H₂O₂ Induces Monocyte Apoptosis and Reduces Viability of *Mycobacterium avium*-*M. intracellulare* within Cultured Human Monocytes

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Mycobacterium avium-*M. intracellulare*, an intracellular parasite of mononuclear phagocytes, rarely causes disease in immunocompetent individuals. In contrast, in human immunodeficiency virus type 1-infected patients, *M. avium*-*M. intracellulare* can infect almost every tissue and organ. This suggests that immunocompetent individuals have a protective mechanism to control or prevent the infection. How mycobacteria may be killed by the host immune response is unclear. We have recently reported that induction of apoptosis of *Mycobacterium bovis* BCG-infected macrophages with ATP⁴⁻ was associated with killing of the intracellular mycobacteria. In the present study, a long-term culture of *M. avium*-*M. intracellulare*-infected monocytes was used to further evaluate the interaction between *M. avium*-*M. intracellulare* and primary human monocytes. In our system, *M. avium*-*M. intracellulare* parasitized the human monocytes and appeared to replicate slowly over 14 days within the host cells. To examine the role of apoptotic mechanisms in survival or death of intracellular mycobacteria, *M. avium*-*M. intracellulare*-infected human monocytes were treated with a monoclonal antibody to Fas receptor (APO-1/CD95) or with various concentrations of H₂O₂. Although both of these exogenous agents induced monocyte apoptosis, optimal killing (65% reduction in CFU) of intracellular *M. avium*-*M. intracellulare* was observed only when *M. avium*-*M. intracellulare*-infected cells were treated with 10 mM H₂O₂. Fas-induced apoptosis did not affect *M. avium*-*M. intracellulare* viability. Our results suggest that not all stimuli of monocyte apoptosis induce killing of intracellular *M. avium*-*M. intracellulare*. Since release of H₂O₂ following phagocytosis of mycobacteria has been documented, H₂O₂-induced apoptotic death of *M. avium*-*M. intracellulare*-infected monocytes and its association with killing of the intracellular bacilli may be a physiological mechanism of host defense against *M. avium*-*M. intracellulare*.

Mycobacterium avium-*Mycobacterium intracellulare* is found ubiquitously in the environment (7, 8). *M. avium*-*M. intracellulare* rarely causes infection in immunocompetent individuals, suggesting that there is some mechanism to control the infection (13). However, in association with human immunodeficiency virus (HIV) infection, *M. avium*-*M. intracellulare* is a common opportunistic pathogen. While antimicrobial drugs against *M. avium*-*M. intracellulare* are available (18, 35), complete eradication of the organism is impossible in the immunosuppressed host, necessitating life-long therapy. Thus, *M. avium*-*M. intracellulare* constitutes a major life-threatening health problem for immunocompromised patients.

M. avium-*M. intracellulare* is a facultative intracellular organism that is phagocytosed by and multiplies in human macrophages (11). How human monocytes/macrophages control the growth of and/or kill intracellular *M. avium*-*M. intracellulare* is unknown (1). We have recently reported that induction of apoptosis of *Mycobacterium bovis* BCG-infected cells with ATP⁴⁻ was associated with killing of the intracellular mycobacteria (30). To further examine the role of apoptotic mechanisms in survival or death of intracellular mycobacteria, other mediators of apoptosis are being investigated. For example, Fas receptor, which belongs to the nerve growth factor receptor/tumor necrosis factor receptor family, is constitutively expressed in many normal tissues and cells, including peripheral blood monocytes. Fas receptor is inducible in other cell types

(23, 24, 27). Treatment with monoclonal antibodies directed against the Fas receptor both in vitro (23) and in vivo (33) triggers apoptosis of Fas receptor-expressing cells. This suggests that Fas ligand may play a role in the regulation of apoptosis in a wide range of tissues. In addition, the role of oxidative stress as a mediator of apoptosis has recently been documented (5), and H₂O₂ has been reported to trigger apoptosis in various cell types (28).

In this study, *M. avium*-*M. intracellulare*-infected human monocytes were treated with a monoclonal antibody (APO-1/CD95) to Fas receptor or with various concentrations of H₂O₂. Although both of these exogenous agents induced apoptosis of *M. avium*-*M. intracellulare*-infected monocytes, Fas-mediated apoptosis did not affect *M. avium*-*M. intracellulare* viability. In contrast, H₂O₂-induced apoptosis of the host cell led to the killing of the intracellular bacilli. This may be physiologically important, since H₂O₂ can be released in vivo upon phagocytosis of the bacilli.

