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Apoptosis of Human Monocytes and Macrophages by *Mycobacterium avium* Sonicate

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Mycobacterium avium **is an intracellular organism which multiplies predominantly within human macrophages. This organism has previously been shown to induce apoptosis in human macrophages. With a view to identifying *M. avium* components that induce cell death in infected host cells, sonicated extracts of *M. avium* as well as individual components isolated from the *M. avium* sonicate were tested in various assays with a human monocytic cell line (THP-1). THP-1 cells incubated with *M. avium* sonicate showed significantly reduced viability after a 2-day exposure compared to control cells incubated with media alone. This effect was dose dependent, with only 6.6% ± 5.2% and 48.8% ± 10.3% of the cells being viable by trypan blue exclusion at 600 and 300 μg/ml, respectively. Control cells, on the other hand, exhibited a viability of 98.8% ± 1.0%. In addition, an 80% ammonium sulfate fraction of the *M. avium* sonicate and the previously characterized 68-kDa protein were found to have similar effects on THP-1 cells. In both cases, the reduction in viability was due to apoptosis characterized by chromatin condensation, DNA fragmentation by agarose gel electrophoresis, or terminal deoxynucleotidyl transferase-mediated d-UTP nick end labeling (TUNEL) and release of nuclear matrix protein (NMP) into the culture medium. *M. avium* sonicate-induced apoptosis of THP-1 cells was completely inhibited by the commonly used antioxidants pyrrolidinedithiocarbamate (PDTC) and butylated hydroxyanisole (BHA), indicating that the generation of free oxygen radicals may be responsible for inducing cell death. *M. avium* sonicate was found to induce apoptosis of monocyte-derived macrophages (MDMs) as well. This effect was not reversed in the presence of PDTC and was not accompanied with DNA fragmentation when determined by agarose gel electrophoresis, as seen in the case of THP-1 cells. However, these MDMs were found to contain fragmented DNA by TUNEL. These findings suggest that the mechanism of cell death in MDMs may be different from that observed with THP-1 cells. Furthermore, these results provide new insight into the effect of *M. avium* components on host cell responses during *M. avium* infection.**

Mycobacterium avium is a common opportunistic pathogen which causes disseminated infection in patients with AIDS (12). This organism predominantly infects and multiplies within macrophages (7). In response to this infection, macrophages are known to secrete several cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (9, 24). Some cytokines, such as TNF-α and GM-CSF, have been known to activate infected macrophages to kill this organism. On the other hand, infection of macrophages with *M. avium* has been shown to result in apoptosis of infected cells (10).

Apoptosis is a morphologically and biochemically distinct form of cell death that regulates cell turnover (32). It is characterized by a distinctive and orchestrated sequence of morphological changes, including cell shrinkage, chromatin condensation, subsequent nuclear segmentation, and eventual cellular disintegration into discrete membrane-bound apoptotic bodies. In addition to *M. avium* (10), recently, several bacterial pathogens or their components have been reported to induce programmed cell death in host cells. These include *Mycobacterium tuberculosis* (14), *Shigella flexneri* (37), *Bordetella pertussis* (15), *Legionella pneumophila* (23), and *Leptospira interrogans* (20) among others (4, 34). Apoptosis can be induced when either a membrane receptor or a cytoplasmic receptor binds to an appropriate ligand, which provokes the generation of second messengers (1). In the case of *S. flexneri* (37) and *L. interrogans* (20), apoptosis is thought to be initiated after entry of the pathogen into the host cell. While the mechanism by which pathogens induce apoptosis is known in some cases, there are several aspects regarding this process that are poorly understood.

Previous studies with *M. avium* and *M. tuberculosis* have used intact organisms to study apoptosis (10, 14). In order to identify specific components of *M. avium* that interact with host cell membrane receptors during apoptosis, we used sonicated extracts of *M. avium* as well as isolated *M. avium* proteins to study apoptosis of monocytes and monocyte-derived macrophages (MDMs) in the present investigation.

MATERIALS AND METHODS

Cell culture. THP-1, a myelomonocytic cell line, was obtained from the American Type Culture Collection, Rockville, Md. (ATCC TIB202), and cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 50 U of penicillin-streptomycin per ml (all from BioWhittaker, Inc., Walkersville, Md.) at 37°C in the presence of 5% CO_2. Cultures were periodically tested for contamination with *Mycoplasma* with a nucleic acid hybridization assay (Mycoplasma T.C. Rapid Detection System; Gen-Probe, Inc., San Diego, Calif.) at the Core *Mycoplasma* testing facility at the University of California, San Diego. Cells were collected by centrifugation at 200 × *g* for 10 min, and the pelleted cells were resuspended in fresh media before use in the various assays described in the study.

Isolation of human monocytes. Monocytes were isolated from human platelet pheresis cell packs obtained from the San Diego Blood Bank by Ficoll-Hypaque and Percoll gradient centrifugation as described previously (26). The purity of the monocytes by this method was greater than 70%. The isolated monocytes were cultured for 5 to 7 days in Iscove's modified Dulbecco's medium (BioWhittaker) supplemented with 10% normal human serum (NHS), 2 mM L-glutamine,

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and 50 U of penicillin-streptomycin per ml in Teflon beakers to yield MDMs. MDMs were further enriched by adherence to tissue culture plates before use in experiments. The purity of MDMs after the second adherence, as assessed by esterase staining and Giemsa staining, was >95%. Viability assessed by trypan blue exclusion was ≥97%.

***M. avium* culture and preparation of *M. avium* sonicate.** *M. avium* (ATCC 25291, serotype 2), obtained from the American Type Culture Collection, was cultured in Middlebrook 7H9 broth (Gibco Laboratories, Detroit, Mich.) at 37°C in the presence of 5% CO_2 for 7 to 10 days with vigorous agitation once a day. Bacteria were harvested by centrifugation at 900 × *g* for 20 min, washed, and used to prepare sonicated extracts as described in our earlier study (26). Briefly, freshly harvested *M. avium* cultures were washed three times with phosphate-buffered saline (PBS) and resuspended in PBS or RPMI 1640 without serum. The suspension was sonicated by a sonic dismembrator (model 550; Fisher Scientific, Tustin, Calif.) for 15 min with 1-min rests between 1-min bursts. The sonicate was centrifuged at 10,000 × *g* for 30 min at 4°C to remove cell debris. The supernatant was filtered through a 0.22-μm-pore-size membrane filter, estimated for protein (bicinchoninic acid protein assay reagent; Pierce Chemical Co., Rockford, Ill.), aliquoted, and stored at −70°C.

Ammonium sulfate precipitation of proteins from *M. avium* sonicate. Proteins in the *M. avium* sonicate were precipitated with ammonium sulfate (80%) by combining the sonicate with four volumes of saturated ammonium sulfate in PBS. The mixture was stirred overnight at 4°C and then centrifuged at 12,000 × *g* for 30 min. The precipitate was washed with 80% ammonium sulfate solution, resuspended, and extensively dialyzed against PBS. The volume was assessed, and the protein concentration was determined. The supernatant after collection of the ammonium sulfate-precipitated proteins was also dialyzed, lyophilized, and resuspended in a volume similar to that used to resuspend the precipitate. This fraction contained only trace amounts of protein (6 μg/ml).

Removal of endotoxin from *M. avium* sonicate. Endotoxin in *M. avium* sonicate was removed with Detoxi-Gel columns (Pierce Chemical Co.). Equilibration and elution were carried out with pyrogen-free saline. *M. avium* sonicate after chromatography on Detoxi-Gel columns was found to contain <0.028 ng of endotoxin/mg of sonicate, as determined by the amebocyte lysate assay (E-Toxate kit; Sigma Chemical Co., St. Louis, Mo.).

