Mycobacterial Growth and Sensitivity to H₂O₂ Killing in Human Monocytes In Vitro

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The intracellular growth and susceptibilities to killing by H₂O₂ in cultured human monocytes of a number of mycobacterial species including laboratory strains and clinical isolates of *Mycobacterium tuberculosis*, and *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) and a clinical isolate of *Mycobacterium avium*-*M. intracellulare* were examined. The clinical isolate of *M. avium*-*M. intracellulare* did not replicate in freshly explanted monocytes (generation time of >400 h); BCG replicated with a generation time of 95 h, and *M. tuberculosis* strains CDC551, H37Rv, and H37Ra replicated with generation times of 24, 35, and 37 h, respectively, during the 4-day growth assay. When cultured in monocytes for 4 days, the mycobacteria were variably sensitive to H₂O₂-induced killing. A positive correlation between the generation time and percent killing of intracellular bacilli was observed. By comparison, mycobacterial strains were similarly sensitive to H₂O₂ treatment in cell-free culture media and in sonicated cell suspensions. Using a number of inhibitors of reactive oxygen intermediates we determined that other than catalase the inhibitors tested did not affect H₂O₂-induced killing of intracellular mycobacteria. Our studies suggest that the killing of mycobacteria growing in human monocytes in vitro by the addition of exogenous H₂O₂ is dependent on the susceptibility to a peroxide-induced killing pathway as well as on the intracellular growth rate of the mycobacteria.

Mycobacteria constitute a large family of organisms with a wide spectrum of virulence as defined by their abilities to establish infection and cause active disease in humans. Among these agents, *Mycobacterium tuberculosis* is the most virulent in humans, with an estimated one billion infected individuals worldwide (40). In comparison, *Mycobacterium avium*-*M. intracellulare*, a common contaminant of soil and water, is usually nonvirulent (i.e., does not establish infection or cause disease) in humans but has now emerged as a life-threatening opportunistic infection in immunocompromised AIDS patients (13, 43).

Mononuclear phagocytes exposed to the T-cell cytokine gamma interferon are primarily responsible for host resistance to mycobacterial infection (5–8, 23). How human monocytes/macrophages kill intracellular mycobacteria is still not known. In fact, mycobacteria survive and/or replicate within phagosomes of human macrophages. Previous studies have suggested that oxygen and reactive oxygen intermediates (ROI) produced by the host cells contribute to the control of mycobacterial growth and survival. For instance, growth of *M. tuberculosis* in culture media is markedly inhibited under high oxygen tension (10), and ROI generated by peroxidase or catalase-H₂O₂-halide have been shown to directly kill *Mycobacterium leprae*, *M. tuberculosis*, and *M. avium*-*M. intracellulare* (14, 16, 41). Human monocytes activated by gamma interferon (39) or by mycobacterial cell wall products (24) have been shown to be more oxidatively active and better capable of killing *M. leprae*. During phagocytosis of mycobacteria by monocytes, release of reactive ROI, including H₂O₂, occurs (9). We have recently reported that low doses of exogenously added H₂O₂ reduce the viability of intracellular *M. avium*-*M. intracellulare* (20). Since

H₂O₂ is bactericidal, resistance to H₂O₂ may be considered a virulence determinant of *M. tuberculosis* (25).

Recently, North and Izzo demonstrated with mice that virulent strains of mycobacteria had faster doubling times in vivo than less-virulent related species (34). To understand the cell biology of the host-parasite interaction in mycobacterial infection of humans, we established an in vitro infection model of human monocytes by using different species of mycobacteria. With this system, we examined the growth rates of *M. tuberculosis*, *Mycobacterium bovis* BCG, and a patient isolate of *M. avium*-*M. intracellulare* within human monocytes maintained in culture for up to 14 days. We also determined the sensitivity to H₂O₂-induced killing of the mycobacteria during growth in human monocytes. Following the addition of various inhibitors of oxidative metabolism, we examined whether reactive oxidative metabolites other than H₂O₂ mediate the mycobacterial killing observed in these studies.

