (100 U/ml) (TNF) and infected with *M. tuberculosis* H37Rv (Rv) or H37Ra (Ra) or infected with either bacterial strain with the addition of TNF- $\alpha$  (100 U/ml) (Rv + TNF and Ra + TNF). Other conditions included infection with strain H37Ra plus the addition of anti-TNF- $\alpha$  antibody (5  $\mu$ g/ml) (Ra + anti-TNF) or pentoxifylline (100  $\mu$ g/ml) to the culture medium (Ra + pentox.). After the cells were stained with ethidium homodimer and calcein, slides were examined by epifluorescence microscopy and one thousand cells were scored as live or dead. Viability is expressed as mean percent dead cells  $\pm$  SD for eight experiments. Infection with H37Ra resulted in significantly greater cytolysis than infection with H37Rv, and the addition of TNF- $\alpha$  to cultures infected with either strain induced a significant increase in cell death, as determined by analysis of variance. The mortality of macrophages infected with H37Rv plus added TNF- $\alpha$  was lower than that of H37Ra-infected cells even without added TNF- $\alpha$ . Treatment of H37Ra-infected cells with anti-TNF- $\alpha$  or pentoxifylline enhanced macrophage survival significantly ( $P < 0.05$  for all comparisons cited). Similar effects with anti-TNF- $\alpha$  and pentoxifylline were observed in H37Rv-infected cells (data not shown).

## RESULTS

Initial experiments tested the effects of infection with *M. tuberculosis* H37Ra (nonpathogenic in mice) and H37Rv (pathogenic in mice) strains on the viability of human alveolar macrophages. Macrophages obtained from healthy volunteers by bronchoalveolar lavage were challenged with strains H37Ra and H37Rv to achieve approximately 5 to 10 bound or internalized bacilli per macrophage, as determined by acid-fast staining of washed cells 3 h after infection. After 5 days in culture, macrophage viability was assessed by staining with ethidium homodimer and calcein. Cells fluorescing green were scored as live, and those fluorescing red were scored dead. Infection with either strain of *M. tuberculosis* resulted in a reduction of macrophage viability compared with the uninfected controls. As shown in Fig. 1, infection with either mycobacterial strain increased macrophage cell death above the control level of 3.8%  $\pm$  0.7% (mean percent dead cells  $\pm$  standard deviation [SD] for eight experiments) ( $P < 0.001$ ). Alveolar macrophage cytotoxicity was significantly greater with H37Ra infection than with H37Rv infection (28.7%  $\pm$  6.9% versus 12.6%  $\pm$  3.1% [ $P < 0.001$ ]). Macrophage viability was not reduced when cells were challenged with *M. tuberculosis* H37Ra which had been heat killed (1 h, 56°C; data not shown).

The cell mortality data we obtained probably underestimated the actual lethality of tuberculosis infection in these cultures, because some dead or dying macrophages detached from the surface of the chamber slide and escaped being counted. A greater fraction of the H37Ra-infected cells detached than that for H37Rv-infected cells, but it was not possible to accurately count detached cells or to count the relative density of cells which remained surface bound. While the difference in cytotoxicity between the two mycobacterial strains may be greater than we report here, we empirically determined conditions which permitted the highest degree of reproducibility between experiments. Growth rates for H37Ra and H37Rv in medium were similar, as determined by plating and colony counting and by Bactec analysis of  $^{14}\text{CO}_2$  production (9; data not shown). While we did not measure growth of intracellular mycobacteria, McDonough et al. (14) reported that intracellular replication of H37Rv was greater than that of H37Ra in human macrophages. It is therefore unlikely that the greater cytotoxicity with H37Ra infection in our experiments was due simply to excessive intracellular proliferation of H37Ra compared with that of H37Rv.