MATERIALS AND METHODS

Reagents. *M. avium*-*M. intracellulare*. A patient isolate of *M. avium*-*M. intracellulare* (a kind gift from Sharon Mannheimer, Cornell University Medical College, New York, N.Y.) was grown in 100 ml of Proskauer-Beck medium containing 0.05% Tween 80. Aliquots were stored in liquid nitrogen and, when thawed, shown to contain ~2 × 10⁸ CFU/ml, capable of growing on solid medium.

All bacteria were lipopolysaccharide free and without reactivity in the *Limulus* amoebocyte lysate assay (Whittaker Bioproducts, Walkersville, Md.). For infection of monocytes, an aliquot was thawed at 37°C, diluted 10-fold in culture medium, and dispersed in a bath sonicator (Laboratory Supplies Co., Hickville, N.Y.) with three 10-s pulses and cooling on ice between pulses. Acid-fast staining

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of the infected cells 2 days postinfection confirmed that most bacilli (>80%) were present as single organisms, not clumps.

Other reagents. Anti-APO-1 hybridoma supernatant was a kind gift from P. H. Krammer (Heidelberg, Germany) (41). Cycloheximide (CHX; Sigma Chemical Co., St. Louis, Mo.), H₂O₂ (Cumberland-Swan, Inc., Smyrna, Tenn.), and recombinant gamma interferon (IFN- γ) (Genzyme, Cambridge, Mass.) were obtained commercially.

Cell preparations. (i) **PBMC.** To obtain peripheral blood mononuclear cells (PBMC), peripheral blood was drawn from donors by venipuncture, collected into heparinized syringes, and processed within 60 min of drawing. Buffy coats were obtained from New York Blood Services Center West (New York, N.Y.). PBMC were isolated as described before (31). Briefly, whole blood and buffy coats were diluted with 0.5 and 1.0 volume of RPMI 1640 medium, respectively, layered on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and centrifuged at $500 \times g$ for 30 min at 25°C. Interface cells were washed three times at 4°C, including a low-speed centrifugation step at $100 \times g$ to remove contaminating platelets. Cells were resuspended in RPMI 1640 (GIBCO BRL, Gaithersburg, Md.) medium supplemented with 1% pooled human AB+ serum obtained commercially (Biocell Ltd., Carson, Calif.) (R-1) and counted in a hemacytometer.

(ii) **Monocytes.** PBMC density was adjusted to 10^7 /ml of R-1, and 100μ l of the cell suspension was plated on each 13-mm Thermanox coverslip (Lux, Naperville, Ill.) in 24-well plates, or PBMC density was adjusted to 6×10^6 /ml of R-1, and 500 or 100 μ l of the cell suspension was plated on Falcon Primaria tissue culture (Becton Dickinson Labware, Lincoln Park, N.J.) 24- and 96-well plates, respectively. Nonadherent cells were washed away after 1 h at 37°C, so that the final density was 6×10^4 (for 96-well plates) and 3×10^5 (for 24-well plates) cells per well, a density which gave rise to an even monolayer after 4 days of culture. A confluent monolayer of monocytes was observed throughout the 14 days of culture whether cells were infected or not.

In order to obtain a large number of monocytes to assay for the presence of a DNA ladder, monocytes were enriched by incubation of PBMC with neuraminidase-treated [*Vibrio cholerae* neuraminidase; Calbiochem-Behring Corp., La Jolla, Calif.] sheep erythrocytes (Cocalco Biologicals, Inc., Reamstown, Pa.) for 1 h on ice and separated by 20 min of centrifugation over a Ficoll-Hypaque gradient from the rosetted population (E⁺ cells). Cells were allowed to adhere directly to 100-mm tissue culture plates and then, after 1 h at 37°C, washed with warm R-1 to remove nonadherent cells. Adherent cells were cultured overnight and detached by replacing the culture medium with ice-cold Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) for 20 min at 4°C. Detached cells were pelleted, resuspended in RPMI with 20% human serum (R-20), and counted in trypan blue for an estimate of viable cells. Cells were >95% viable. Cell density was adjusted to 10^6 /ml of R-20, and monocytes were cultured in Teflon beakers. All cells were incubated at 37°C and 5% CO₂. Cells isolated by either protocol were 90 to 95% monocytes by the criterion of CD14 expression. Fluorescence-activated cell sorting (FACS) analysis demonstrated that approximately 10 to 15% of the PBMC isolated were monocytes. For infection, monocytes were exposed immediately after removal of nonadherent cells to a single-cell suspension of *M. avium-M. intracellulare* in R-20 containing about one viable bacillus per cell.

