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Effects of Mycobacteria on Regulation of Apoptosis in Mononuclear Phagocytes

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Since apoptosis is observed in tuberculous granulomata, we investigated the molecular mechanisms underlying the apoptotic pathway in an in vitro model of mycobacterial infection of mononuclear phagocytes. We postulated that *Mycobacterium tuberculosis* could trigger the apoptotic pathway in macrophages, resulting in death of the microorganism by modulating the expression of *bcl-2*, *bax*, *bcl-x*$_L$, and *bcl-x*$_S$. We found that the mRNA of *bcl-2*, an inhibitor of apoptosis, was downregulated in peripheral blood monocytes (PBM) between 2 and 6 h following infection with *M. bovis* BCG or induction with heat-killed *M. tuberculosis* H37Ra. Western analysis showed a downregulation of the Bcl-2 protein, with a half-life of 24 h. At the same time points, there was no change in the expression of Bax or Bcl-x$_S$, inducers of apoptosis, but Bcl-x$_L$, another inhibitor of apoptosis, was minimally upregulated by BCG. To determine if apoptosis could be a mechanism for growth inhibition in vivo, we obtained alveolar macrophages by bronchoalveolar lavage from involved sites in patients with active pulmonary tuberculosis. Using the TUNEL (terminal deoxynucleotidyltransferase mediated nick end labeling) technique, we observed significantly more apoptosis in involved segments of five tuberculosis patients (14.8 ± 1.9%) than in those of normal controls (<1%, $P = 0.02$) or in uninvolved segments (4.3 ± 0.9%, $P < 0.05$). We conclude that apoptosis of mononuclear phagocytes induced by *M. tuberculosis* occurs in vivo and that in an in vitro model of mycobacterial infection, apoptosis may be mediated by downregulation of Bcl-2.

The resurgence of tuberculosis (TB) secondary to human immunodeficiency virus type 1 (HIV-1) infection and poor socioeconomic conditions has crystallized the need for new therapies and greater understanding of the human host response to *Mycobacterium tuberculosis* (4, 5, 37, 39). The vast majority (90%) of infected individuals who convert their tuberculin skin test from negative to positive do not develop tuberculous disease, and prior to chemotherapy, approximately half of the patients did well with bed rest alone (40). Growth inhibition and killing of *M. tuberculosis* by mononuclear phagocytes is important for the elimination of inhaled mycobacteria or containment within granulomata.

M. tuberculosis binds mononuclear phagocytes by using complement receptors, especially CR4 for human alveolar macrophages and CR1 and CR3 for peripheral blood monocytes (PBM), and is rapidly phagocytosed (16, 47, 48). Mycobacteria are observed to reside within phagosomes surrounded by a membrane within the cytoplasm (31). Although it is well known that mycobacteria inhibit phagosome-lysosome fusion, mononuclear phagocytes are capable of inhibiting the growth of mycobacteria (2, 51). Potential mechanisms for interdicting the growth and metabolism of *M. tuberculosis* include apoptosis, reactive oxygen species, or nitric oxide (NO). In a murine model of TB, reactive oxygen species have been shown to be uninvolved but NO was shown to kill *M. tuberculosis* and was regulatable by gamma interferon (8). These data were supported by the demonstration of strikingly increased expression of inducible nitric oxide synthase in the alveolar macrophages lavaged from the site of disease in pulmonary TB (33). In support for the role of apoptosis, Cree and colleagues have demonstrated morphological changes consistent with apoptosis in epithelioid cells in granulomata from patients with TB, leprosy, and sarcoidosis (11). They postulated that apoptosis exceeded mitosis as a cause of rapid turnover of granulomata and was a major cause of their regression. Mycobacteria are normally difficult to find in granulomata of immunocompetent hosts, consistent with growth inhibition.