Previous experiments by Filley and Rook (8) suggested that mycobacterial infection could render fibroblast and monocytoic cell lines susceptible to the cytotoxic effects of TNF- $\alpha$ . To determine whether TNF- $\alpha$  activity was involved in the observed alveolar macrophage cell death, macrophages were challenged with *M. tuberculosis* in the presence of recombinant TNF- $\alpha$ , anti-TNF- $\alpha$  antibody, or the TNF- $\alpha$  inhibitor pentoxifylline (Fig. 1). The addition of TNF- $\alpha$  enhanced killing of both H37Ra- and H37Rv-infected macrophages, while macrophages treated with TNF- $\alpha$  in the absence of mycobacterial infection demonstrated only a slight increase in mortality compared with untreated cells that did not meet statistical significance. The proportion of dead cells in the H37Ra group rose from  $28.6\% \pm 6.2\%$  without added TNF- $\alpha$  to  $47.9\% \pm 4.0\%$  with the addition of TNF- $\alpha$  ( $P < 0.0001$ ). In the H37Rv-infected cultures, cell death rose from  $12.6\% \pm 3.1\%$  to  $17.4\% \pm 2.3\%$  ( $P = 0.02$ ) with the addition of TNF- $\alpha$ . Mortality was significantly higher in the H37Ra-infected cells not treated with TNF- $\alpha$  than in the H37Rv-infected cells, even when the H37Rv-infected cells had additionally been treated with TNF- $\alpha$  ( $P < 0.001$ ). The addition of anti-TNF- $\alpha$  antibody or pentoxifylline to H37Ra-infected cells reduced cell death to  $8.2\% \pm 4.7\%$  and  $6.5\% \pm 2.0\%$ , respectively, compared with macrophages infected with H37Ra in the absence of a TNF- $\alpha$  inhibitor ( $P < 0.0001$  for both comparisons). Levels of immunoreactive TNF- $\alpha$  in culture supernatants of *M. tuberculosis*-infected macrophages were measured by ELISA (Fig. 2). There was no difference in alveolar macrophage TNF- $\alpha$  production following infection with H37Ra or H37Rv. Together, these data indicated that infection with *M. tuberculosis* sensitized the alveolar macrophages to a cytotoxic effect of TNF- $\alpha$ . The increased mortality of cells infected with H37Ra compared with H37Rv did not appear to be due simply to higher levels of TNF- $\alpha$  release, suggesting there may be a differential sensitization to TNF- $\alpha$  cytotoxicity by the two *M. tuberculosis* strains.

Tumor necrosis factor can induce both apoptotic and necrotic forms of cell lysis (12). The ethidium homodimer and calcein staining technique does not provide specific information about the mechanism of cell death. We had observed free nuclei expelled from macrophages in the cytotoxicity experiments, suggesting that apoptosis was occurring. To confirm the presence of apoptosis, DNAs harvested from *M. tuberculosis*-infected alveolar macrophages and uninfected control cells were analyzed by agarose gel electrophoresis (Fig. 3). A char-

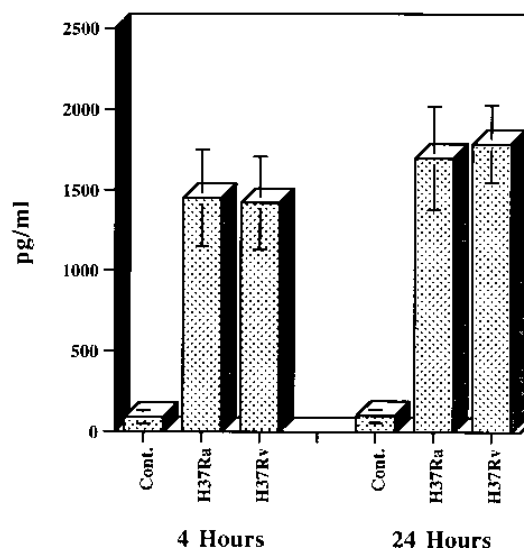


FIG. 2. TNF- $\alpha$  production by *M. tuberculosis*-infected alveolar macrophages. Alveolar macrophages were cultured with control medium alone or with the addition of mycobacterial strain H37Ra or H37Rv. Supernatants were harvested at 4 and 24 h, and TNF- $\alpha$  levels were measured by ELISA. Each value is the mean  $\pm$  SD for seven individuals tested. Cont., control.