Anti-APO-1- and H₂O₂-induced cytotoxicity. Monolayers of infected and uninfected monocytes were cultured for 4 days in flat-bottomed Primaria 96-well tissue culture plates. Some cultures were treated with 100 U of human IFN- γ per ml immediately after removal of nonadherent cells. Adherent cells were loaded with 0.5 μ Ci of sodium [⁵¹Cr]chromate (New England Nuclear, Boston, Mass.) for 16 to 18 h at 37°C, washed twice with warm R-1, incubated for 2 h in R-20, and washed once more before the addition of 0.1 ml of R-20, solutions of anti-APO-1, CHX, or both, or various dilutions of H₂O₂ in R-20. Plates were incubated for either 6 or 18 h at 37°C in 5% CO₂. Supernatants were removed, and the cells were lysed by incubation in 0.1 ml of 1% Triton X-100 for 1 h. Supernatants and cell lysates were counted separately in a gamma counter (Packard Instrument Company, Downers Grove, Ill.). Spontaneous ⁵¹Cr release was calculated as counts per minute (cpm) in supernatants of control wells, and total ⁵¹Cr release was calculated as total cpm in supernatants and lysates of control wells. Specific ⁵¹Cr release was calculated as $100 \times [(\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release})/(\text{total } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release})]$.

Flow cytometric analysis of expression of Fas receptor. Immunofluorescence analysis of Fas receptor expression on human monocytes was performed as follows. Briefly, 3×10^5 monocytes purified by adherence were incubated with 10 μ g of mouse anti-human Fas receptor monoclonal antibody (immunoglobulin M [IgM]; Kamiya Biomedical Company, Thousand Oaks, Calif.) per ml on ice for 45 min and washed with ice-cold 2% fetal bovine serum (FBS) in PBS twice before a 20-min incubation on ice with (1:500) fluorescein isothiocyanate (FITC)-conjugated goat F(ab')₂ fragment of IgG anti-mouse IgM (μ chain) (Organon Teknica Corp., West Chester, Pa.). Treated cells were washed twice in 2% FBS in PBS and fixed in 10% buffered Formalin for 20 min. The stained cells were washed, resuspended in 2% FBS in PBS, and analyzed with a FACScan (Becton Dickinson).

CFU assay. Monolayers of 3×10^5 infected monocytes in 0.5 ml of culture medium were cultured for 2 to 14 days in wells of flat-bottomed Primaria 24-well tissue culture plates. After removal of nonadherent cells, some cultures were treated with 100 U of human IFN- γ per ml. For the *M. avium-M. intracellulare*

killing assay, 4-day-old infected monocytes were treated with R-20 alone, R-20 containing anti-APO-1, CHX, or anti-APO-1 plus CHX, or various dilutions of H₂O₂ in R-20. All infected cultures were examined by light microscopy to determine monocyte viability prior to each treatment. All cultures were found to be more than 90% adherent and viable. Plates were incubated for an additional 6 (H₂O₂) or 18 (anti-APO-1 plus CHX) h at 37°C in 5% CO₂. After the incubation period, cells were lysed by adding 0.5 ml of PBS containing 0.016% digitonin (Sigma Chemical Co.) and 0.25% Tween 80 to each well, incubated at 37°C for 10 min, and sonicated with six 0.5-s pulses at low output power to disperse clumps of bacilli. This schedule of sonication had been previously determined to be optimal for dispersing bacteria without causing any loss of viability. Serial 10-fold dilutions were made by vigorously mixing in PBS, and 0.01-ml drops of appropriate dilutions were plated on Middlebrook and Cohn 7H9 or 7H10 agar plates. Plates were sealed in plastic bags and kept at 37°C for 7 to 10 days. Dilutions containing 10 to 100 colonies arising from single mycobacteria were counted with the aid of a dissecting microscope. For each culture, six replicate samples were counted, and the mean was calculated.

Acid-fast staining. The monolayers of infected monocytes on 13-mm Thermanox coverslips (Lux, Naperville, Ill.) were fixed for 30 min in 10% Formalin (Fisher Scientific), dried, and stained with auramine-rhodamine (TB Fluorescent Stain, Set T; Fisher Scientific) according to the manufacturer's instructions. After drying and mounting, bacteria were counted with a Nikon BG-12 excitation and OG-1 barrier filter for epifluorescence.

In situ nick translation. Monolayers of monocytes on coverslips were fixed in methanol-acetone (3:1) and processed as described before (30). Briefly, a reaction mixture containing nick translation buffer, digoxigenin-labeled nucleotides, and DNA polymerase I was added to each coverslip, which was incubated at 37°C for 60 min. The reaction was washed four times with Tris-buffered saline (TBS) and incubated with FITC-labeled anti-digoxigenin Fab for 40 min at room temperature. The coverslips were washed three times, mounted in glycerol, and examined by epifluorescence microscopy (Nikon Microphot Fx; Nikon, Garden City, N.Y.).