MATERIALS AND METHODS

Reagents. (i) **Mycobacteria.** The mycobacterial strains used for these studies were as follows: a patient isolate of *M. avium*-*M. intracellulare* (a kind gift from Sharon Mannheimer, Cornell University Medical College, New York, N.Y.); *M. tuberculosis* H37Ra, Pasteur strain 201 (Trudeau Institute, Saranac Lake, N.Y.); *M. tuberculosis* H37Rv (Trudeau Institute); *M. tuberculosis* CDC551 (T. M. Shinnick, Centers for Disease Control and Prevention, Atlanta, Ga.); and BCG, Pasteur strain 1011 (Trudeau Institute).

Mycobacteria were grown in 100 ml of lipopolysaccharide-free Proskauer-Beck medium containing 0.05% Tween 80. Aliquots were stored in liquid nitrogen and, when thawed, were shown to contain $\sim 2 \times 10^8$ CFU capable of replication on 7H10 solid medium per ml.

(ii) **Other reagents.** Cycloheximide (CHX), actinomycin D, H₂O₂, catalase, superoxide dismutase (SOD), L-histidine, 1,3-dimethyl-2-thiourea (DMTU), deferoxamine mesylate, EDTA, and EGTA were obtained from Sigma Chemical Co., St. Louis, Mo.

Cell preparations. (i) **PBMC.** Peripheral blood was obtained from volunteer 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.). Peripheral blood mononuclear cells (PBMC) were isolated as described previously (27). Briefly, whole blood and buffy coats were diluted with RPMI 1640 medium, layered on Ficoll-Hypaque

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(Pharmacia, Uppsala, Sweden), and centrifuged. Interface cells were washed, resuspended in RPMI 1640 (GIBCO BRL, Gaithersburg, Md.) medium supplemented with 1% pooled human AB+ serum obtained commercially (R-1; Biocell Ltd., Carson, Calif.), and counted in a hemacytometer.

Monocytes. PBMC density was adjusted to 6×10^6 /ml in R-1 medium, and 500 ml of the cell suspensions was plated on Falcon Primaria tissue culture (Becton Dickinson Labware, Lincoln Park, N.J.) 24-well plates. For morphologic studies parallel cell suspensions were plated on 13-mm-diameter Thermanox coverslips (Lux, Naperville, Ill.) placed in 24-well plates. Nonadherent cells were washed away after 2 h to give a final density of 3×10^5 to 5×10^5 cells/well, which generated an even monolayer in the well or on the coverslips after 4 days of culture. Monocytes were cultured in RPMI 1640 medium supplemented with 20% pooled human AB+ serum (R-20) and were incubated at 37°C and 5% CO₂. Monolayers were >95% monocytes as assessed by fluorescence-activated cell sorter analysis of CD14 expression.

(iii) Infection of monocytes with mycobacteria. Infection of monocyte monolayers was performed as described previously (26). Briefly, single-cell suspensions of mycobacteria were sonicated with six half-second pulses at low output power to disperse clumps of bacilli. This amount of sonication had previously been determined to be optimal for dispersing bacteria without causing any loss of viability (26). Sonicated mycobacteria were added to freshly isolated adherent monocytes that had been plated for 2 h at a multiplicity of infection (MOI) of one viable bacillus per cell and were incubated in R-20 medium for 0 to 14 days. At 6 h postinfection, 30 to 50% of the monocytes were infected with a mean of 1.4 to 2.5 mycobacteria per infected cell as determined by direct counting of acid-fast bacilli (see below).