acteristic internucleosomal banding pattern was detected in DNA extracted from the *M. tuberculosis*-infected cells but was not present in identical preparations from control cells. EM analysis revealed nuclear fragmentation and condensation consistent with apoptosis in *M. tuberculosis*-infected alveolar macrophages (Fig. 4). Similar evidence of apoptosis was identified by EM in H37Rv-infected cells, though at frequencies lower than those of H37Ra-infected cells (not shown). An apoptotic process was further confirmed by TUNEL analysis of fragmented DNA. Nuclear staining indicative of apoptosis was seen in alveolar macrophages infected with strain H37Ra in vitro but not in uninfected control cells (Fig. 5). Quantitative analysis of apoptosis by in situ TUNEL was performed on alveolar macrophages infected with H37Ra or H37Rv at 1 to 5 bacilli per macrophage. Negative and positive controls con-

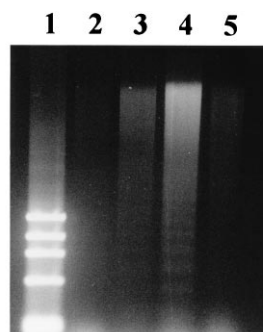


FIG. 3. Internucleosomal DNA fragmentation in *M. tuberculosis*-infected alveolar macrophages. Human alveolar macrophages were infected with strain H37Ra or cultured in control medium and then harvested at days 3 and 6. Genomic DNA was prepared for agarose gel electrophoresis as described in Materials and Methods. Lane 1, *Hae*III-digested  $\phi$ X174 DNA markers; lane 2, genomic DNA from uninfected macrophages at 3 days; lane 3, genomic DNA from H37Ra-infected macrophages at 3 days (a very faint apoptotic ladder was observed); lane 4, genomic DNA from H37Ra-infected macrophages at 6 days, demonstrating a more prominent banding pattern; lane 5, genomic DNA from uninfected macrophages at 6 days, showing no bands.



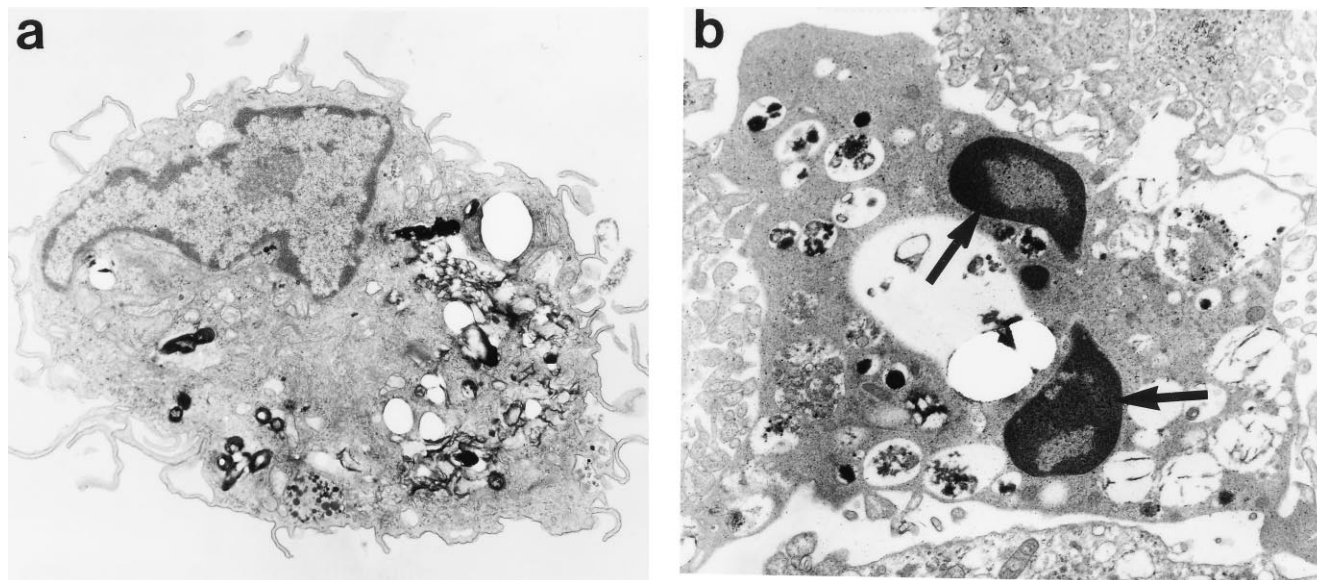


FIG. 4. EM of *M. tuberculosis*-infected alveolar macrophages. Electron micrographs of uninfected alveolar macrophages (a) or H37Ra-infected macrophages (b) were prepared after 2 days in culture. Nuclear fragmentation and condensation (arrows) were seen only in cells from the H37Ra-infected group. Magnification,  $\times 7,056$  in both photomicrographs.