DNA agarose gel electrophoresis. Monocytes harvested as described above were cultured for 4 days in Teflon beakers. Monocytes (12×10^6) were used for each treatment in the experiment. Various dilutions of H₂O₂ in R-20 were added to cultured monocytes to give final concentrations of 2, 5, 10, and 20 mM. Anti-APO-1 hybridoma supernatant and CHX were added to give final concentrations of 0.1 and 1 μ g/ml, respectively. Both treated and untreated monocytes were incubated for 6 h. DNA was harvested as described before (30). The DNA solution was subjected to electrophoresis in 1.5% agarose for 2 h at 60 V. DNA was visualized with ethidium bromide.

RESULTS

Growth of *M. avium-M. intracellulare* in human monocytes. Immediately after nonadherent cells were washed away, monolayers of adherent monocytes were infected at a multiplicity of approximately one viable bacillus per cell. At 2 days postinfection, when the first CFU assay was performed, about 30% of monocytes were infected with a mean of 2.2 bacilli per infected cell, as evaluated by direct light microscopic counting of acid-fast bacilli. At the time points indicated, numbers of viable bacilli were determined by CFU (as described in Materials and Methods) (Fig. 1). No significant change in bacillus count was observed during the first 7 days in culture. However, over the next 7 days, the mean number of viable intracellular *M. avium-M. intracellulare* (CFU) increased somewhat (Fig. 1). Infected monocyte monolayers remained confluent and viable throughout the 14-day assay. The total number of viable infected monocytes did not change significantly during the 14-day culture, as determined by direct microscopic examination. By the end of the assay, about 20% of cells had detached from the tissue culture surface. Both adherent and nonadherent cells were assayed for CFU. Parallel cultures of R-20 medium alone were inoculated with the same number of *M. avium-M. intracellulare* as cultures containing monocytes. The number of viable bacilli in culture medium alone continued to fall for the 2-week duration of the experiment. Thus, in the absence of human monocytes, *M. avium-M. intracellulare* could not survive. This result indicates that this patient isolate of *M. avium-M. intracellulare* is an obligate intracellular organism that survives and may even replicate slowly in human monocytes.

Effect of H₂O₂ or anti-APO-1 treatment on 4-day-cultured monocytes. To determine whether oxidative stress induced

FIG. 1. *M. avium-M. intracellulare* growth in cultured monocytes. Viable *M. avium-M. intracellulare* (3×10^5) were added to wells containing 0.5 ml of culture medium alone (circles) or 0.5 ml of culture medium containing 3×10^5 freshly isolated adherent monocytes (squares). At the time points indicated, the number of CFU was assayed. Results are expressed as CFU per well and are means $\pm$ standard deviation (SD) of 2 experiments, each done in triplicate.

death of human monocytes in culture, 4-day-cultured cells were treated for 6 h with H_2O_2 -diluted in R-20 at final concentrations ranging from 2 to 20 mM. Parallel assays with uninfected and *M. avium-M. intracellulare*-infected cells were performed. Cell death occurred in a dose-dependent manner, and the dose-response curve for H_2O_2 -induced cell death was reproducible with cells from different donors (Fig. 2A). The results suggest that intracellular *M. avium-M. intracellulare* rendered cultured monocytes more sensitive to death induced by H_2O_2 (P values for 5 and 10 mM H_2O_2 are 0.001 and 0.002, respectively) (Fig. 2A). Because IFN- γ has been shown to be important in expression of protective immunity to mycobacterial infection in IFN- γ gene-disrupted mice (10), the effect of IFN- γ treatment on target monocytes treated with H_2O_2 was investigated. The results revealed that pretreatment with IFN- γ had no effect on the kinetics of H_2O_2 -induced cell death of monocytes. Furthermore, the dose-response curve was almost identical to that of untreated monocytes (Fig. 2B).