Preparation of purified *M. avium* proteins. *M. avium* 68- and 48- to 52-kDa proteins were purified from *M. avium* sonicate by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (28). Briefly, *M. avium* sonicate (6 to 8 mg of protein) was incubated with SDS-PAGE sample buffer for 2 h at 37°C and electrophoresed on 10% polyacrylamide gels under nonreducing conditions with the help of a Prep Cell preparative electrophoresis apparatus (model 491; Bio-Rad, Hercules, Calif.). Proteins were eluted from the gel with Tris-glycine buffer and collected as 8-ml fractions. A 400-μl aliquot of every third fraction was lyophilized and analyzed by SDS-PAGE to identify fractions containing the 68- and 48- to 52-kDa proteins. Fractions containing these proteins were pooled, extensively dialyzed against PBS, and lyophilized. The lyophilized fraction was desalted with Econo-Pac 10DG desalting columns (Bio-Rad, Richmond, Calif.), and the purity of the desalted protein was confirmed by SDS-PAGE. The purified proteins were tested for the presence of endotoxin and were found to contain <0.6 ng of endotoxin/ml at the concentration used in the experiments described in the present study. Purified proteins were aliquoted and stored at −20°C until further use.

Antibodies and other reagents. Monoclonal antibodies (MAbs) against TNF-α receptor I (TNF-αRI) (clone 16803.1), which are known to neutralize the biological effects of TNF-αRI without inducing apoptosis, were purchased from R&D Systems, Minneapolis, Minn. MAbs against TNF-α and mouse immunoglobulin G1 κ chain were purchased from Pharmingen, San Diego, Calif. All antibodies were free of azide and contained only trace amounts of endotoxin (<10 ng/mg). Pyrrolidinedithiocarbamate (PDTC), butylated hydroxyanisole (BHA), propidium iodide (PI), and *Escherichia coli* lipopolysaccharide (LPS) were purchased from Sigma Chemical Co.

Culture of cells with *M. avium* sonicate and *M. avium* proteins. THP-1 cells (2×10^5/ml) were cultured in the presence of *M. avium* sonicate at final concentrations of 600, 300, or 100 μg/ml in 96-well tissue culture plates for up to 7 days. Equal volumes of PBS or RPMI 1640 without serum were added to the control wells instead of the sonicate. In experiments with purified *M. avium* and *Mycobacterium leprae* proteins (*M. avium* 68- and 48- to 52-kDa proteins and *M. leprae* 18- and 65-kDa proteins), the protein concentration used was 25 μg/ml. The viability of cells was assessed on days 1, 2, 3, 5, and 7 by trypan blue exclusion. In the case of MDMs 2×10^5 to $\sim 3 \times 10^5$ cells were adhered to wells of a 24-well tissue culture plate. Nonadherent cells were removed by washing wells with prewarmed RPMI 1640, and adherent MDMs were cultured in RPMI 1640 (1 ml/well) containing 300 μg of *M. avium* sonicate per ml for up to 7 days. Heat-inactivated NHS was used instead of FBS for culture of MDMs.

Culture of cells with *M. avium* sonicate in the presence of antioxidant. THP-1 cells (2×10^5/ml) were cultured in the presence or absence of *M. avium* sonicate (300 μg/ml) or purified mycobacterial proteins (25 μg/ml) in 96-well tissue culture plates for 2 days. To some cells exposed to *M. avium* sonicate, PDTC (100 μM) was added simultaneously. Another batch of cells were first pretreated with BHA (200 μM) for 2 h and then exposed to *M. avium* sonicate. On day 2, the viability of cells exposed to *M. avium* sonicate or purified mycobacterial proteins alone as well as in the presence and absence of PDTC or BHA was assessed by trypan blue exclusion.

Culture of cells with *M. avium* sonicate in the presence of MAbs against TNF-αRI and TNF-α. THP-1 cells were cultured in the presence or absence of *M. avium* sonicate in 96-well tissue culture plates for 2 days as described above. To neutralize TNF-αRI, cells were preincubated with MAbs specific for TNF-αRI (50 μg/ml) at 37°C for 1 h before addition of *M. avium* sonicate. Cells incubated with normal mouse IgG at the same concentration served as a control. In the case of TNF-α, MAbs against this molecule (50 μg/ml) were added immediately before addition of *M. avium* sonicate. The cells were incubated at 37°C for 2 days, and the viability was assessed by trypan blue exclusion.

Assessment of apoptosis and apoptosis-associated events. Apoptosis and apoptosis-associated changes were assessed by the following methods.

(i) Determination of cell viability. The viability of THP-1 cells exposed to *M. avium* sonicate in the presence and absence of other agents such as antioxidants and TNF-αRI- or TNF-α-specific MAbs was assessed by trypan blue exclusion with the help of a hemocytometer. An aliquot of the cells was mixed with an equal volume of 0.4% trypan blue solution (Sigma Chemical Co.) and observed under a light microscope. At least 100 cells were counted, and the number of viable cells that excluded trypan blue was determined. The results were expressed as percent viability.

(ii) Morphological change. Nuclear morphology was studied by phase-contrast microscopic examination of PI-stained cells. *M. avium* sonicate-treated cells (300 μl) were centrifuged at 400 × *g*, washed with PBS, and fixed with 70% ethanol for 1 h at 4°C. Fixed cells were washed with PBS, collected by centrifugation at 900 × *g*, and resuspended in 10 μl of PI solution (25 μg/ml in PBS). Two microliters of the stained cell suspension was dropped onto a glass slide and observed under a fluorescence microscope equipped with a rhodamine-fluorescein isothiocyanate (FITC) filter set. In the case of MDMs, 10^5 cells adhered to Lab-Tek plates were exposed to *M. avium* sonicate, washed, fixed with an acetone-methanol mixture (1:1) [vol/vol]), stained with PI, and observed as described above.

(iii) Detection of DNA fragmentation by agarose gel electrophoresis. DNA was isolated from *M. avium* sonicate-treated cells as described previously, but with minor modifications (22). Cells were harvested by centrifugation (200 × *g*) and washed with PBS. In the case of MDMs, culture supernatants of *M. avium* sonicate-exposed cells were first collected and then combined with the adhered cells, which were recovered with the help of a cell scraper. The cells were collected by centrifugation and lysed with 300 μl of lysis buffer (0.5% Triton X-100, 10 mM Tris-HCl [pH 8.0], 10 mM EDTA). The lysate was centrifuged at 10,000 × *g* for 30 min to remove cell debris and high-molecular-weight DNA. The supernatant containing low-molecular-weight DNA was placed in a fresh tube, treated with RNase A (Sigma Chemical Co.) at a final concentration of 0.4 μg/ml, and incubated at 37°C for 30 min. Proteinase K (Sigma Chemical Co.) was then added at a final concentration of 0.4 μg/ml and incubated for 1 h. DNA was precipitated overnight at −20°C in 50% isopropanol and 0.5 M NaCl. The precipitate was pelleted, washed with 70% ethanol, and air dried. The DNA pellet was resuspended in Tris-EDTA solution (10 mM Tris-HCl [pH 8.0], 1 mM EDTA), analyzed by agarose gel electrophoresis (2%), and detected by ethidium bromide staining. A 100-bp DNA ladder (Gibco Laboratories) was used as a marker. The presence or absence of fragmented DNA in different samples was visualized under UV illumination.

(iv) Assay for release of NMP. For a quantitative analysis of apoptosis, soluble nuclear matrix protein (NMP) in culture medium was quantitated by enzyme-linked immunosorbent assay (ELISA). Cells were incubated with different concentrations of *M. avium* sonicate. At various time points (days 1, 2, and 3), the culture supernatant was collected after centrifugation to remove cells (200 × *g*, 15 min) and analyzed with NMP ELISA kits (Oncogene Research Products, Cambridge, Mass.) according to the manufacturer's instructions.