sisted of uninfected cells cultured in the presence or absence of actinomycin D, respectively. The proportions of apoptotic macrophages in the H37Ra- and H37Rv-infected cultures identified by the TUNEL method were similar to the proportion of dead cells counted in the calcein- and ethidium homodimer-stained preparations at similar time points (Fig. 6), indicating that apoptosis was the primary mechanism of cytotoxicity in these experiments.

While we were able to observe macrophage apoptosis *in vitro*, it was not certain that a similar response to *M. tuberculosis* infection occurred *in vivo*. We therefore obtained archival tissue samples from four cases of tuberculosis (gift of E. A. Gaensler, Boston, Mass.) for *in situ* TUNEL experiments. Widespread apoptosis was observed in tuberculous granulomas present in three of the cases, one of which is presented in Fig. 7. Although cytotoxic processes other than mycobacterium-induced apoptosis may also occur in these lesions, the

presence of numerous TUNEL-positive cells within these sections suggested that the effects of tuberculosis infection we observed *in vitro* were relevant to the host response against tuberculosis *in vivo*. One of the four tuberculosis tissue samples we studied contained only inactive hyalinized granulomas; no active granulomas were found in this lung tissue section, and no TUNEL-positive cells were identified (Fig. 7). On the basis of this limited sample, it appears that high rates of apoptosis occur only during the host response to active tuberculosis infection and that apoptosis is not a feature of quiescent lesions.

## DISCUSSION

We found that *M. tuberculosis* infection reduced the viability of primary human alveolar macrophages *in vitro*. The role of TNF- $\alpha$  in this process was demonstrated by inhibition of cell

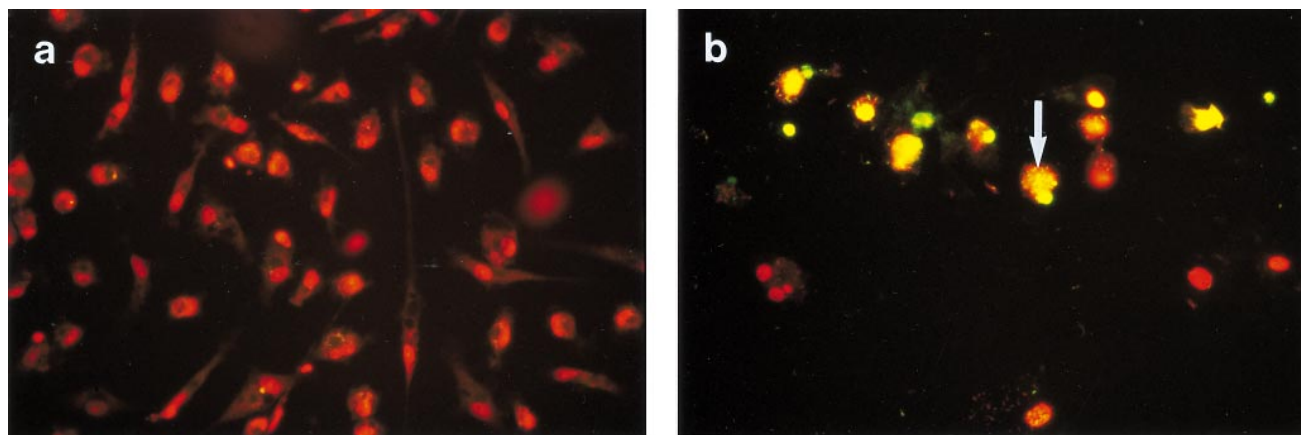


FIG. 5. Demonstration of alveolar macrophage apoptosis following infection with *M. tuberculosis* H37Ra by *in situ* TUNEL. Macrophages were cultured in control medium (a) or infected with H37Ra (b), and DNA strand breaks were detected by TdT end labeling of 3' hydroxyl ends with digoxigenin-dUTP, followed by anti-digoxigenin-fluorescein antibody staining and ethidium bromide counterstaining. Cells were examined by epifluorescence microscopy using fluorescein excitation. Apoptotic cells in the *M. tuberculosis*-infected culture are identified by yellow-orange nuclear fluorescence (arrow). Cells which are not apoptotic are identified by the red nuclear propidium iodide counterstain. No TUNEL-positive cells are seen in the control group. Magnification,  $\times 200$ .