Monocytes were then treated with anti-APO-1 to determine whether Fas receptor was involved in induction of death of these cells. Cell death (Fas induced) was seen only when cells were treated with anti-APO-1 in the presence of CHX (Fig. 3A). This is consistent with a previous report that Fas-induced death was enhanced when protein synthesis was blocked (14). Unlike H_2O_2 treatment, Fas-induced death of monocytes required a longer period of treatment (18 h) (Fig. 3A). When cultured monocytes were pretreated with IFN- γ , the killing of cultured monocytes induced by anti-APO-1 plus CHX treatment was enhanced (Fig. 3A). IFN- γ has been shown to up-regulate Fas receptor expression in human colon carcinoma HT-29 cells (23); by FACS analysis, we have observed similar results in our cultured monocytes. IFN- γ treatment of monocytes slightly enhanced the expression of Fas receptor, as measured by anti-APO-1 staining of the cell surface (Fig. 3C). When parallel assays were carried out with uninfected and *M. avium-M. intracellulare*-infected cells, the dose response to Fas-induced killing was similar, indicating that the presence of intracellular *M. avium-M. intracellulare* neither protected nor predisposed host monocytes to death induced by anti-APO-1 plus CHX (Fig. 3B).

FIG. 2. ^{51}Cr release from 4-day-infected monocytes treated with H_2O_2 for 6 h. (A) Assay of 6×10^4 uninfected monocytes (squares) and *M. avium-M. intracellulare*-infected monocytes (circles). (B) Assay of 6×10^4 uninfected monocytes treated with 100 U of IFN- γ per ml in R-20 (triangles) or with R-20 alone (squares) before H_2O_2 treatment. The monocytes in panels A and B were obtained from different donors. Results are means $\pm$ SD of triplicate cultures.

Effect of H_2O_2 and anti-APO-1 plus CHX treatment on the viability of intracellular *M. avium-M. intracellulare*.

M. avium-M. intracellulare-infected monocytes were treated with H_2O_2 or anti-APO-1 plus CHX to determine the effects of host cell death on bacillus viability. Survival of bacteria was assayed by counting CFU after the various treatments. When *M. avium-M. intracellulare*-infected monocytes were treated with various doses of H_2O_2 for 6 h, killing of intracellular mycobacteria (CFU) was observed (Fig. 4). Bacillary killing was observed at doses of between 2 and 20 mM H_2O_2 . The optimal dose of 10 mM H_2O_2 resulted in the killing of approximately 65% of intracellular bacteria (Fig. 4). When infected monocytes were lysed by sonication before the beginning of the assay, which rendered the bacteria extracellular, the presence of H_2O_2 up to 10 mM had no significant effect on the viability of the bacilli (Fig. 4). However, treatment of extracellular bacilli with H_2O_2 at 20 mM resulted in the killing of about 20% of the organisms (Fig. 4).

Treatment of parallel cultures of infected monocytes with anti-APO-1 plus CHX for 18 h had no effect on recovery of viable bacilli; intact monolayers treated with culture medium (control) and anti-APO-1 plus CHX showed 100% and 106.2% $\pm$ 3.5% viable *M. avium-M. intracellulare*, respectively (means

FIG. 3. Effect of anti-APO-1 plus CHX (1 μg/ml) on cultured monocytes. (A) Anti-APO-1 plus CHX treatment of monocytes for 18 h without pretreatment (solid circles) or pretreated with IFN-γ (solid diamonds). *P* values for 0.001, 0.01, 0.1, and 0.5 μg of anti-APO-1 per ml plus CHX versus anti-APO-1 plus CHX plus IFN-γ are 0.03, 0.02, 0.08, and 0.009, respectively. In some cultures, cells were treated for 6 h with anti-APO-1 plus CHX with IFN-γ pretreatment (open diamonds) or without IFN-γ pretreatment (open circles) or treated with anti-APO-1 without CHX for 18 h (solid triangles). (B) Anti-APO-1 plus CHX treatment of monocytes infected with *M. avium-M. intracellulare* (circles) or uninfected (squares). The results in panels A and B are means ± SD of triplicate cultures, expressed as percent specific release of ⁵¹Cr from labeled monocytes. (C) Measurement of Fas receptor expression by FACS analysis of monocytes pretreated with IFN-γ (circles) or untreated (squares). Results are expressed as mean fluorescence units.

± standard error of the mean [SEM]). These results suggest that Fas-mediated cytotoxicity is not involved in killing of intracellular mycobacteria. Similarly, when infected cells were sonicated before treatment with culture medium or anti-APO-1 plus CHX, there was no reduction in mycobacterial CFU (109.6% ± 1.7% and 113.3% ± 1.2% viable *M. avium-M.*

FIG. 4. Effect of H₂O₂-induced monocyte apoptosis on killing of *M. avium-M. intracellulare*. Monocytes (3×10^5) were infected immediately after isolation and cultured for 4 days. H₂O₂ diluted in culture medium was added to intact monolayers (intracellular *M. avium-M. intracellulare*) (open bars) or to monolayers that had first been sonicated (extracellular *M. avium-M. intracellulare*) (shaded bars). Cultures were harvested for the CFU assay after 6 h. Results are means ± SEM of three to five experiments in triplicate, expressed as percent killing of *M. avium-M. intracellulare*.

intracellulare, respectively). Even when infected monocytes were pretreated with IFN-γ, which led to enhanced expression of Fas receptor on these cells (Fig. 3C) and resulted in increased killing of infected monocytes by anti-APO-1 plus CHX treatment (Fig. 3A), no significant effect on the viability of intracellular bacteria was observed (Table 1). Treatment of *M. avium-M. intracellulare*-infected monocytes with IFN-γ for the 4-day culture period did not increase bacterial growth or survival in this system.