We have previously demonstrated that human alveolar macrophages can rapidly and progressively inhibit mycobacteria over 48 h in vitro and that in vitro matured PBM but not fresh PBM approach but do not equal this growth inhibition (2). Molloy and colleagues have demonstrated that apoptosis, but not necrosis, is coupled with killing of intracellular *M. bovis* BCG (31). Apoptosis was induced in human PBM by the addition of ATP, and intracellular BCG showed a threefold decline as demonstrated by CFU. We have also shown increased immunostaining in TB granuloma for a 15-kDa protein in lymphocytes that induces apoptosis in permeabilized thymocytes (27, 53). Lymphocytes recovered by bronchoalveolar lavage from eight patients with active pulmonary TB demonstrated three- to fourfold increased immunostaining with TIA-1, a cytoplasmic protein associated with cytotoxicity and apoptosis.

Apoptosis is a genetically regulated program for cell death that can be inhibited and promoted by members of the family of Bcl-2 proteins (24, 38). The *bcl-2* gene is conserved through evolution from nematodes to mammals and was originally identified as a translocated gene from chromosome 18q21 to 14q32 in human B-cell lymphomas (9, 34, 54). In several cancer systems, it has been shown that the ratio of Bcl-2 to Bax

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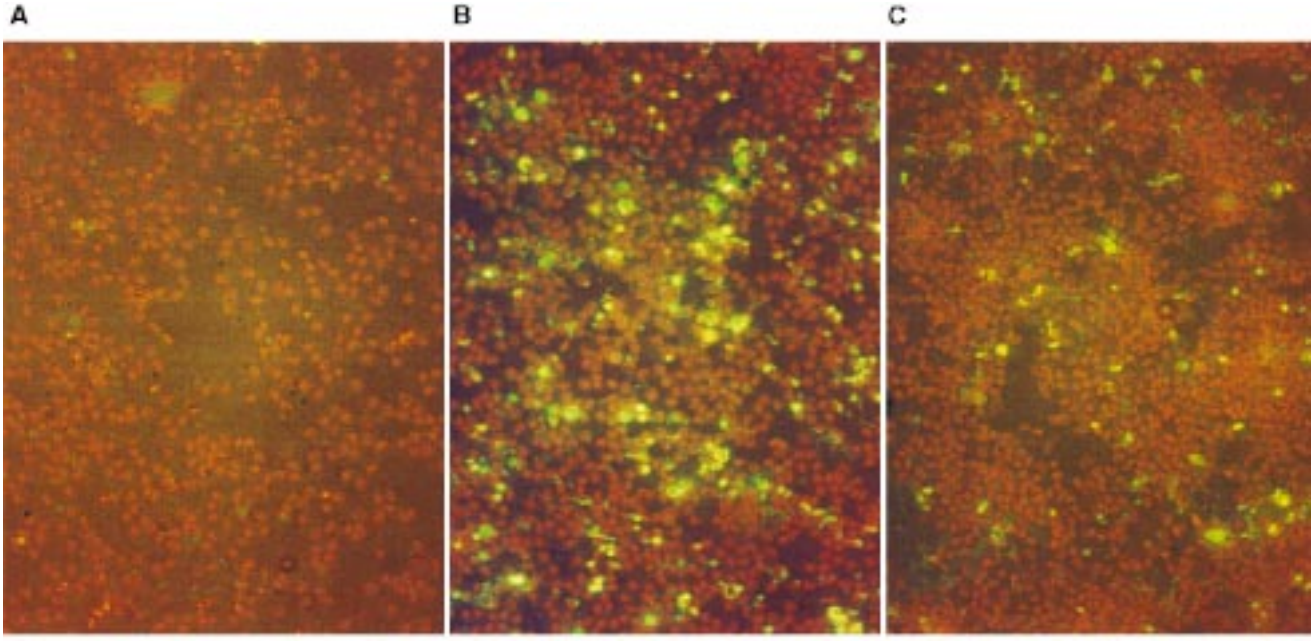

FIG. 1. TUNEL assay of apoptosis in PBM after BCG or heat-killed *M. tuberculosis* H37Ra treatment. (A) Untreated, 24 h; (B) BCG infected, 24 h; (C) heat-killed *M. tuberculosis* H37Ra, 24 h. The green-yellow cells demonstrate striking apoptosis after mycobacterial treatment.

dictates susceptibility of cells to undergo apoptosis as Bcl-2–Bax heterodimers can neutralize the cell death-promoting function of Bax (6, 23, 35). We investigated the role that apoptosis could play in the control of *M. tuberculosis* by establishing an in vitro model of infection whereby we could induce apoptosis and evaluate the role of the Bcl-2, Bax, and Bcl-x proteins. Our in vitro model demonstrated that mycobacterial infection of PBM downregulated Bcl-2, thereby potentially disrupting the balance between antiapoptotic and proapoptotic signals. We used bronchoalveolar lavage to investigate the role of apoptosis in alveolar macrophages from lobes involved with pulmonary tuberculosis.