(v) TUNEL assay. Individual cell apoptosis was demonstrated by the terminal deoxynucleotidyl transferase-mediated d-UTP nick end labeling (TUNEL) assay. THP-1 cells treated with *M. avium* components were centrifuged at 400 × *g* and washed with PBS. Cell smears were prepared on glass slides. In the case of MDMs, cells were adhered to Lab-Tek plates and exposed to *M. avium* sonicate. Cells were then washed with PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Samples were labeled with an in situ cell death detection kit (fluorescein; Boehringer, Mannheim, Germany) and observed under a fluorescence microscope. In some cases, cells were subsequently counterstained with PI to examine nuclear morphological changes.

Statistical analysis. Results are expressed as means ± standard deviations. Statistical differences were determined with Student's *t* test or the Mann-Whitney rank sum test. A *P* value below 0.05 was considered to be statistically significant.

RESULTS

Cytotoxic effect of *M. avium* sonicate on THP-1 cells. The cytotoxic effect of *M. avium* sonicate on THP-1 cells was assessed by trypan blue exclusion. Cells (2×10^5/ml) were incubated with various concentrations of *M. avium* sonicate up to 7

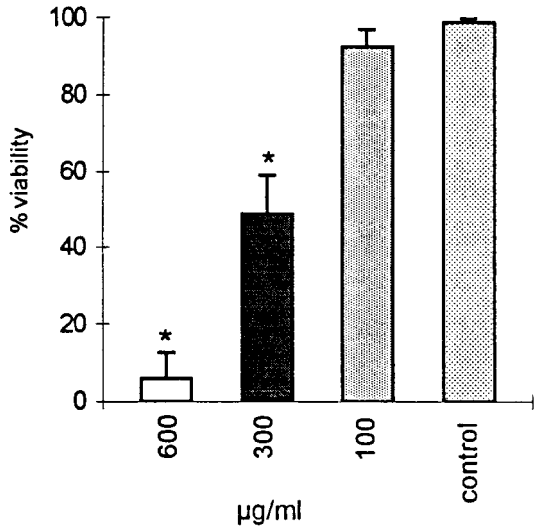

FIG. 1. Effect of *M. avium* sonicate on viability of THP-1 cells. Cells were incubated with 600, 300, or 100 μg of *M. avium* sonicate per ml at 37°C. Control cells were incubated with culture medium without any sonicate. The viability of the cells was determined by trypan blue exclusion on day 2. Results are expressed as means ± standard deviations of four experiments in duplicate. *, $P < 0.001$.

days, and their viability was assessed at different time points. Maximum cell death was observed by day 3, with no further changes up to day 7. *M. avium* sonicate was found to reduce the viability of these cells in a dose-dependent manner (Fig. 1). On day 2, the viabilities of *M. avium* sonicate-treated THP-1 cells were 6.6% ± 5.2% at 600 μg/ml ($P = 0.0005$), 48.8% ± 10.3% at 300 μg/ml ($P = 0.005$), and 92.5% ± 4.4% at 100 μg/ml ($P > 0.05$) compared to control THP-1 cells, which showed a viability of 98.8% ± 1.0%.

Cytotoxic effect of various preparations of *M. avium* sonicate. In order to identify the causative agent in the *M. avium* sonicate responsible for cytotoxicity of THP-1 cells, the sonicate was subjected to ammonium sulfate precipitation as described in Materials and Methods. The ammonium sulfate-precipitated fraction of *M. avium* sonicate (*M. avium* sonicate-AS-Ppt) and the resulting supernatant (*M. avium* sonicate-AS-Sup) were tested in the cytotoxicity assay. The viability of cells incubated with 300 μg of *M. avium* sonicate-AS-Ppt per ml was 47.4% ± 19.6% identical to that of cells treated with *M. avium* sonicate before ammonium sulfate precipitation (48.8% ± 10.3%). THP-1 cells treated with *M. avium* sonicate-AS-Sup exhibited a viability of 94.8% ± 5.0%, similar to that of control untreated cells (Fig. 2a).

Endotoxin at a concentration of 0.1 μg/ml is known to induce cytotoxic effects in monocytic cells (2). Therefore, to remove endotoxin, the *M. avium* sonicate was passed through a Detoxi-Gel column and compared with native *M. avium* sonicate with respect to its effect on cell viability (Fig. 2b). The endotoxin contents of *M. avium* sonicate before and after treatment with Detoxi-Gel were 0.24 and 0.03 ng per milligram (0.08 and 0.01 ng/300 μg of *M. avium* sonicate), respectively. The viability of THP-1 cells incubated with 300 μg of Detoxi-Gel-treated *M. avium* sonicate per ml was found to be identical to that of cells incubated with the same concentration of native *M. avium* sonicate (47.4% ± 5.2% and 48.8% ± 10.3%, respectively).

The effect of the *M. avium* 68- and 48- to 52-kDa proteins on the viability of THP-1 cells was also tested (Fig. 2c). The 68-kDa protein has previously been shown to play a role in the attachment of *M. avium* to human macrophages (27). In the present study, this protein was found to be highly cytotoxic to THP-1 cells. Cells incubated with purified *M. avium* 68-kDa protein (25 μg/ml) for 2 days exhibited a viability of 26.2% ± 5.9% compared to control cells, which were 98.8% ± 1.0% viable. Cells incubated with *M. avium* 48- to 52-kDa protein at the same concentration exhibited a viability of 83.4% ± 1.0%.