Since CHX was necessary for induction of monocyte killing by anti-APO-1, CHX could potentially abolish the cytotoxicity-coupled death of the mycobacteria. Therefore, H₂O₂ was added together with CHX to cultures of infected monocytes to determine whether CHX inhibited the H₂O₂-induced killing of intracellular *M. avium-M. intracellulare*. The extent of bacillus killing observed under these conditions was identical to that in cultures treated with H₂O₂ alone (Table 2). The results suggested that only H₂O₂-induced killing of monocytes was coupled with reduced viability of intracellular *M. avium-M. intracellulare*. Moreover, protein synthesis did not appear to be required for the mycobacterial killing observed in H₂O₂-induced host cell death.

Induction of DNA fragmentation in cultured monocytes by anti-APO-1 with CHX and by H₂O₂. DNA fragmentation is a

TABLE 1. Effect of IFN-γ pretreatment on anti-APO-1 plus CHX-induced killing of mycobacteria^a

Treatment	Amt (μg/ml)	CFU (10 ⁴)	
		Control	IFN-γ
Culture medium		21.1 ± 1.2	23.6 ± 1.0
Anti-APO-1	0.1	22.1 ± 1.5	20.2 ± 1.1
CHX	1	19.8 ± 1.3	23.2 ± 1.5
Anti-APO-1 + CHX	0.1 + 1	23.5 ± 1.5	28.1 ± 2.8

^a Four-day-infected monocytes were incubated for 18 h at 37°C in 5% CO₂ and treated as indicated. Parallel cultures were incubated with IFN-γ (100 U/ml) from the time of monocyte infection. Results are means ± SEM for triplicate cultures.

TABLE 2. Bacillus killing after treatment of infected monocytes with H₂O₂ and CHX

Treatment	Concn	CFU ^a (10 ⁴)	
		Intact monolayers	Sonicated cultures
None (control)		21.6 ± 1.1	21.9 ± 1.1
H ₂ O ₂	10 mM	11.5 ± 0.6	18.8 ± 1.3
CHX	1 µg/ml	21.8 ± 1.2	21.9 ± 1.2
H ₂ O ₂ + CHX	10 mM + 1 µg/ml	10.6 ± 0.8	18.3 ± 0.6

^a Results are means ± SEM of triplicate cultures.

key feature of apoptotic cell death (9, 42). In the experiments reported here, phase contrast microscopy of treated cells revealed a decrease in cell density compared with control cultures (Fig. 5B) and alterations in morphology induced by anti-APO-1 plus CHX (Fig. 5F) as well as alterations induced by H₂O₂ (Fig. 5D). With either treatment, many of the monocytes which remained adherent were rounded and small. In particular, pyknotic nuclei were apparent as dense shrunken bodies of condensed chromatin. Monocyte death induced by either H₂O₂ or anti-APO-1 plus CHX was accompanied by fragmentation of chromatin, which was not observed in control cells (Fig. 5A). When free ends of DNA were assayed by fluorescent labeling, pyknotic nuclei induced by both treatments were brightly fluorescent (Fig. 5C and E), indicating that the chromatin had been fragmented.

Discrete nucleosome-sized DNA fragments were detected by agarose gel electrophoresis of treated cells (Fig. 6). Anti-APO-1 treatment in the absence of CHX did not induce fragmentation of target cell DNA (data not shown). H₂O₂ treatment at 2, 5, and 10 mM but not 20 mM resulted in internucleosomal DNA fragmentation. It was conceivable that 20 mM H₂O₂ might initially induce DNA fragmentation, but rapid secondary necrosis might lead to degradation of the nucleosome-sized fragments, making detection of the fragments difficult. Therefore, we attempted to document DNA ladder formation at an earlier time point. Even as early as 2 h after treatment with 20 mM H₂O₂, no DNA fragmentation was observed (Fig. 6).