MATERIALS AND METHODS

Study population. Bronchoalveolar lavage was performed on five patients with active pulmonary TB confirmed by culture of *M. tuberculosis* in the sputum. The protocol was approved by Human Subjects Review Committees at NYU Medical Center and Bellevue Hospital. The five patients included four smokers and one nonsmoker; all were males (mean age, 36 ± 6 years) and were all HIV negative. We also evaluated four HIV-negative healthy volunteers with normal chest radiographs, pulmonary function tests, and physical examinations. There were three smokers and one nonsmoker (mean age, 38 ± 4 years). Bronchoalveolar lavage was performed in involved and uninvolved lobes as described above. A total cell count was done in a hematocytometer; cell differentials were performed on cytocentrifuge slides stained with Diff-Quick, and 500 cells were counted. Apoptosis of alveolar macrophages was performed by fluorescence microscopy to detect localized green fluorescence of apoptotic cells (fluorescein-12-dUTP) in a red background (propidium iodide) as instructed by the manufacturer (Promega, Madison, Wis.). Comparisons between TB patients and healthy controls used the Wilcoxon rank sum test.

Cell cultures. PBM were allowed to adhere in RPMI 1640 supplemented with 10% fetal calf serum (FCS) for 1 h at 37°C. Adherent cells were scraped off with a sterile rubber policeman. The purity of the monocyte fraction was approximately 90%, as assessed by morphology by May-Grünwald-Giemsa staining and myeloperoxidase. PBM were cultured in polypropylene tubes (Falcon) at a density of 5×10^5 to 2×10^6/ml in RPMI 1640 with 10% FCS at 37°C. THP-1 (myelomonocytic leukemia cell line) and U-937 cells were obtained from the American Type Culture Collection (Rockville, Md.) and cultured in RPMI 1640 supplemented with 10% FCS.

Reagents. Hydrogen peroxide (H_2O_2) (Sigma, St. Louis, Mo.) was used to induce apoptosis; GTP (Boehringer Mannheim, Indianapolis, Ind.) was used as a control.

Identification of apoptotic cells. Apoptotic cells were identified by light microscopy on cytospin slides with May-Grünwald-Giemsa (Sigma) staining at a magnification of ×1,000. Apoptotic cells were identified by in situ terminal deoxynucleotidyltransferase-mediated nick end labeling (TUNEL) of DNA strand breaks (Promega) (28). We detected green fluorescence of apoptotic cells (fluorescein-12-dUTP) in a red background (propidium iodide) by fluorescence microscopy. The percentage of apoptotic cells were estimated by counting 500 cells at several randomly selected fields.

Flow cytometry (FACScan) (Becton Dickinson) was performed on propidium iodide-stained cells (10^6) for cell cycle analysis.

All experiments were performed three or more times.

DNA fragmentation. Mononuclear phagocytes (2×10^6) were washed with phosphate-buffered saline, and the cell pellet was lysed (0.6% sodium dodecyl sulfate [SDS], 10 mM EDTA, 10 mM Tris [pH 7.4], 20 μg of RNase A per ml) at 37°C for 1 h. Then 5 M NaCl (100 μl) was added, and the preparation was

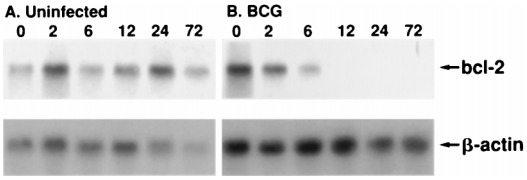

FIG. 2. Northern analysis of *bcl-2* gene expression in PBM after infection with BCG. (A) Uninfected; (B) infection with BCG. A time course from 0 to 72 h demonstrates a decline in *bcl-2* gene expression beginning at 2 h that is striking by 6 h and undetectable from 12 to 72 h after BCG infection. β-Actin was used to control for RNA loading in the lanes.