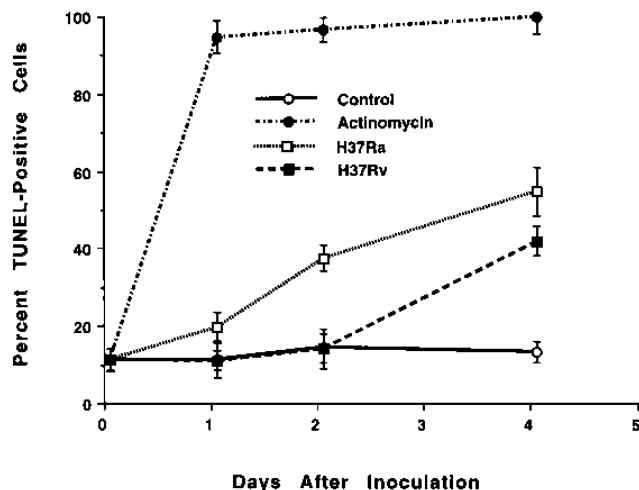


FIG. 6. Quantitative analysis of apoptosis in *M. tuberculosis*-infected alveolar macrophages. Alveolar macrophages were cultured in duplicate on glass coverslips and either infected with *M. tuberculosis* H37Ra or H37Rv (1 to 5 bacilli per macrophage) or cultured in control medium in the presence or absence of actinomycin D. At various time points, cells were fixed and prepared for TUNEL using biotinylated dUTP, incubated with avidin peroxidase, and developed with 3-amino 9-ethyl carbazole in dimethylformamide and hydrogen peroxide. Control slides were prepared in an identical fashion but in the absence of TdT. Approximately 200 cells per field in three fields were counted on each coverslip, and the number of positively stained cells was subtracted from the background values for the TdT-negative controls. Results of duplicate experiments using macrophages from three different donors are expressed as mean percent TUNEL-positive cells  $\pm$  SD.

death in *M. tuberculosis*-infected macrophages treated with anti-TNF- $\alpha$  or pentoxifylline, while augmented cytotoxicity was observed in the infected cells, but not controls, following the addition of exogenous TNF- $\alpha$ . The predominant mechanism of cytolysis was shown to be apoptosis by three methods as follows. Electrophoresis of genomic DNA revealed internucleosomal banding in the *M. tuberculosis*-infected but not control macrophages. Nuclear fragmentation and condensation were identified by EM in the *M. tuberculosis*-infected cells but not in uninfected controls. Macrophage DNA fragmentation was identified in situ by TUNEL analysis of *M. tuberculosis*-infected cells.

Alveolar macrophages are the primary host cell for inhaled *M. tuberculosis*. Following phagocytosis, the mycobacterium is capable of intracellular survival and replication within the alveolar macrophage which appears poorly equipped for mycobacterial killing in the absence of cytokine stimulation from activated lymphocytes (3). The macrophage response to *M. tuberculosis* ultimately results in the induction of cell-mediated immunity which is relatively effective in containing the infection. Intracellular mycobacteria rely on the host cell to evade