Role of nitric oxide production in killing of intracellular *M. avium-M. intracellulare*. Nitric oxide production has been demonstrated to be an important part of the antimicrobial activities of activated murine macrophages against many pathogens, including mycobacteria (15, 19). We therefore investigated whether NO played a role in the control of *M. avium-M. intracellulare* viability in human monocyte cultures in vitro. The antimicrobial activities of NO have been shown to be reversed by antagonists of L-arginine oxidation, such as N^G-monomethyl-L-arginine [L-NMMA]. Therefore, *M. avium-M. intracellulare*-infected monocytes were treated with L-NMMA for 16 h before treatment with 10 mM H₂O₂ (Table 3). L-NMMA alone at 500 µM had no effect on the viability of *M. avium-M. intracellulare* and did not affect H₂O₂-induced killing of intracellular *M. avium-M. intracellulare*. The results suggest that the H₂O₂-mediated killing of intracellular *M. avium-M. intracellulare* in explanted human monocytes did not involve NO production.

DISCUSSION

M. avium-M. intracellulare, an intracellular parasite of mononuclear phagocytes, rarely causes infection in immunocompetent hosts, suggesting that survival of the organism is limited by the host immune response. To study the intraphagosomal killing of *M. avium-M. intracellulare*, we examined whether two

different inducers of host cell apoptosis affected the viability of the intracellular organisms. In the in vitro system described here, the isolate of *M. avium-M. intracellulare* used was an obligate intracellular pathogen. *M. avium-M. intracellulare* failed to replicate measurably in human monocytes within the first 7 days of culture, suggesting that human monocytes could transiently restrict the growth of this particular strain of *M. avium-M. intracellulare*. However, the mean viable-bacillus count on day 14 increased from that on day 7, indicating that the *M. avium-M. intracellulare* infection persisted over time, rendering human monocytes somewhat more permissive for the growth of the intracellular organism (Fig. 1). Since *M. avium-M. intracellulare* survive in monocytes and gradually replicate with a doubling time of 2 weeks in this system, any significant reduction in CFU indicates a bactericidal effect. In our present studies, only H₂O₂-induced killing of the host cells is associated with reduced viability of the intracellular *M. avium-M. intracellulare*. Thus, the mean 65% loss in viability observed after 6 h of treatment with a low dose of H₂O₂ suggested that killing rather than stasis of the intracellular bacilli was induced.

H₂O₂-induced apoptotic death of *M. avium-M. intracellulare*-infected monocytes/macrophages and its association with killing of the intracellular bacilli may be a physiological mechanism of host defense against *M. avium-M. intracellulare*, since release of H₂O₂ and other reactive oxygen metabolites has been documented following phagocytosis of mycobacteria, including *M. avium-M. intracellulare* (16, 17, 36). Different *M. avium-M. intracellulare* strains have different resistances to the killing effects of the oxygen burst of macrophages in vitro. The susceptible strains are readily killed, while the resistant strains are capable of parasitizing the host cell and even replicating intracellularly (3). H₂O₂ has been shown to induce apoptosis in cell types other than monocytes (28). The doses used in these reports ranged from 10 to 100 µM H₂O₂. In our study, the dose of H₂O₂ that was efficacious in monocyte killing is much higher, and this may reflect the relative resistance of peripheral blood monocytes to oxidative damage. Macrophages employ oxidative bactericidal mechanisms to combat many intracellular pathogens, such as *Candida albicans* and *Listeria monocytogenes* (29, 37, 38). Reactive oxygen intermediates can damage many cellular macromolecules or initiate a chain reaction in which the free radical is shuttled from one molecule to the next, causing extensive damage to cellular structures (20). Many apoptotic stimuli are either oxidants (e.g., H₂O₂) or initiators of cellular oxidative metabolism, and many antiapoptotic agents either exhibit antioxidant activity or enhance cellular antioxidant defenses (5). Therefore, the survival of host cells, with or without an intracellular infection, may depend on an appropriate balance between oxidants and antioxidants.

Nitric oxide production has been demonstrated to be an important component of the antimicrobial capacity of activated murine macrophages (15, 19). The existence of similar bactericidal pathways in human monocytes/macrophages has been difficult to demonstrate. A number of studies (6, 25, 32, 34) failed to report NO synthesis when human macrophages were stimulated with either bacterial products or lymphokines. In addition, the induction of NO synthase has not yet been clearly observed in human peripheral blood monocytes or peritoneal macrophages (39). Our present results suggest that in the *M. avium-M. intracellulare*-infected human monocyte, NO does not appear to play a role in the H₂O₂-induced apoptosis-associated death of the intracellular mycobacteria (Table 3).