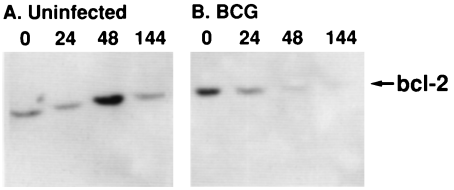

FIG. 3. Western analysis of Bcl-2 protein expression in PBM after infection with BCG. (A) Uninfected; (B) infection with BCG. Bcl-2 protein in PBM infected with BCG declines after 24 h as compared to uninfected cells.

incubated on ice for 1 h and then centrifuged at 13,000 × *g* for 30 min. The supernatant containing fragmented DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and the low-molecular-weight DNA was precipitated in ethanol. Samples were electrophoresed in a 1.5% agarose gel and visualized by ethidium bromide staining.

DNA fragments were also estimated by the diphenylamine reagent method (0.15 g of diphenylamine, 0.15 ml of sulfuric acid, 0.05 ml of acetaldehyde, 10 ml of glacial acetic acid). After overnight color development, optical density at 570 nm was measured with a microplate reader (Bio-Rad Laboratories, Hercules, Calif.) (36).

Northern blot analysis. Mononuclear phagocytes were lysed in 5.5 M guanidinium isothiocyanate buffer, and total RNA was collected by $CsCl_2$ gradient ultracentrifugation. An equal amount of RNA in each lane was fractionated by electrophoresis through a 1% agarose–6% formaldehyde denaturing gel and transferred onto a Hybond-N nitrocellulose filter (Amersham Life Science, Arlington Heights, Ill.). The UV-cross-linked filter was prehybridized at 65°C for 12 h. For hybridization, the *bcl-2* cDNA (gift of Stanley Korsmeyer, St. Louis, Mo.) was labeled with [α-^{32P}]dCTP and hybridized at 65°C for 18 h. The filter was washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% SDS at room temperature for 10 min, followed by 2× SSC–0.1% SDS for 15 min at room temperature and 0.1× SSC–0.5% SDS for 30 min at 68°C. The filter was exposed to radiographic film at −80°C.

Western blot analysis. Mononuclear phagocytes were lysed (1% Triton-X 100, 150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride) for 30 min on ice. The lysate was centrifuged for 10 min at 15,000 × *g*, and the supernatant was diluted in Laemmli's buffer. Samples were electrophoresed on a 15% SDS-polyacrylamide gel for 18 h and electroblotted on an Immobilon-P transfer membrane (Millipore, Bedford, Mass.). Blots were blocked with 5% skim milk in phosphate-buffered saline plus 0.5% Tween 20 for 1 h and incubated with anti-Bcl-2 antibody (Pharmingen, San Diego, Calif.) at a dilution of 1:1,000 for 1 h, with anti-Bax antibody (Santa Cruz Biotechnology, Santa Cruz, Calif.) at a dilution of 1:2,000 for 45 min, or with anti-Bcl-$x_{L/S}$ antibody (Santa Cruz Biotechnology) at a dilution of 1:1,000 for 30 min. After primary antibody incubation, the blots were incubated with peroxidase-conjugated anti-mouse immunoglobulin G antibodies (Amersham Life Science) (1:1,000 for Bcl-2 for 1 h, 1:10,000 for Bax for 30 min, and 1:5,000 for Bcl-$x_{L/S}$ for 45 min). Immunoblots were developed by the ECL Western blotting detection system (Amersham Life Science). Autoradiographs were scanned with a laser densitometer (Ultroscan XL; LKB Bromma, Sweden).

RESULTS

We first demonstrated the induction of apoptosis in PBM infected with BCG or heat-killed *M. tuberculosis* H37Ra at 24 h by the TUNEL technique (Fig. 1). Apoptosis was assessed by typical morphological changes, including cell shrinkage, condensed chromatin, and fragmented nuclei, and generation of the typical 196-bp DNA ladder upon gel electrophoresis (data not shown).