Procedures. (i) CFU assay. Monolayers of 3×10^5 to 5×10^5 infected monocytes in 0.5 ml of culture medium were cultured for 0 to 14 days in flat-bottom, Falcon Primaria, 24-well tissue culture plates (Becton Dickinson Labware) under varying conditions as described below. After the incubation period, 0.5 ml of phosphate-buffered saline containing 0.016% digitonin (Sigma Chemical Co.) and 0.25% Tween 80 was added to each well and the organisms were processed for the CFU assay as described previously (26). The cultures were sonicated (as described above) to disperse clumps of bacilli. This degree of sonication was confirmed to be optimal for breaking up clumps of bacilli to yield a single cell suspension without causing any loss in bacillary viability. Serial 10-fold dilutions of the bacterial suspension were made and plated on Middlebrook and Cohn 7H10 agar plates. After 7 to 21 days, dilutions containing 10 to 100 colonies each arising from single mycobacteria were counted with a dissecting microscope. For each culture, six replicate samples were counted and the mean was calculated. Doubling time for the mycobacterial species was calculated from a best-fit straight line through a logarithmic plot of the CFU-versus-time data.

For the intracellular killing assay, 4-day-old infected monocytes were treated with R-20 medium alone or with various dilutions (2 to 20 mM) of H₂O₂ in R-20 for 6 h. To evaluate the killing of mycobacteria in cellular lysate, 4-day-old infected monocytes were sonicated with six half-second pulses at low output power to disrupt the cells and render the mycobacteria extracellular. The lysates were then treated with R-20 alone or with various dilutions (5 to 80 mM) of H₂O₂ in R-20 for 6 h.

For the killing assay in cell-free medium, frozen aliquots of mycobacteria were thawed, diluted, and sonicated in the bath sonicator as described previously (26). The bacteria were resuspended at 6×10^5 bacilli per ml of R-20 and cultured at 0.5 ml/well on Falcon Primaria tissue culture 24-well plates for 1 h before treatment with various dilutions (5 to 80 mM) of H₂O₂ for 6 h.

(ii) Auramine-rhodamine staining for mycobacteria. For morphology determination, parallel cultures similar in all respects to those prepared on Primaria 24-well plates were prepared on 13-mm-diameter Thermanox coverslips. The monolayers of infected monocytes 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 being dried and mounted, stained bacteria were counted with a Nikon BG-12 excitation and an OG-1 barrier filter for epifluorescence.

(iii) Electron microscopy. Monolayers of monocytes cultured on Thermanox plastic coverslips were fixed in 2% glutaraldehyde–0.1 M sucrose–0.1 M cacodylate buffer and processed for electron microscopy as described previously (26). Sections were examined with a Jeol JEM 100CX transmission electron microscope. Photographs were taken on Kodak electron imaging film. About 35 infected monocytes per culture were evaluated for direct counting of the total number of bacilli and the number of degraded mycobacteria.

RESULTS

Growth of mycobacteria in freshly isolated human monocytes. Monolayers of adherent human monocytes plated for 2 h were infected with either *M. avium-M. intracellulare* or H37Ra at an MOI of one viable bacillus per cell. Infection at the MOI of 1 was chosen because at this MOI monolayers of infected monocytes remained fully intact for at least the first 7 days of culture. Our previous studies have shown that when the mycobacteria were added to adherent monocytes at this MOI, the

FIG. 1. Mycobacterial growth in cultured monocytes. A total of 3×10^5 viable mycobacteria were added to wells containing 0.5 ml of culture medium alone (open symbols) or 0.5 ml of culture medium containing 3×10^5 freshly isolated adherent monocytes (solid symbols). At the time points indicated, CFU were assayed. For comparison, CFU data for each mycobacterial species were normalized to the number of CFU at the start of the experiment and expressed as the fold change in CFU over time. The baseline CFU were $(34 \pm 2) \times 10^4$ for *M. avium-M. intracellulare* (MAI) and $(49 \pm 1) \times 10^4$ for H37Ra. Results are expressed as means $\pm$ standard errors of the means of two experiments, each done in triplicate. Numbers in parentheses denote the mean generation times for both strains of mycobacteria.