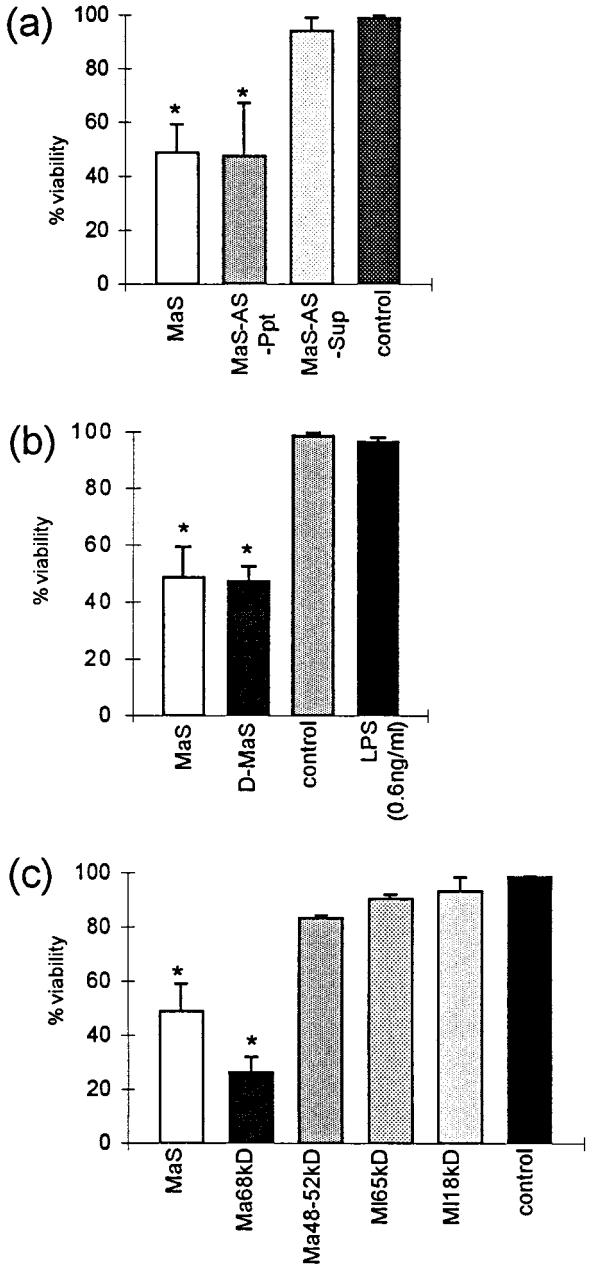

FIG. 2. Cytotoxic effect of *M. avium* sonicate (MaS) (a), endotoxin-free *M. avium* sonicate (b), and purified *M. avium* proteins (c) on THP-1 cells. Cells were incubated with *M. avium* sonicate 80% ammonium sulfate precipitate of *M. avium* sonicate (MaS-AS-Ppt), 80% ammonium sulfate supernatant of *M. avium* sonicate (MaS-AS-Sup), Detoxi-Gel-treated *M. avium* sonicate (D-MaS), *M. avium* 68-kDa protein (Ma68kD), *M. avium* 48- to 52-kDa protein (Ma48-52kD), *M. leprae* 65-kDa protein (Ml65kD), *M. leprae* 18-kDa protein (Ml18kD), media alone (control), or LPS (0.6 ng/ml) for 2 days at 37°C. Cell viability was determined by trypan blue exclusion. Results are expressed as means ± standard deviations of four experiments in duplicate. *, $P < 0.01$.

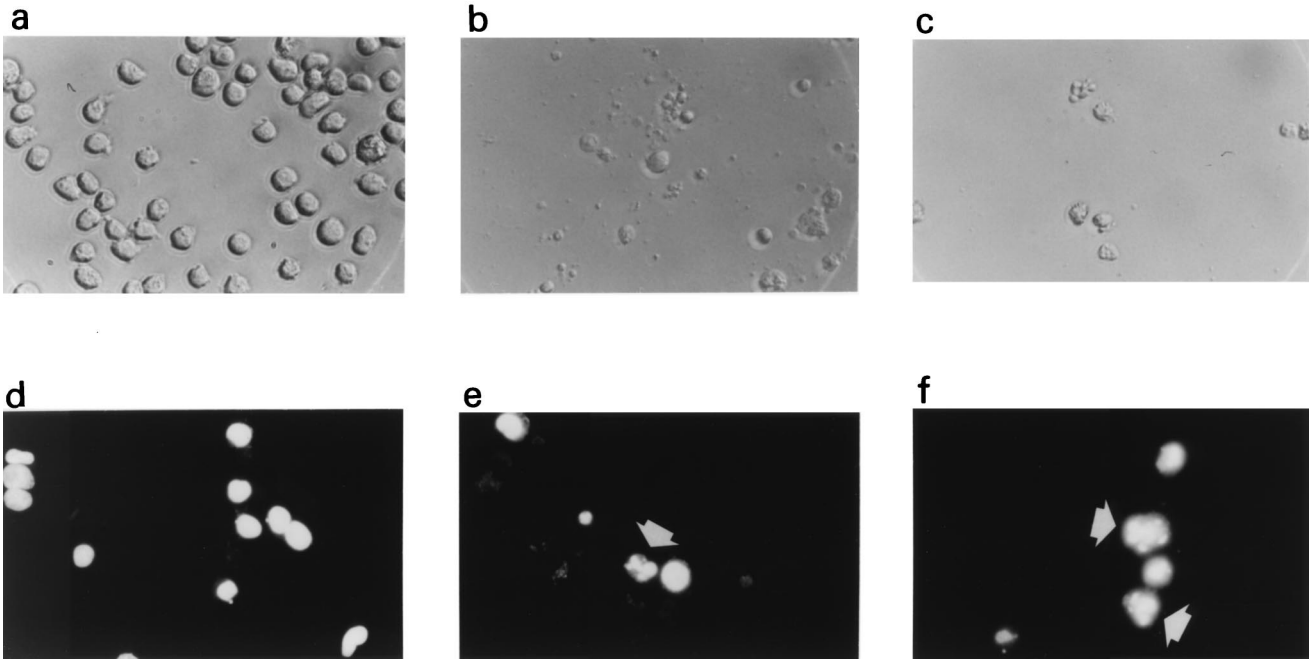

FIG. 3. Effect of *M. avium* sonicate and *M. avium* 68-kDa protein on the morphology of THP-1 cells. Cells were cultured at 37°C for 2 days in the presence of *M. avium* sonicate (300 μg/ml) or *M. avium* 68-kDa protein (25 μg/ml) and examined under a phase-contrast microscope (upper panel). Cells in some wells were fixed, stained with PI, and examined with a rhodamine-FITC filter set (lower panel). Cells cultured in medium without sonicate or 68-kDa protein served as a control. a and d, control THP-1 cells; b and e, THP-1 cells treated with *M. avium* sonicate; c and f, THP-1 cells treated with *M. avium* 68-kDa protein (photographed at a magnification of ×600). Arrows indicate apoptotic cells.

The *M. leprae* 65- and 18-kDa proteins which were used as controls had very little effect on the viability of THP-1 cells. Cells incubated with these proteins exhibited viabilities of 90.5% ± 1.5% and 93.3% ± 5.1%, respectively. Since *M. avium* 68-kDa protein was found to contain trace amounts of endotoxin (0.6 ng/ml), we examined the cytotoxic effect of endotoxin at various concentrations (1.0 to 0.1 ng/ml). The viability of cells incubated with 0.6 ng of *E. coli* LPS per ml (96.5% ± 1.6%) was found to be similar to that of control cells (98.8% ± 1.0%) (Fig. 2b).

The results described above suggest that the cytotoxic agent(s) present in *M. avium* sonicate is proteinaceous in nature, with all of the cytotoxic activity being retained in the ammonium sulfate-precipitated fraction. The cytotoxic effect of *M. avium* sonicate was not due to endotoxin present in the sonicate. Finally, *M. avium* 68-kDa protein may be one of several agents present in the sonicate that are cytotoxic to monocytic cells.

THP-1 cells exposed to *M. avium* sonicate undergo apoptosis. In order to determine whether the cytotoxic effect of *M. avium* sonicate described above was due to apoptosis, cells exposed to *M. avium* sonicate were evaluated for apoptotic markers.

(i) Morphology. THP-1 cells with and without exposure to *M. avium* sonicate (300 μg/ml) were examined under a phase-contrast microscope. Control cells had a well-defined, almost spherical, smooth plasma membrane (Fig. 3a). In contrast, the cells incubated with *M. avium* sonicate (Fig. 3b) or *M. avium* 68-kDa protein (Fig. 3c) were found to have an irregular shape and decreased cell size. Furthermore, individual cells appeared as clusters of small, round bodies characteristic of apoptosis. Nuclear changes were examined under a fluorescence microscope after the cells had been stained with PI, which specifically stains nucleic acids. *M. avium* sonicate- and *M. avium* 68-kDa protein-treated cells (Fig. 3e and f, respectively) showed chromatin condensation, which was absent in control cells (Fig. 3d).

These findings indicate that THP-1 cells exposed to *M. avium* sonicate exhibit morphological features characteristic of apoptosis.

(ii) DNA fragmentation by agarose gel electrophoresis. Since fragmentation of cellular DNA into low-molecular-weight oligomers is characteristic of apoptosis, the effect of *M. avium* sonicate on THP-1 cellular DNA was evaluated by agarose gel electrophoresis (Fig. 4A). DNA isolated from cells incubated with *M. avium* sonicate and *M. avium* sonicate-AS-Ppt for 2 days showed distinctive DNA ladder formation, with bands ranging from 150 to 1,500 bp (Fig. 4A, lanes 2 and 3), while there was no evidence of DNA fragmentation in control cells (Fig. 4A, lane 1). However, THP-1 cells exposed to *M. avium* 68-kDa protein were found to contain only a single band of fragmented DNA (800 bp), in contrast to the distinctive DNA laddering observed with *M. avium* sonicate (Fig. 4B, lane 2).