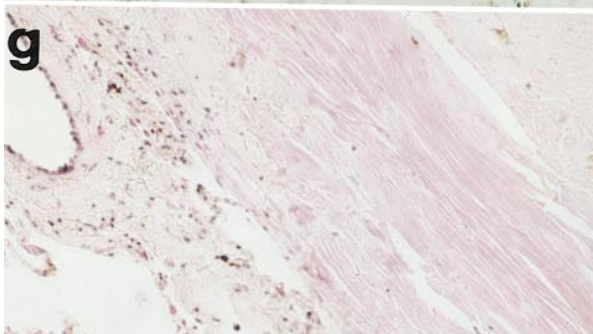
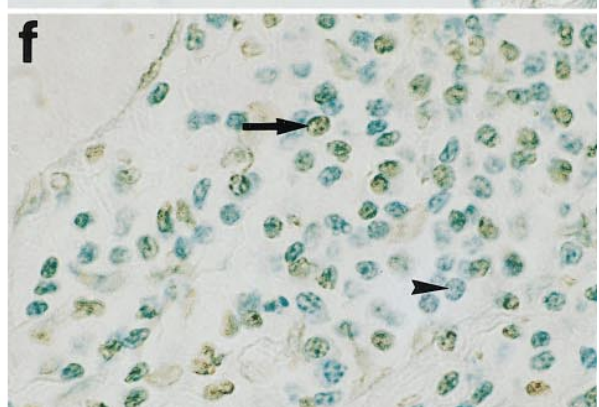
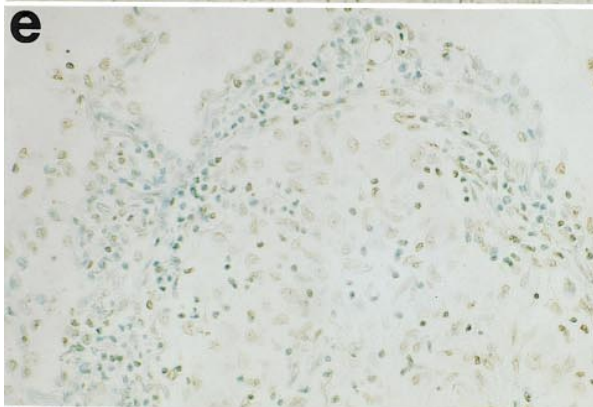
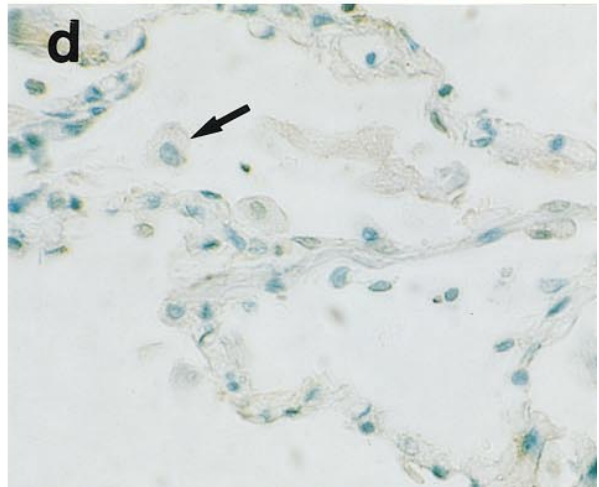
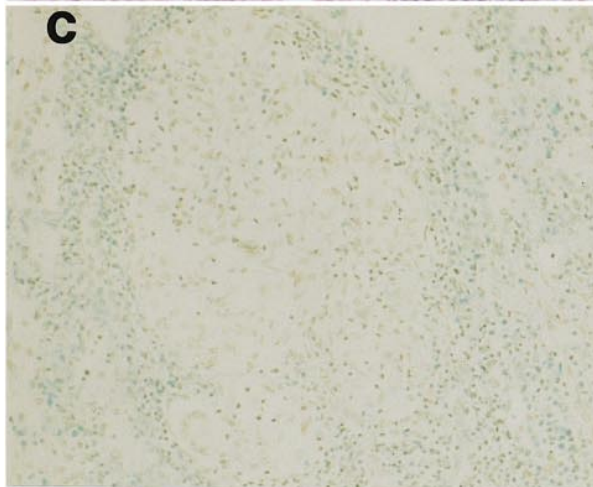
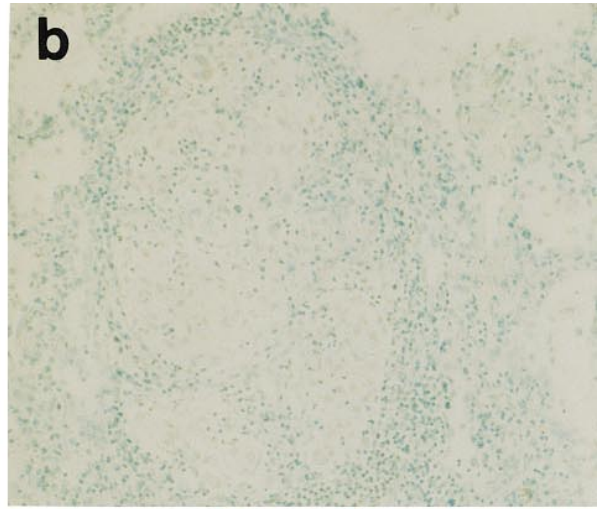
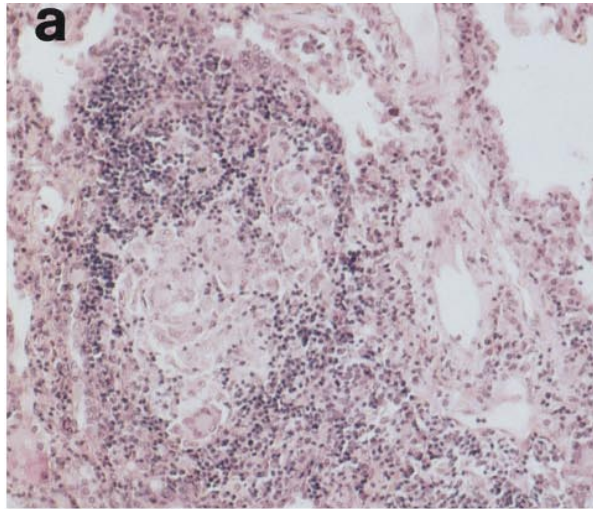
immune effector responses (such as more-effective activated phagocytes) that might otherwise destroy them. The hallmark of an adequate immunological response to *M. tuberculosis* infection is the caseating granuloma, and granuloma formation in mice coincides with local TNF- $\alpha$  synthesis (11). The effect of TNF- $\alpha$  to induce apoptosis of alveolar macrophages which we observed suggests one mechanism contributing to development of the caseous focus and expulsion of intracellular bacilli into that hostile environment.