bacilli were phagocytosed by the cells and they survived and sometimes replicated within a unique phagocytic vacuole (26, 35). As a control, parallel cultures containing R-20 medium alone without monocytes were inoculated with the same number of organisms as for the cultures with monocytes. At the time points indicated, cells and medium were harvested and CFU assays were performed to determine the numbers of viable bacilli in the cultures. In the absence of human monocytes, H37Ra survived but did not replicate in the culture medium while *M. avium-M. intracellulare* neither replicated nor remained viable in the culture medium (Fig. 1). In the presence of human monocytes, only a slight change in the number of viable *M. avium-M. intracellulare* organisms was observed during the first 7 days in culture, while H37Ra appeared to replicate in cultured monocytes without any apparent lag time (Fig. 1).

After the first week of culture, H37Ra-infected monocytes began to show microscopic signs of disruption of the monolayer, detachment of some infected cells, and a slight reduction in the rate of replication. These changes were not observed in *M. avium-M. intracellulare*-infected cultures during the 14-day assay. The calculated doubling times of H37Ra and *M. avium-M. intracellulare* during 14 days of culture in human monocytes were 48 and 408 h, respectively. The doubling times for the two mycobacterial species were not affected by increasing the infecting inoculum up to an MOI of 10:1 (data not shown). Thus, within cultured human monocytes the intracellular growth of this selected isolate of *M. avium-M. intracellulare* was restricted compared to the intracellular growth of H37Ra. Because of the changes in the growth rate of H37Ra over time, all further growth experiments were limited to a maximum of 4 days of infection.

Effects of exogenously added H₂O₂ on the survival of intracellular and extracellular mycobacteria. Previously, we reported that treatment of infected human monocytes with low doses of H₂O₂ was associated with the killing of intracellular *M. avium-*

FIG. 2. Killing of mycobacteria by H₂O₂ treatment. Symbols: diamond, *M. avium-M. intracellulare*; circle, H37Ra. (A) A total of 3×10^5 newly explanted human monocytes were infected with one viable bacillus per cell and cultured for 4 days. H₂O₂ diluted in culture medium was added to intact monolayers (intracellular mycobacteria). Cultures were harvested for CFU assay after 6 h. (B) A total of 3×10^5 newly explanted human monocytes were infected with one viable bacillus per cell and cultured for 4 days. On day 4 postinfection, H₂O₂ diluted in culture medium was added to monolayers that had first been sonicated (extracellular mycobacteria). Cultures were harvested for CFU assay after 6 h. Results (A and B) are expressed as percent killing of mycobacteria and are means $\pm$ standard errors of the means of three to seven experiments each performed in triplicate. (C) A total of 3×10^5 bacilli were added to R-20 culture medium for 1 h before treatment with various dilutions of H₂O₂. All cultures were harvested for CFU assay after 6 h of treatment. Results are expressed as percent killing of mycobacteria and are the means $\pm$ standard deviations of two experiments, each done in triplicate.

M. intracellulare (20). To further explore the H₂O₂-induced killing of intracellular mycobacteria, we assessed the susceptibilities of two mycobacterial species to exposure to exogenous H₂O₂. Monocytes were infected with either *M. avium-M. intracellulare* or H37Ra immediately after introduction into culture and then incubated for 4 days. When monocytes infected with mycobacteria and incubated for 4 days were treated for 6 h with various doses of H₂O₂ (2 to 20 mM), a reduction in the number of CFU was observed, indicating killing of intracellular bacilli (Fig. 2A). For *M. avium-M. intracellulare*, maximal killing was observed (mean of 60% of bacilli killed per culture) when infected cells were treated with 10 mM H₂O₂. When H37Ra-infected cells were treated with 10 mM H₂O₂, the percentage of intracellular mycobacteria killed was 15% (Fig. 2A). Because of the difference in the total number of bacilli per culture (resulting from the differences in the growth rates of the two mycobacterial species during 4 days of culture), the numbers of bacilli killed within infected cells at 10 mM H₂O₂ were $(1.7 \pm 0.2) \times 10^5$ for *M. avium-M. intracellulare* and $(1.2 \pm 0.2) \times 10^5$ for H37Ra. To establish whether a given monocyte culture could kill only a certain number of intracellular organisms, we varied the infecting inoculum of *M. avium-M. intracellulare*. When human monocytes were infected with *M. avium-M. intracellulare* at MOIs of 5:1 and 10:1, the percentage of *M. avium-M. intracellulare* cells killed by 10 mM H₂O₂ was similar to that observed at an MOI of 1:1. Killing of