Expression of Bcl-2, Bax, Bcl-x_L and Bcl-x_S in PBM after infection with mycobacteria. After infection with BCG, *bcl-2* mRNA expression was downregulated in PBM between 2 and 6 h and was no longer detectable after 12 h (Fig. 2). We found a similar time course of Bcl-2 downregulation in the THP-1 myelomonocytic cell line, but downregulation in U937 cells was delayed, occurring between 12 and 24 h. Western analysis of Bcl-2 protein in PBM after infection with BCG revealed a decrease beginning at 24 h and Bcl-2 protein was barely detectable at 48 h (Fig. 3). Since Bcl-2 was downregulated and apoptosis was detected by TUNEL, we next evaluated the expression of Bax since Bax promotes apoptosis. We infected PBM with BCG over a 96-h time course and found no change in bax protein expression compared to the baseline level (Fig. 4A). In contrast, under in vitro conditions with treatment with H_2O_2 (10 mM), which induces apoptosis as demonstrated by the generation of DNA ladders (data not shown), we were able to see a progressive increase in Bax protein beginning at 24 h (H_2O_2 [Fig. 4B]). Similar results were observed in THP-1 and U937 cells as well as in uninfected controls.

Differential splicing of *bcl-x* mRNA gives rise to short (*bcl-x_S*) and long (*bcl-x_L*) splice variants with different functions: *bcl-x_L* has an antiapoptotic effect, whereas *bcl-x_S* suppresses *bcl-2* function, thus promoting apoptosis. Western analysis of uninfected PBM or PBM infected with BCG over 72 h showed a strong protein signal for Bcl-x_S, with no modulation by mycobacteria (Fig. 5); however, Bcl-x_L was weakly induced at 24 h after BCG infection.

We found similar results with heat-killed *M. tuberculosis* H37Ra in PBM as with BCG for Bcl-2, Bax, Bcl-x_L, and Bcl-x_S (Fig. 6). Western blots demonstrated a similar time course over 72 h.

Evaluation of apoptosis in alveolar macrophages from patients with pulmonary TB. The patients with TB had more total cells recovered than normal controls ([525 ± 49] × 10^3/ml versus [263 ± 37] × 10^3/ml; $P < 0.01$). The cell differentials from TB-involved segments were as follows: macrophages, 83%; lymphocytes, 15%; and neutrophils, 2%. Using fluorescence microscopy and TUNEL to evaluate apoptotic cells (fluorescein-12-dUTP), we found a significant increase in apoptotic alveolar macrophages from TB-involved lobes compared to normal controls (14.8% ± 1.9% [TB] versus <1% [normals] [$P < 0.02$] or 4.3% ± 0.9% [uninvolved lobes] [$P < 0.05$] [Fig. 7]). Figure 8A illustrates an apoptotic alveolar macrophage, and Fig. 8B identifies several apoptotic cells in the tuberculous granuloma, using fluorescence microscopy and the TUNEL technique.

DISCUSSION

Apoptosis is a physiological process of programmed cell death that is important for pulmonary homeostasis and resolution of pathophysiologic processes (24, 34, 38). Granulomata in tuberculosis, leprosy, and sarcoidosis have a high turnover of epithelioid cells due to apoptosis and an influx of mononuclear phagocytes (11). In vitro mycobacterial infection of PBM followed by induction of apoptosis has resulted in striking growth inhibition (31). We examined the regulatory signals of apoptosis in an in vitro model of BCG-infected or H37Ra-induced mononuclear phagocytes and found that Bcl-2, an inhibitor of apoptosis, was downregulated at the protein and mRNA levels after 6 to 24 h, but the levels of proapoptotic signals of Bax and Bcl-x_S were unchanged (9). Apoptotic signals were strikingly out of balance and correlated with apoptosis in BCG-infected PBM or PBM induced with heat-killed *M. tuberculosis* H37Ra in vitro. We also observed that mycobacteria are killed by the apoptotic process when PBM are treated with H_2O_2. We demonstrated that BCG-infected PBM are capable of undergoing apoptosis by TUNEL staining and morphology and induction of DNA fragments of 196 bp (25). Keane et al. demonstrated that H37Ra induced 29% macrophage cytotoxicity versus 13% by H37Rv and that heat-killed H37Ra did not reduce macrophage viability in vitro after 5 days of incubation, which is much longer than our time course (21). Over this long interval, tumor necrosis factor alpha (TNF-α) actually enhanced apoptosis rather than inhibited it as has been reported by others (28, 29).