(iii) NMP ELISA. Cells undergoing apoptosis are known to release NMP (21). NMP in the culture supernatant of THP-1 cells incubated with *M. avium* sonicate for up to 3 days was quantitated by ELISA (Fig. 5). The amount of NMP in the culture supernatant of *M. avium* sonicate-treated cells was significantly larger than that released by control cells. Furthermore, the amount of NMP released into the culture supernatant by these cells increased with time. Cells exposed to *M. avium* sonicate after removal of endotoxin, *M. avium* sonicate-AS-Ppt, and *M. avium* 68-kDa protein were also found to release NMP (Table 1).

(iv) TUNEL assay. To further demonstrate that THP-1 cells treated with *M. avium* components contain nuclei with fragmented DNA, cells incubated with *M. avium* sonicate or purified *M. avium* 68-kDa protein were assessed with the TUNEL

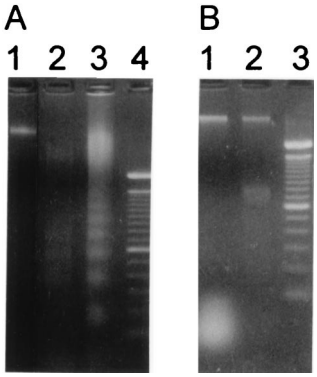

FIG. 4. DNA fragmentation of *M. avium* sonicate- and *M. avium* 68-kDa-treated THP-1 cells. Cells were incubated with *M. avium* sonicate (300 μg/ml) or *M. avium* 68-kDa protein (25 μg/ml) for 2 days, lysed, and used to isolate DNA. The isolated DNA was subjected to agarose gel electrophoresis (2%). (A) Lanes: 1, control THP-1 cells; 2, THP-1 cells treated with 80% ammonium precipitate of *M. avium* sonicate; 3, THP-1 cells treated with *M. avium* sonicate; 4, 100-bp ladder. (B) Lanes: 1, control THP-1 cells; 2, THP-1 cells treated with *M. avium* 68-kDa protein; 3, 100-bp ladder.

assay (Fig. 6). A large number of *M. avium* sonicate-treated (Fig. 6b) and *M. avium* 68-kDa protein-treated (Fig. 6c) cells were found to take up the label, while very few control cells were positive (Fig. 6a).

This observation further substantiates the findings presented above, confirming that THP-1 cells undergo apoptosis when treated with *M. avium* sonicate or *M. avium* 68-kDa protein.

Inhibition of cytotoxic effect of *M. avium* sonicate and *M. avium* 68-kDa protein. Oxidative stress is known to be a common mediator of apoptosis (3). Antioxidants have been reported to prevent apoptosis induced by various stimuli (31). We therefore examined whether antioxidants could inhibit *M. avium* sonicate-induced apoptosis of THP-1 cells. PDTC and BHA, which are commonly used antioxidants, were found to inhibit the cytotoxic effect of *M. avium* sonicate. The viability of THP-1 cells incubated with *M. avium* sonicate (300 μg/ml) for 2 days in the presence of 100 μM PDTC (89.1% ± 4.6%; $P < 0.001$) and of 200 μM BHA (71.2% ± 5.5%; $P < 0.001$) was partially restored to that observed for control cells (98.8% ± 1.0%), while the viability of cells treated with *M. avium* sonicate in the absence of antioxidants was 48.6% ± 8.3%. PDTC was found to be more effective in inhibiting the cytotoxic effect of *M. avium* sonicate. In addition, the viability of THP-1 cells incubated with 25 μg of *M. avium* 68-kDa protein per ml in the presence of PDTC and BHA at the concentrations presented above was also partially restored (74.6% ± 7.0% and 71.0% ± 5.3%, respectively; $P < 0.001$) to that observed in the case of control cells, which are not exposed to this protein (98.8% ± 1.0%). The viability of cells treated with *M. avium* 68-kDa protein in the absence of antioxidants was 34.6% ± 9.2%.

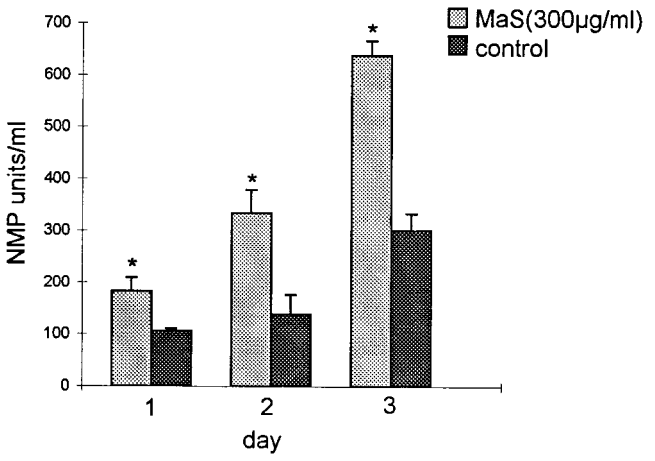

FIG. 5. Kinetics of NMP release by *M. avium* sonicate-treated THP-1 cells. Cells were incubated with 300 μg of *M. avium* sonicate (MaS) per ml for 3 days at 37°C. Cells cultured in medium without *M. avium* sonicate served as control. On days 1, 2, and 3, the culture supernatant from appropriate wells was collected and assayed for NMP by ELISA. Results are expressed as means ± standard deviations of four experiments performed in duplicate. *, $P < 0.001$.

TABLE 1. NMP released by THP-1 cells and MDMs incubated with various preparations of *M. avium* sonicate

Cell type	Condition[a]	NMP released (U/ml)[b]
THP-1	Control	94.7 ± 33.9
	MaS (300 μg/ml)	327.4 ± 50.3*
	68-kDa protein	290.1 ± 56.5*
	MaS (300 μg/ml) + PDTC (100 μM)	173.7 ± 56.3**
	MaS (300 μg/ml) + BHA (200 μM)	243.4 ± 56.8**
	68-kDa protein + PDTC (100 μM)	174.4 ± 15.5**
	68-kDa protein + BHA (200 μM)	263.9 ± 47.4
MDMs	Control	109.7 ± 46.6
	MaS (300 μg/ml)	288.3 ± 106.1*
	MaS (300 μg/ml) + PDTC (100 μM)	312.9 ± 97.9***
	MaS (300 μg/ml) + BHA (200 μM)	399.7 ± 96.2***

[a] Cells were incubated with *M. avium* sonicate (MaS) or *M. avium* 68-kDa protein in the presence or absence of an antioxidant at 37°C for 2 days. The amount of NMP released in the culture supernatant was measured by ELISA.

[b] Data for THP-1 cells are expressed as means ± standard deviations of three independent experiments in duplicate. Data pertaining to MDMs are expressed as means ± standard deviations of four different experiments with MDMs from four different donors. *, $P < 0.03$ compared with control; **, $P < 0.008$ compared with cells cultured with *M. avium* sonicate in the absence of antioxidants; ***, $P > 0.1$ compared with cells cultured with *M. avium* sonicate in the absence of antioxidants.

Inhibition of apoptosis was accompanied by a reduction in the amount of NMP released into the culture supernatant. The amount of NMP released by cells treated with *M. avium* sonicate in the presence of PDTC and BHA was significantly reduced compared to that released by cells incubated with *M. avium* sonicate alone (Table 1). However, while PDTC was found to reduce the release of NMP by cells incubated with the 68-kDa protein, BHA did not inhibit the release of NMP from those cells.