$58 \pm 13\%$ and $56 \pm 6\%$ of intracellular *M. avium-M. intracellulare* was observed at MOIs of 5:1 and 10:1, respectively. This represents a larger number of *M. avium-M. intracellulare* cells killed in the cells infected at the higher MOI. Thus, it appears that the percentage of intracellular mycobacteria killed is specific for a particular mycobacterial species rather than being determined by the number of organisms within the monocytes at the time of the killing stimulus. Moreover, those mycobacteria which replicate intracellularly more efficiently, e.g., H37Ra, have a survival advantage over time.

The ability of the mycobacteria to withstand the toxic effects of H₂O₂ when the infected monocytes were disrupted by sonication, which rendered the bacilli extracellular in the culture medium containing monocyte fragments, was investigated. Under these conditions, both species of mycobacteria were more resistant to H₂O₂-induced killing. The addition of H₂O₂ at concentrations of up to 20 mM to the cell sonicates had little effect on the viability of extracellular H37Ra (Fig. 2B). At 10 and 20 mM H₂O₂, sonicated cultures of *M. avium-M. intracellulare*-infected cells showed a slight decrease in the viability of the bacilli ($\sim 20\%$). As the concentration of exogenously added H₂O₂ was increased above 20 mM, both mycobacterial species were similarly affected (Fig. 2B) and were completely killed at 80 mM H₂O₂. Our results indicated that H₂O₂-induced killing of mycobacteria was more pronounced when the infected host cells were intact and the bacilli were within phagocytic vacuoles.

To determine the effect of H₂O₂ on the viability of mycobacteria in the culture medium in the absence of any monocyte fragments or components, frozen aliquots of *M. avium-M. intracellulare* and H37Ra were thawed, resuspended in R-20 medium, and cultured for 1 h before treatment with various dilutions of H₂O₂ for 6 h. CFU assays were performed at the end of 6 h, and the percent killing at different concentrations of H₂O₂ was determined for the two species of mycobacteria (Fig. 2C). Dose-dependent killing of mycobacteria in R-20 medium alone without any residual cellular components was observed between 5 and 40 mM H₂O₂. Extracellular *M. avium-M. intracellulare* appeared to be only slightly more sensitive to 20 mM H₂O₂ treatment than extracellular H37Ra. It is noteworthy that under these conditions the relative sensitivities of the two extracellular organisms to H₂O₂ were the same. The results also show that extracellular mycobacteria in medium containing monocyte lysates were less sensitive to H₂O₂ treatment than extracellular mycobacteria in cell-free culture media.