It is important to note, however, that not all agents that induce monocyte apoptosis also induce killing of intracellular bacilli. Fas-mediated apoptosis of *M. avium-M. intracellulare*-

FIG. 5. Fragmentation of chromatin following treatment of monocytes with H₂O₂ or anti-APO-1 plus CHX. Monocytes (10⁵) on coverslips were cultured for 4 days and not treated (A and B), treated with 2 mM H₂O₂ for 6 h (C and D), or treated with anti-APO-1 (0.1 µg/ml) and CHX (1 µg/ml) for 6 h (E and F). Free ends of DNA (arrows) were visualized by FITC-conjugated antibodies. (A, C, and E) Fluorescent micrographs; (B, D, and F) corresponding phase micrographs. Magnification, ×250.

FIG. 6. Internucleosomal DNA fragmentation. The DNA ladder was visualized by agarose gel electrophoresis of monocytes treated for 6 h with either anti-APO-1 plus CHX or low doses (2 to 10 mM) or a high dose (20 mM) of H_2O_2 .

infected cells did not lead to killing of intracellular *M. avium-M. intracellulare*, even after IFN- γ treatment, which up-regulated the expression of Fas receptors on human monocytes and enhanced killing of the cells. A recent published report indicated that Fas-triggered apoptosis did not involve reactive oxygen intermediates (22). Results to date indicate that only the apoptotic pathways induced by ATP $^{4-}$ (30) and low-dose H_2O_2 (this study) were associated with killing of intracellular mycobacteria.

Many studies have used cytokines in an attempt to modulate the survival of *M. avium-M. intracellulare* in human monocytes/macrophages (1). In a previously reported study, treatment of monocytes with either granulocyte macrophage-colony stimulating factor, tumor necrosis factor alpha, or both cytokines in combination rendered monocytes bacteriostatic for a virulent strain of *M. avium-M. intracellulare* (12). In contrast, the effects of IFN- γ treatment on *M. avium-M. intracellulare*-infected monocytes are controversial at present. One study (40) reported that growth of *M. avium-M. intracellulare* in human monocytes was restricted if IFN- γ was present in the culture after infection. Another study (4) found that IFN- γ stimulated the growth of intracellular *M. avium-M. intracellulare* within human monocytes. In the experiment reported here, IFN- γ treatment of *M. avium-M. intracellulare*-infected monocytes did not enhance growth of the intracellular bacilli. At present, it is still not clear how cytokines produced in response to *M. avium-M. intracellulare* infection may affect the course of the infection in the human host.

Monocytes treated with tumor necrosis factor alpha have been shown to kill an avirulent strain of *M. avium-M. intracellulare* (2). Results from a previously published study suggest

strongly that tumor necrosis factor alpha-induced cell death may also involve an oxidative mechanism (21). We are currently investigating whether tumor necrosis factor alpha or other cytokine treatment induces apoptosis of *M. avium-M. intracellulare*-infected human monocytes in vitro and whether cytokine-induced apoptosis is also associated with killing of intracellular *M. avium-M. intracellulare*.

The question of how *M. avium-M. intracellulare* infection is resolved in immunocompetent individuals while HIV-1-infected patients usually succumb to the infection remains unanswered. One study has shown that monocytes obtained from healthy individuals and from AIDS patients were comparable in their abilities to phagocytose and restrict the growth of *M. avium-M. intracellulare* (26). In the immunocompromised host, other factors involved in the activation of the resistance mechanism against *M. avium-M. intracellulare* may be reduced or inoperative. Whether killing of *M. avium-M. intracellulare* via H_2O_2 -induced apoptosis is compromised in HIV-1-infected individuals is not known. Further investigations into the susceptibility to oxidative stress of *M. avium-M. intracellulare*-infected monocytes obtained from AIDS patients will shed additional light on the role of H_2O_2 -induced apoptosis-associated killing.

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TABLE 3. Bacillus killing after treatment of infected monocytes with H_2O_2 and L-NMMA

Treatment	Concn	CFU ^a (10 ⁴)	
		Intact monolayers	Sonicated cultures
None (control)		27.1 ± 1.1	30.5 ± 1.1
L-NMMA	500 μ M	30.7 ± 1.4	31.9 ± 1.3
H_2O_2	10 mM	14.7 ± 0.9	27.3 ± 1.4
H_2O_2 + L-NMMA	10 mM + 50 μ M	14.9 ± 0.8	26.5 ± 1.0
H_2O_2 + L-NMMA	10 mM + 500 μ M	16.8 ± 0.9	27.8 ± 1.2

^a Results are means ± SEM of triplicate cultures.

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