THP-1 cells are known to produce TNF-α in response to mycobacteria (35). Furthermore, TNF-α at 5 ng/ml is known to induce cell death and DNA fragmentation in THP-1 cells (33). Therefore, we investigated the potential role of TNF-α in *M. avium* sonicate-induced apoptosis of THP-1 cells by using eutralizing MAbs against TNF-α and TNF-αRI. MAbs against both molecules did not reverse apoptosis induced by *M. avium* sonicate despite the high concentration (50 μg/ml) of antibodies used in our studies (data not shown) compared to those in studies by other investigators (14).

These results suggest that *M. avium* sonicate-induced apoptosis of THP-1 cells may be due to the generation of free oxygen radicals.

***M. avium* sonicate promotes apoptosis of MDMs.** To determine whether *M. avium* sonicate induces apoptosis in MDMs, we assessed the morphological changes brought about in these cells by exposure to *M. avium* sonicate. Adherent MDMs ap-

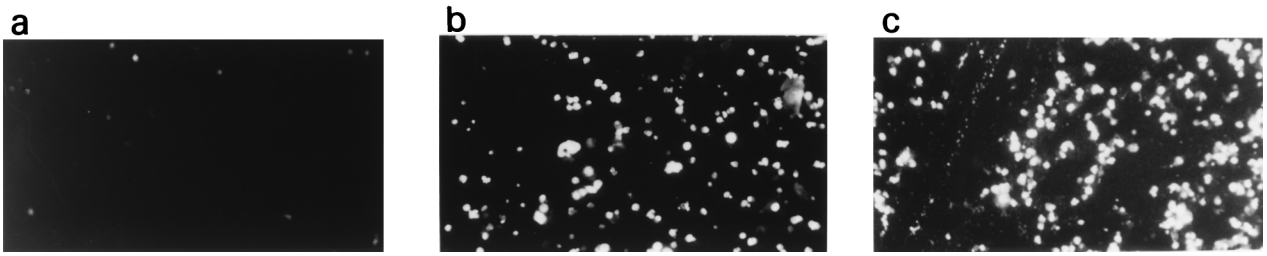

FIG. 6. TUNEL assay of THP-1 cells treated with *M. avium* sonicate or purified 68-kDa protein. Cells were cultured at 37°C for 2 days in the absence (a) or presence (b) of *M. avium* sonicate (300 μg/ml) or *M. avium* 68-kDa protein (25 μg/ml) (c) and assessed by the TUNEL assay (photographed at a magnification of ×150).

peared as clumps and were found to detach from wells (Fig. 7b). Control cells, on the other hand, appeared as a uniform adherent monolayer with no clumps (Fig. 7a). The nuclear morphology on day 2 assessed by PI staining showed a decrease in the size of the nucleus (Fig. 7d) compared to that in control cells (Fig. 7c).

M. avium sonicate-treated cells were found to contain fragmented DNA when analyzed by the TUNEL assay. As seen in the case of THP-1 cells, a large number of the sonicate-treated MDMs were found to take up the stain (Fig. 7f) compared to control cells (Fig. 7e). Since MDMs showed a decrease in the size of the nucleus, but did not exhibit chromatin condensation, as seen in the case of THP-1 cells, the cells were counterstained with PI to differentiate the nuclear morphology of TUNEL-positive cells versus TUNEL-negative cells after the TUNEL assay had been performed (Fig. 8). Most of the MDMs in clumps were found to be TUNEL positive, appearing yellow and exhibiting decreased nuclear size (Fig. 8b) compared to control cells, which were TUNEL negative and appeared red (Fig. 8a).

NMP release by MDMs in the presence of *M. avium* sonicate was compared to that of control MDMs up to 3 days. Concentration of NMP in the culture supernatant of MDMs incubated with *M. avium* sonicate was significantly elevated ($P < 0.04$) compared to that of control cells (Fig. 9). MAbs against TNF-α and TNF-αRI (50 μg/ml each) did not reverse NMP release by MDMs incubated with *M. avium* sonicate (data not shown). Furthermore, release of NMP by MDM was not inhibited by PDTC and BHA, as seen in the case of THP-1 cells (Table 1). In addition, there was no evidence of DNA fragmentation on agarose gel electrophoresis. These results suggest that although *M. avium* sonicate appears to induce apoptosis of MDM, it is likely that the pathway by which it occurred is different from that observed with THP-1 cells.

DISCUSSION

Infection of a cell by a pathogen may follow one of two events: containment of the infection within the infected cells, favoring the host, or spread of infection from the infected cells to other cells, favoring the pathogen. In recent years, there have been several reports of pathogens inducing cell death or apoptosis of host cells. In most instances, it is unclear whether this process favors the host or the pathogen. Depending on the pathogen, apoptosis may help contain the infection by depriving the pathogen of an intracellular sanctuary or it may help in enhancing the infection by uptake of infected apoptotic bodies by noninfected phagocyte cells. Previous studies by Gan et al. (10) have demonstrated that *M. avium* can induce apoptosis of MDMs. In the present study, we have further added to these observations by identifying *M. avium* components that play a role in this process. We have also identified a probable mechanism by which these components induce apoptosis of monocytes.

Pathogen-induced apoptosis can occur either directly after entry of bacteria or bacterial toxin or ligand(s) into the host cell or indirectly by binding to a receptor on the cell and subsequent activation of second messengers within the cell (36). THP-1 cells and MDMs exposed to *M. avium* sonicate or *M. avium* 68-kDa protein show significant reduction in viability compared to nonexposed control cells. A further evaluation revealed that this effect was due to cells undergoing apoptosis, which was characterized by morphological changes, nuclear degradation, DNA fragmentation by TUNEL, and release of NMP. The 68-kDa protein of *M. avium* has previously been shown to play a role in attachment of the organism to MDM via the integrin receptor $\alpha_V\beta_3$ on these cells (11, 26). From additional studies in our laboratory, it is known that the 68-kDa protein is not secreted (data not shown). These data suggest that uptake of the organism may not be required for apoptosis to be initiated and that the 68-kDa protein may initiate receptor-mediated apoptosis by binding to host cell surface receptors.

Apoptosis is characterized by specific morphological and nuclear changes within the cell. Release of NMP is specific to apoptotic, but not necrotic cells (21). Assay of NMP in culture supernatants has previously been used as a direct measure of the number of apoptotic cells in culture (21). NMP is insoluble and tightly bound to chromatin in intact cells and is only released into the medium in a soluble form during early apoptosis (21). Both *M. avium* sonicate and the *M. avium* 68-kDa protein induced NMP release by THP-1 cells as well as MDMs. With MDMs, exposure to *M. avium* sonicate showed evidence of DNA fragmentation by TUNEL assay, but did not show evidence of DNA fragmentation on agarose gel electrophoresis, while THP-1 cells exposed to the sonicate exhibited the characteristic DNA ladder formation. Certain cell types are known to undergo apoptosis in the absence of intrachromosomal DNA fragmentation or with fragmentation of DNA into larger 50- to 300-kb oligomers and chromatin condensation, which are not detectable by conventional agarose gel electrophoresis (6, 25).