The infection studies carried out with H37Ra and *M. avium-M. intracellulare* suggested a relationship between intracellular growth rate and sensitivity to H₂O₂-induced killing. We investigated this further with additional mycobacterial strains, as shown in Table 1. Two additional strains of virulent *M. tuberculosis*, H37Rv and the clinical isolate CDC551, as well as BCG, were compared to *M. avium-M. intracellulare* and H37Ra. We observed that those organisms with a shorter generation time when growing within human monocytes, e.g., *M. tuberculosis* CDC551 and H37Rv, were killed less efficiently following exposure of the infected cells to exogenous H₂O₂. H37Rv and H37Ra were similar both in intracellular growth rates and susceptibilities to intracellular killing following addition of exogenous H₂O₂. BCG, which grew more slowly than the *M. tuberculosis* strains but more rapidly than *M. avium-M. intracellulare* was intermediate in its susceptibility to H₂O₂-induced killing. *M. avium-M. intracellulare*, which survived intracellularly without significant replication, was killed most efficiently by the addition of H₂O₂. These results confirmed the inverse relationship between intracellular rate of growth and efficiency of intracellular killing of mycobacteria by exogenous H₂O₂.

TABLE 1. Intracellular mycobacterial growth and sensitivity to H₂O₂-induced killing in human monocytes^a

Mycobacteria	Generation time (h) ^b	H ₂ O ₂ -induced killing (%)
<i>M. tuberculosis</i> CDC551	24 ± 4	20.8 ± 0.4
<i>M. tuberculosis</i> H37Rv	35 ± 3	22.8 ± 0.3
<i>M. tuberculosis</i> H37Ra	37 ± 2	21.7 ± 2.9
<i>M. bovis</i> BCG	95 ± 4	37.3 ± 5.5
<i>M. avium-M. intracellulare</i> clinical isolate	>400	60.4 ± 8.3

^a Monocytes infected for 4 days were incubated for 6 h at 37°C and 5% CO₂ and treated as described in Materials and Methods. Results are means of three to five experiments ± one standard error.

^b Generation time was calculated with monocytes infected on day 0 and cultured for 4 days.

The effect of inhibitors of ROI on H₂O₂-induced killing of intracellular mycobacteria. Since the addition of H₂O₂ induced the killing of intracellular bacteria, the potential role of other ROI in the killing of intracellular mycobacteria was examined by using various inhibitors. Figure 3 shows the major pathways involved in the generation of ROI as well as the inhibitors used in our study to block their production. In our experiments, the addition of 5 µg of catalase per ml to *M. avium-M. intracellulare*-infected monocytes 30 min before the addition of 10 mM H₂O₂ inhibited the killing of the intracellular mycobacteria by 85%. The addition of catalase after 30 min of H₂O₂ treatment or the removal of H₂O₂ from the culture supernatant by washing after 30 min of H₂O₂ treatment did not halt the killing of intracellular *M. avium-M. intracellulare* (Fig. 4). These experiments suggest that the addition of exogenous H₂O₂ initiates a killing mechanism that does not require the continued extracellular presence of H₂O₂.

To investigate whether H₂O₂-induced killing of intracellular *M. avium-M. intracellulare* requires transcription of new genes or new protein synthesis, *M. avium-M. intracellulare*-infected monocytes were treated with either 5 µg of actinomycin D per ml, 20 µg of CHX per ml, or 10 mM H₂O₂ or a combination of either actinomycin D plus H₂O₂ or CHX plus H₂O₂ for 6 h. Our results revealed no significant reduction in the numbers of CFU in actinomycin D-treated cultures or in CHX-treated cultures compared to the numbers in the untreated control cultures. Moreover, H₂O₂-induced killing of intracellular *M. avium-M. intracellulare* was unaffected when the infected cells were treated simultaneously with H₂O₂ and actinomycin D or with H₂O₂ and CHX (Table 2). Taken together, our results indicated that transcription and new protein synthesis did not appear to be required for the killing of intracellular *M. avium-M. intracellulare* by H₂O₂.