The mechanism of growth inhibition of mycobacteria is not known, but NO is a likely candidate since induction of inducible nitric oxide synthase (38) and increased NO production in animal models of TB correlate with survival (8, 44). Apoptosis is associated with the release of interleukin-1β (IL-1β) from mononuclear phagocytes, probably from activation of the IL-1β-converting enzyme that is also linked to the apoptotic process (44, 17). *M. tuberculosis* stimulates the release of IL-1β and IL-6 from macrophages by activating the transcription factors NF-κB and NF-IL6, which positively regulate transcription of these two genes (61, 62). We have demonstrated that IL-1β and IL-6 as well as TNF-α and IL-8 are released spontaneously in vivo from involved lung segments in active pul-

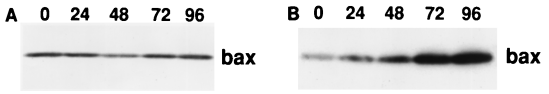

FIG. 4. Western analysis of Bax protein in PBM after infection with BCG. (A) Time course with BCG infection; (B) time course with H_2O_2 (10 mM) treatment. BCG infection does not induce Bax protein, but H_2O_2 induces Bax protein at 1 to 3 days when apoptosis is also induced.

monary TB (26, 63, 64). In this report we now demonstrate that alveolar macrophages from TB-involved sites have significantly increased apoptosis compared to normal volunteers and are increased threefold over uninvolved lobes. Keane et al. found a high percentage of apoptotic cells (50 to 70%) in the periphery of three active TB granulomata (21), which is consistent with the concept that in vivo, apoptosis may be an important mycobacterial killing process.

Several investigators (12, 28, 29) demonstrated that TNF-α added in vitro to PBM inhibited apoptosis, which may be mediated through NF-κB, a known inhibitor of apoptosis (1). Mycobacteria can directly activate NF-κB but can also release significant amounts of the antioxidants catalase and superoxide dismutase, which are inhibitors of NF-κB (1). Thus, it is important to demonstrate in vivo that the dominant effect of *M. tuberculosis* is to promote apoptosis at the site of infection.

The apoptotic pathway has been shown to be a mechanism for removing infected cells and microorganisms from various viral infections and malignancies. Macrophages have been shown to phagocytose neutrophils, T cells, and eosinophils and to process the apoptotic cells without releasing their toxic granule or lysosomal components. Anti-Fas monoclonal antibody blocks this process in vitro, and the $\alpha_v\beta_3$ integrin receptor on the surface of macrophages recognizes apoptotic neutrophils and lymphocytes (45, 46). Apoptosis occurs in neutrophils that accumulate in bacterial pneumonia and the adult respiratory distress syndrome (36).

The process of activation of macrophages appears to be closely linked to apoptosis. For example, IL-1β and NO are released during apoptosis, and both are important for control of mycobacteria (17, 64). Cytotoxic $CD4^+$ T-cell clones can induce apoptosis in target cells, and inflamed granulomata of *M. tuberculosis* contain increased $CD4^+$ cells (27). $CD4^+$ cells of the Th1 phenotype express the Fas ligand and can undergo apoptosis, whereas Th2 cells do not express Fas ligand and are more resistant to apoptosis (55). $CD8^+$ cells contain perforin granules that can lyse *M. tuberculosis*-infected macrophages, resulting in mycobacterial growth inhibition (50). TNF/TNF receptor is a major pathway for the induction of apoptosis, and TNF-α is increased both in bronchoalveolar lavage from involved sites of pulmonary TB and in our in vitro model of BCG-infected PBM (18, 58). Mice lacking TNF-α obtained by administration of neutralizing antibody against TNF-α or gene knockout of the 55-kDa TNF receptor are highly susceptible to *M. tuberculosis* infection, and their granulomata are deficient in