M. avium sonicate was found to significantly reduce the viability of THP-1 cells in a dose-dependent manner at 300 and 600 μg/ml, although maximum NMP release was observed at 300 μg/ml, with no further increase at 600 μg/ml (data not shown). Analysis of cells incubated with 600 μg of *M. avium* sonicate per ml by agarose gel electrophoresis showed a band of DNA corresponding to very low-molecular-weight DNA fragments rather than a DNA ladder (data not shown). These findings allow us to speculate that at high concentrations (600 μg/ml) of *M. avium* sonicate, cells may undergo rapid and extensive damage of the plasma membrane, resulting in necrosis rather than apoptosis, which was observed at the lower

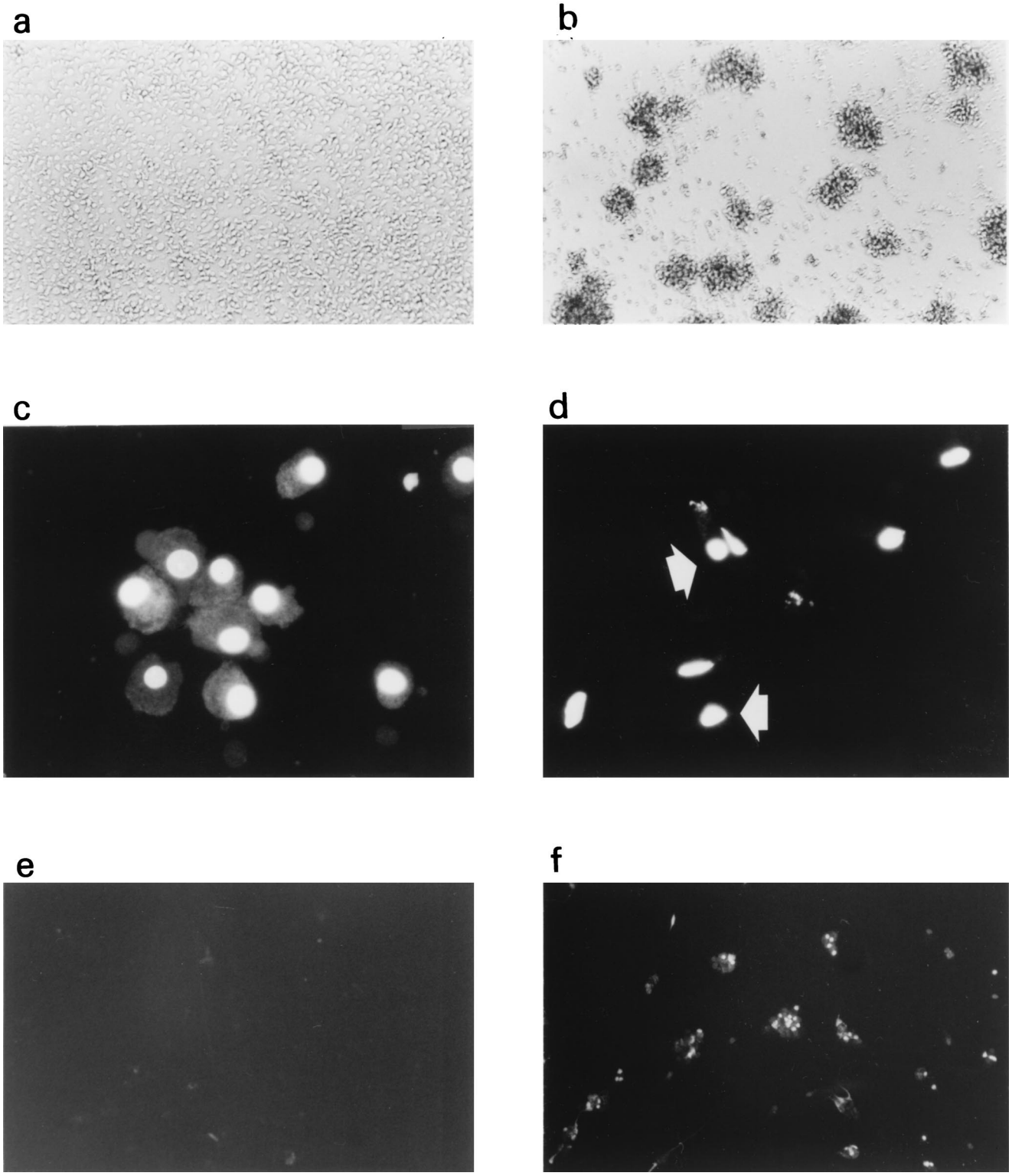

FIG. 7. Effect of *M. avium* sonicate on the morphology of MDMs. Cells were cultured at 37°C for 2 days in the presence of *M. avium* sonicate (300 μg/ml) and examined under a phase-contrast microscope (upper panel). Cells in some wells were fixed, stained with PI, and examined with a rhodamine-FITC set filter (middle panel). Cells in other wells were fixed, assessed by the TUNEL assay, and examined with an FITC filter (lower panel). a, c, and e, control MDMs; b, d, and f, MDMs treated with *M. avium* sonicate. Arrows indicate apoptotic cells (photographed at a magnification of ×150 (a, b, e, and f) or ×600 (c and d).

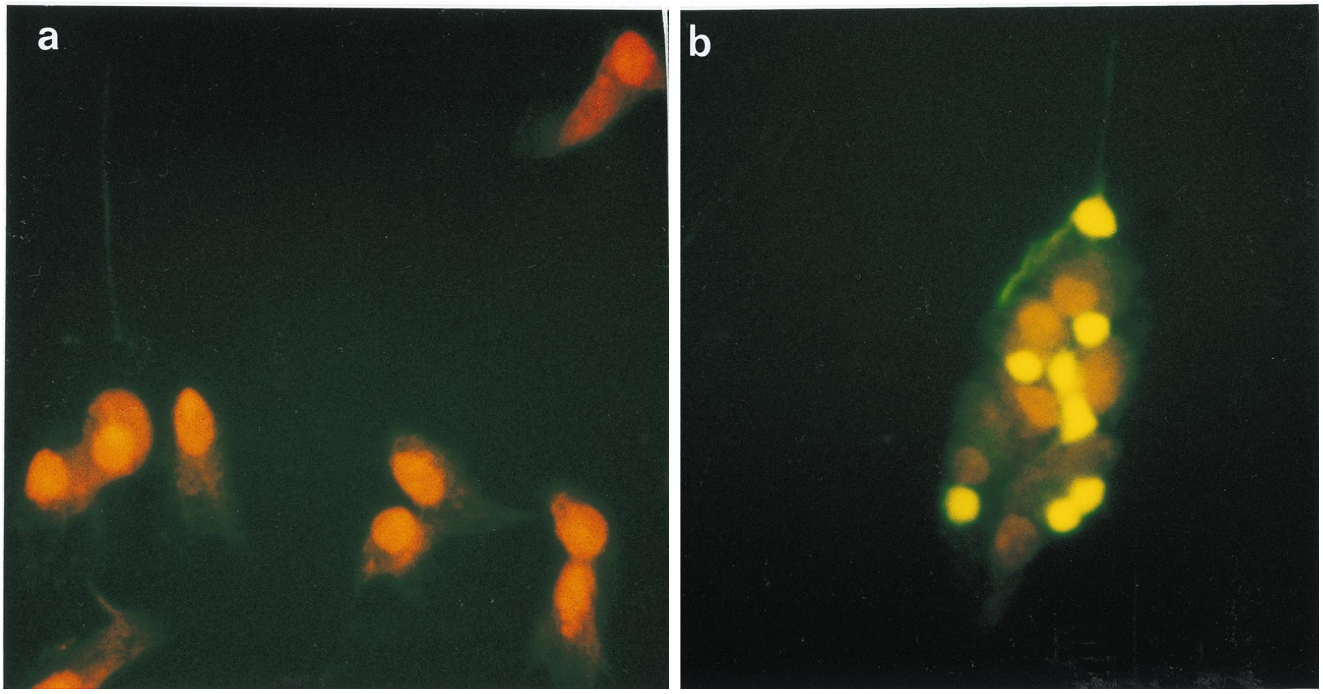

FIG. 8. TUNEL assay of *M. avium* sonicate-treated MDMs. Cells were cultured at 37°C for 2 days in the presence of *M. avium* sonicate (300 μg/ml), assessed by the TUNEL assay, and counterstained with PI. Samples were examined with a rhodamine-FITC set filter. (a) Control MDMs; (b) MDMs treated with *M. avium* sonicate. Apoptotic nuclei in the *M. avium* sonicate-treated MDMs can be identified by the yellow fluorescence. Cells which are not apoptotic can be identified by the red nuclear staining (photographed at a magnification of ×600).

concentration (300 μg/ml) used in this study. Certain bacterial components, such as pore-forming toxins, are known to exert this type of effect, inducing necrosis at high concentrations and apoptosis at lower concentrations (13, 19). *M. avium* sonicate, which is a mixture of several molecules, may contain toxin-like components capable of inducing necrosis and apoptosis, depending on the concentration.