Animal studies have suggested a role for gamma interferon in activating bactericidal functions of macrophages including the generation of toxic radicals. While *M. tuberculosis* appears to be refractory to the effects of reactive oxygen intermediates, it is sensitive to the effects of reactive nitrogen intermediates in murine macrophages stimulated with gamma interferon (4, 7). Inducible nitric oxide synthase mRNA has been identified in human peripheral blood monocyte-derived macrophages (17), although its role in the antimicrobial response of human macrophages remains controversial (2, 19). The in vivo murine model of tuberculosis differs significantly from human infection. Mice infected with virulent *M. tuberculosis* by delivery to the airways develop progressive disease in the lungs (16), whereas human infection is more frequently associated with persistent asymptomatic infection. Studies in animal models have not reported any cytotoxic effect of *M. tuberculosis* on macrophages. While nitric oxide has been implicated in apoptosis in stimulated murine macrophages (18), we found no change in macrophage cytotoxicity when they were infected with *M. tuberculosis* in the presence of the nitric oxide synthase inhibitor  $N^G$ -methyl-L-arginine or 2-amino guanidine (data not shown).

Apoptosis has previously been associated with intracellular infection by other pathogens. The first intracellular pathogen shown to cause apoptosis was *Shigella flexneri* (23). *Bordetella pertussis* was subsequently also shown to cause apoptosis of infected macrophages (10). Both of these infections are acute, and apoptosis of the host cell appears to propagate the infection. In contrast, *Leishmania donovani* causes a chronic visceral disease protected from the immune system in its primary host cell, the macrophage. Intracellular infection by *L. donovani* inhibits macrophage apoptosis (induced by growth factor withdrawal) and thus perpetuates its chronic infection (15). Tuberculosis is also a chronic infection in humans, which suggests that alveolar macrophage apoptosis might favor the host by depriving the pathogen of its intracellular sanctuary. Our data indicate that the virulent strain H37Rv was less cytotoxic for alveolar macrophages than the avirulent strain H37Ra. Also, the addition of exogenous TNF- $\alpha$  augmented killing more in the H37Ra-infected group than in the H37Rv-infected group. The viability of H37Ra-infected cells without added TNF- $\alpha$  was lower than that of H37Rv-infected cells which had also been treated with TNF- $\alpha$ . If this trend for an inverse relationship between virulence and the induction of apoptosis