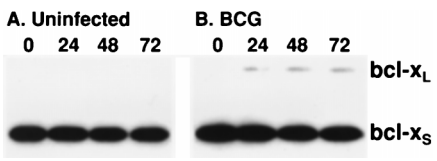

FIG. 5. Western analysis of Bcl-x_L and Bcl-x_S proteins in PBM after infection with BCG. (A) Uninfected; (B) infection with BCG. BCG infection increased Bcl-x_L protein from 24 to 72 h, but Bcl-x_S was unchanged after BCG infection.

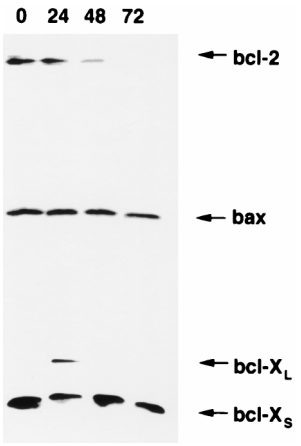

FIG. 6. Western analysis of Bcl-2, Bax, Bcl-x_L, and Bcl-x_S in PBM treated with heat-killed *M. tuberculosis* H37Ra.

epithelioid cells (15). Macrophages in this model appear not to be activated to destroy mycobacteria, which may be due to imbalance of apoptosis-regulatory genes.

Bcl-2 inhibits apoptosis and was originally identified in B-cell lymphoma, where the *bcl-2* gene is translocated from chromosome 18 to chromosome 14 juxtaposed to the immunoglobulin heavy-chain gene (37, 56). The Bax protein has 20% amino acid identity with Bcl-2 and accelerates apoptosis when overexpressed by gene transfer (3). For Bcl-2 to function, it must heterodimerize with Bax (60). Both are expressed in a tissue-specific fashion, and deregulation with increased Bax occurs in genotoxic stress induced, for example, in cancer or stroke (10). A model in which the inherent ratio of Bcl-2 to Bax determines the susceptibility to death following an apoptotic signal has been proposed (30, 35). A quantitative analysis within a hematopoietic cell line indicated that when half or more of the endogenous Bax formed heterodimers with either Bcl-2 or Bcl-x_L, apoptosis was repressed (22, 59). The more recently described *bcl-x* gene, which is 28% identical to *bcl-2* (19), has two splice variants; *bcl-*x_L (expressed in differentiated cells) is antiapoptotic, and *bcl-*x_S is expressed in cells with high turnover resembling functional *bax* (7). *bcl-*x_L inhibits the ability of *bcl-2* to enhance survival in growth factor-deprived cells. We postulated that phagocytosis of microorganisms could modu-

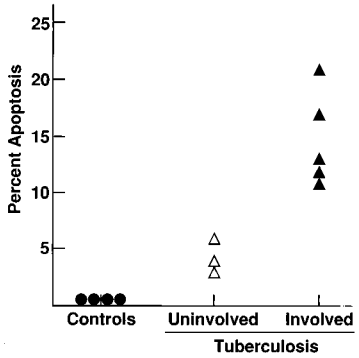

FIG. 7. Percent apoptosis in alveolar macrophages. Apoptosis was determined on cytospin slides by using the TUNEL technique. There was a significant increase in percent apoptosis from TB-involved segments compared to normal controls or uninvolved segments. Circles, normal controls; open triangles, uninvolved lobes; closed triangles, involved lobes with pulmonary TB.