Bacterium-induced apoptosis is a relatively new concept that is being actively investigated. As described earlier, the *M. avium* 68-kDa protein which mediates attachment of the organism to MDM is also capable of inducing apoptosis. In the recent past, there have been several reports of bacterial components or toxins that induce apoptosis in host cells (4, 29). These include adenylate cyclase-hemolysin of *B. pertussis* (15), diphtheria toxin of *Corynebacterium diphtheriae* (16), *Pseudomonas aeruginosa* exotoxin A (16), *Clostridium difficile* toxin (17), and the invasion antigen of *S. flexneri* (5). In each case, the mechanism is distinct from the other. In the case of bacteria such as *S. flexneri* (37) and *L. interrogans* (20), apoptosis is induced after entry into host cells. Previous studies have shown that apoptosis by bacterial components can be mediated by direct activation of enzymes which induce apoptosis (5) or by stimulation of the production of cytokines which are involved in apoptosis (18). It is likely that in the case of *M. avium*, attachment to MDMs is required for induction of apoptosis, since the 68-kDa protein is not secreted by the organism. The 68-kDa protein of *M. avium* may activate second messengers after attachment to host cell surface receptors or after entry of the organism into the cell.

Both *M. avium* sonicate and *M. avium* 68-kDa protein have been shown to induce the production of TNF-α by MDMs (28). This cytokine has been implicated in the apoptosis of monocytes as well as MDMs when added exogenously to cultures (14, 33). However, in our studies, *M. avium* sonicate-induced apoptosis of THP-1 cells did not appear to be mediated by TNF-α. Experiments using neutralizing MAbs against TNF-αRI or TNF-α at concentrations 10-fold higher than that used in other similar studies (14) did not inhibit apoptosis in THP-1 cells. On the other hand, PDTC and BHA, commonly used antioxidants, were found to prevent this process. In the presence of PDTC and BHA, the viability of *M. avium* sonicate-treated THP-1 cells and release of NMP in the culture medium by these cells were partially reversed to mimic what was observed with control cells. On the other hand, the cyto-

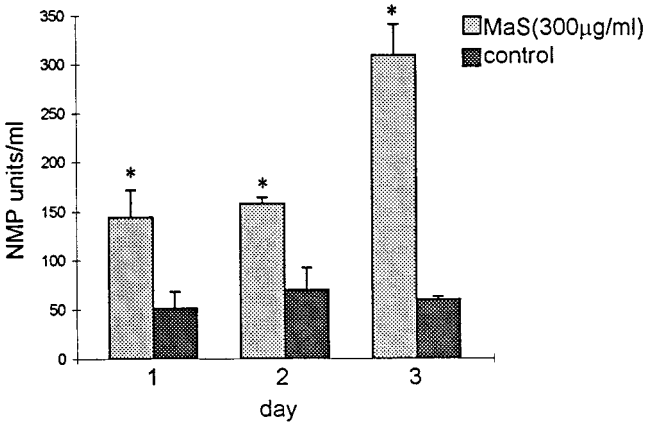

FIG. 9. Kinetics of NMP release by MDMs cultured with *M. avium* sonicate. Adherent MDMs were incubated with 300 μg of *M. avium* sonicate (MaS) per ml for up to 3 days at 37°C. Cells cultured in medium without *M. avium* sonicate served as a control. On days 1, 2, and 3, the culture supernatant was collected and assayed for NMP by ELISA. The data shown represent one of four donors studied. *, $P < 0.04$.

toxicity of cells treated with *M. avium* 68-kDa protein and release of NMP in the culture medium by these cells were inhibited by PDTC, but not by BHA. *M. avium* sonicate is a mixture of several components, more than one of which could be cytotoxic to THP-1 cells. It is likely that PDTC and BHA inhibit cytotoxicity mediated by different components present in the sonicate. With the 68-kDa protein on the other hand, cytotoxicity mediated by a single purified component is inhibited by a single agent (PDTC, not BHA). PDTC has been shown to inhibit apoptosis by various stimuli, such as UV irradiation, H_2O_2, and actinomycin D in the human leukemia cell line HL-60 (31). It is also known to be a potent scavenger of nitrogen radicals (30). BHA, on the other hand, is reported to be an oxygen radical scavenger (30). These results suggest that *M. avium* sonicate-induced apoptosis of THP-1 cells may be mediated by generation of free oxygen or nitrogen radicals. Surprisingly, apoptosis of MDMs was not inhibited by PDTC and BHA. This finding, together with the observation that *M. avium* sonicate-induced apoptosis of MDMs is not accompanied by DNA fragmentation on agarose gel electrophoresis, as seen in THP-1 cells, is suggestive of different mechanisms of apoptosis for THP-1 cells and MDMs.

The role of apoptosis in the pathogenesis of *M. avium* infection remains to be understood. It is still unclear whether apoptosis of host cells by pathogens is beneficial to the host or the pathogen. *M. avium*-induced apoptosis may favor the pathogen by inhibiting host immune responses, enabling bacterial survival by reducing the number of immunoregulatory cells. On the other hand, *M. avium*-induced apoptosis of MDMs may be a part of normal immune responses depriving the pathogen of a sanctuary and restricting bacterial replication. In addition, apoptosis of MDMs might play a role in removing unwanted cells producing proinflammatory cytokines induced by the pathogen which might cause local tissue damage. Macrophages are known to phagocytose cells undergoing apoptosis (8). Apoptotic bodies containing this organism formed as a result of cell death may be taken up and killed by other healthy noninfected MDMs, thereby limiting the spread of infection. These data are provocative and serve as a good foundation for additional studies with MDMs from human immunodeficiency virus-infected individuals. Studies to determine whether MDMs from these individuals undergo apoptosis similar to that seen with MDMs from non-human immunodeficiency virus-infected individuals are needed.

Histopathological sections from *M. avium*-infected patients often show macrophages containing many bacteria (34). It is therefore quite paradoxical that the organism, as well as its components, is able to induce cell death. It is possible that in tissue sections of *M. avium*-infected patients, cells containing organisms may be apoptotic but appear intact. Previous studies have shown that it is very difficult to identify apoptotic cells in tissue sections by routine histopathological staining (14). At this point, it is not clear whether the findings reported here are relevant to host response in vivo. However, during infection with *M. tuberculosis*, Keane et al. (14) reported that numerous macrophages in tuberculous granulomas are TUNEL positive. Based on this, they suggest that the macrophages are apoptotic, although they did not exhibit structural changes suggestive of apoptosis. Further studies along these lines to determine whether cells in tissue sections from *M. avium*-infected patients are normal or near cell death by sensitive methods, such as the TUNEL assay, will help to clarify this issue.

In summary, the results presented here provide new insight into the effect of *M. avium* components on host cell response during *M. avium* infection. The identification of individual apoptosis-inducing *M. avium* components and the mechanism by which they induce apoptosis of monocytes and MDMs further adds to existing knowledge regarding *M. avium* pathogenesis.

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