FIG. 7. Identification of apoptosis in human tuberculosis. Paraffin sections from clinical cases of pulmonary tuberculosis were prepared for in situ TUNEL analysis using anti-digoxigenin-peroxidase and methyl green counterstaining as described in Materials and Methods. (a) Lung tissue section from a tuberculosis case stained with hematoxylin and eosin. An active granuloma is seen in the left side of the section. Magnification,  $\times 19$ . (b) Negative control for the TUNEL assay. A section from the same clinical case of tuberculosis was prepared for TUNEL, but TdT was not added to the reaction mixture. No brown nuclear staining indicative of apoptosis is identified. Magnification,  $\times 19$ . (c) TUNEL preparation of a section from the same case of tuberculosis. A high proportion of apoptotic cells are identified by brown staining nuclei. Magnification,  $\times 19$ . (d) TUNEL preparation of a healthy lung. A healthy alveolar macrophage with no nuclear staining is identified by the arrow. Magnification,  $\times 154$ . (e and f) Higher-power views from the edge of the TUNEL-positive tuberculous granuloma. An apoptotic cell with marginalized and clumped brown-staining chromatin is identified by the arrow in panel e. A representative green-staining healthy cell in the same field is identified by an arrowhead. Magnifications,  $\times 64$  (for panel e) and  $\times 192$  (for panel f). Tissue samples from two additional cases of active tuberculosis (not shown) revealed identical patterns and proportions of apoptotic cells in regions of granulomatous inflammation. (g) Hematoxylin-and-eosin-stained lung tissue from an inactive tuberculosis case. A hyalinized lesion with no active granulomatous inflammation is present. Magnification,  $\times 40$ . (h) TUNEL analysis of the inactive tuberculosis lesion showing no apoptotic cells.





is confirmed with other clinical *M. tuberculosis* strains and strains with attenuated virulence, the ability to avoid triggering apoptosis or to otherwise inhibit the apoptotic response once triggered could be a mycobacterial virulence factor.

TNF- $\alpha$  has been reported to prevent apoptosis in maturing monocytes (13), but *M. tuberculosis* infection of fibroblast and monocytoid cell lines has also been shown to sensitize the cells to the cytotoxic effects of TNF- $\alpha$  (8). We have shown TNF- $\alpha$  to be a potent promoter of apoptosis in *M. tuberculosis*-infected human alveolar macrophages, while TNF- $\alpha$  added in the absence of *M. tuberculosis* had no significant effect on cell viability. Both H37Ra and H37Rv infections induced the release of comparable amounts of TNF- $\alpha$  into the culture supernatant in our experiments. The difference in cell killing observed between these strains therefore may represent a difference in their ability to sensitize the macrophages to the cytotoxic effects of TNF- $\alpha$ . This could reflect differences in TNF receptor expression, differential activation of a later step in the apoptotic pathway by H37Ra or H37Rv, or the induction of an anti-apoptosis mechanism by H37Rv. While TNF- $\alpha$  is recognized as an important inducer of apoptosis in many cell types, our results do not exclude the possibility that the role of TNF- $\alpha$  in *M. tuberculosis*-induced macrophage cytotoxicity is indirect.

A previous light and electron microscopy study of pulmonary granulomas, including tuberculous granulomas, reported the infrequent occurrence of structural changes suggestive of apoptosis (5). Using the more sensitive in situ TUNEL technique, we found that 50 to 70% of cells in the peripheries of tuberculous granulomas from three clinical cases of tuberculosis were apoptotic. A fourth case, with no active granuloma present in the biopsy specimen, was TUNEL negative. Containment of active tuberculosis infection in humans relies on the development of the characteristic caseous granuloma. Within the necrotic center of this structure, mycobacterial growth is inhibited (6). The development of the caseating granuloma and the exposure of mycobacteria to phagocytes with more effective microbicidal function may be facilitated by the orderly induction of cell death in responding alveolar macrophages. A greater understanding of the role of alveolar macrophage apoptosis in the macrophage-tuberculosis interaction may suggest new approaches to enhancing the host response to this important human infection.

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