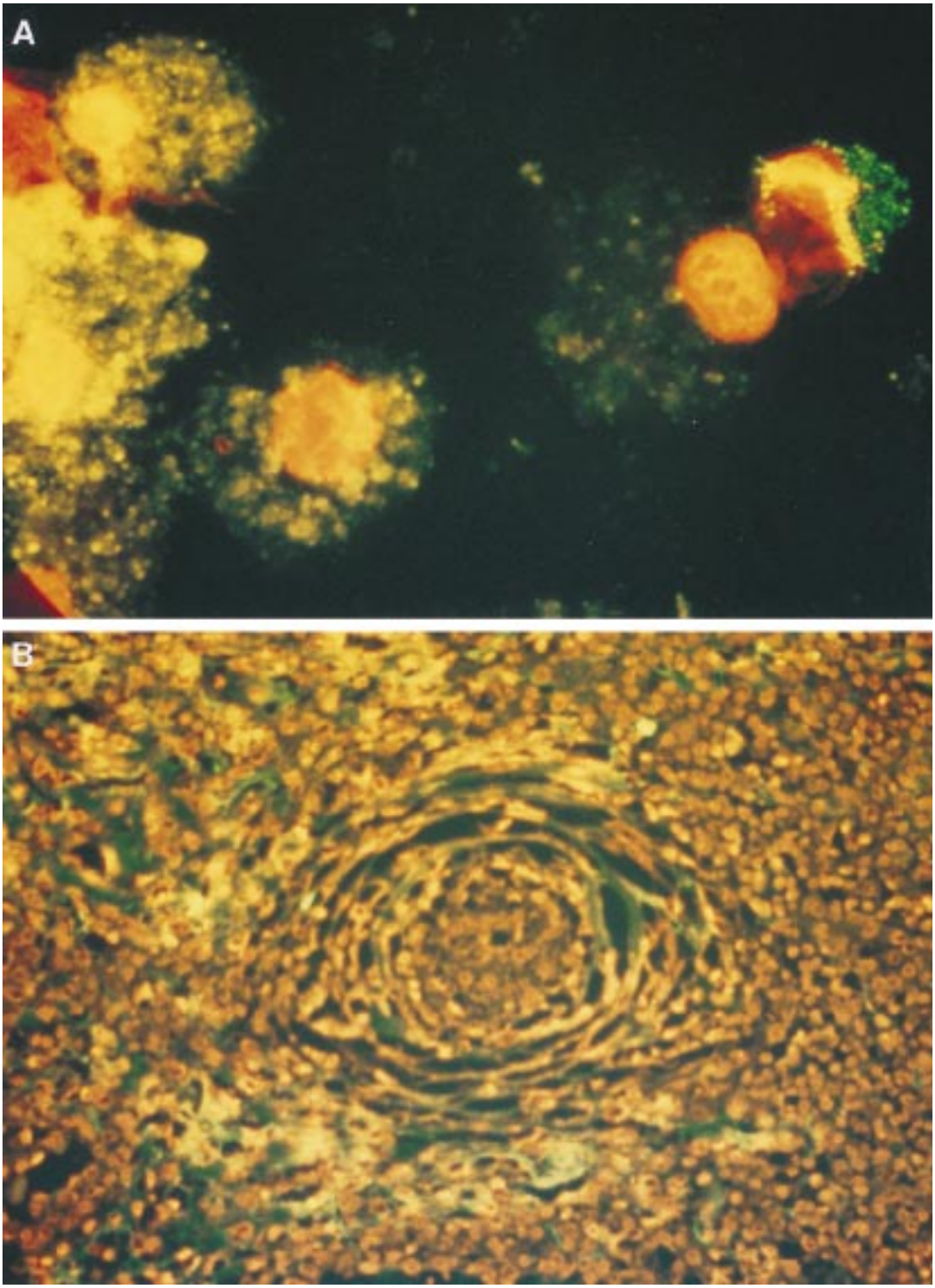

FIG. 8. Apoptosis in pulmonary TB. (A) Cytospin slide from pulmonary TB illustrating apoptosis in alveolar macrophage (green granules; magnification, ×890); (B) TB granuloma illustrating prevalence of apoptosis in vivo (bright yellow-green cells are undergoing apoptosis; magnification, ×356).

late the genes regulating apoptosis and inhibit the pathogen concurrent with apoptosis, which is consistent with our observation of downregulation of *bcl-2*. Since both live BCG and heat-killed *M. tuberculosis* H37Ra downregulate *bcl-2* and trigger apoptosis in the mononuclear phagocyte, mycobacteria, like leishmaniae (32), must evolve other strategies to enable them to survive in vivo. Enrichment of signals that induce apoptosis might be a therapeutic strategy (52).

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