Previously, we have shown that H₂O₂-induced killing of intracellular *M. avium-M. intracellulare* was independent of nitric oxide production (20). Here, we investigated whether pretreatment of *M. avium-M. intracellulare*-infected monocytes with other inhibitors of ROI would affect the killing of intracellular *M. avium-M. intracellulare* induced by H₂O₂. The antioxidants used in this study were as follows: 10 µg of SOD (detoxifying superoxide anion) per ml, 10 µg of L-histidine (detoxifying singlet oxygen and hydroxy radical) per ml, and 5 to 50 mM DMTU (detoxifying hydrogen peroxide, hydroxyl radical, and hypochlorous acid) (Table 3 and Fig. 3). For all experiments, these scavengers of ROI were added to *M. avium-M. intracellulare*-infected monocytes 1 h prior to the addition of 10 mM H₂O₂ and were maintained in the culture through the course of the experiment. We observed that these antioxidants failed to inhibit the killing of intracellular *M. avium-M. intracellulare*.

Since treatment of cells with L-histidine and DMTU has been shown to result in the accumulation of these molecules in the cytoplasm of the treated cells (2, 4, 37), the lack of inhibitory effect of these scavengers in this system suggests that H₂O₂-induced killing of intracellular mycobacteria did not involve either singlet oxygen, hydroxyl radicals, or hypochlorous acid (Fig. 3).

The role of iron in H₂O₂-induced killing of intracellular mycobacteria was examined (Fig. 3). Iron ions have been shown to catalyze the Haber-Weiss reaction to generate hydroxyl radicals (11). When cells are treated with deferoxamine mesylate, a powerful chelator of Fe³⁺, the intracellular concentration of Fe³⁺ is decreased and interferes with this iron-dependent free-radical reaction (2, 12). In our experiments, 100 mM deferoxamine mesylate was added to *M. avium-M. intracellulare*-infected monocyte cultures 2 h before treatment with 10 mM H₂O₂ for 6 h. The same percentage of killing of intracellular *M. avium-M. intracellulare* in cultures treated with deferoxamine mesylate as in untreated cultures following 10 mM H₂O₂ treatment was observed (data not shown). Therefore, Fe³⁺ does not appear to be involved in the H₂O₂-induced killing of intracellular *M. avium-M. intracellulare*.

Activation-induced respiratory burst of human monocytes has been shown to be dependent on both Ca²⁺ and Mg²⁺ (18, 19) (Fig. 3). We therefore studied the effect of the chelation of divalent cations by the addition of 1 to 10 mM EDTA or 10 mM EGTA to *M. avium-M. intracellulare*-infected monocyte cultures 1 h prior to the addition of 10 mM H₂O₂. Both EDTA and EGTA treatment of *M. avium-M. intracellulare*-infected monocytes resulted in partial detachment of the infected cells, but the percent killing of intracellular *M. avium-M. intracellulare* was unaffected (data not shown). Taken together, our results indicate that other than catalase, scavengers of ROI and chelators of Fe³⁺, Ca²⁺, and Mg²⁺ did not affect H₂O₂-induced killing of intracellular *M. avium-M. intracellulare*.

Morphology of intracellular mycobacteria. Electron microscopy of mycobacteria-infected monocytes cultured on coverslips and infected with an MOI of 1:1 revealed that at all times the bacilli were localized within perinuclear vacuoles (Fig. 5). Electron-dense bacilli were surrounded by an electron-translucent zone, which was bounded by a tightly apposed lipid bilayer, and the bacteria appeared intact inside the vacuoles (Fig. 5a and c). Some bacilli appeared to be in the process of dividing, coincident with division of the vacuoles (Fig. 5C). Direct counting of electron micrographs of infected monocytes cultured for 4 days before fixation and processing revealed that H37Ra-infected and *M. avium-M. intracellulare*-infected monocytes contained an average of 10.3 and 2.5 bacilli per infected cell, respectively. Since the monocyte cultures were infected with similar numbers of mycobacteria, this observation is consistent with our findings by CFU assay that the growth of *M. avium-M. intracellulare* inside human monocytes is more restricted than that of H37Ra.

Following treatment of infected cells with a low dose (2 to 10 mM) of H₂O₂, changes in the phagocytic vacuoles containing *M. avium-M. intracellulare* were observed. *M. avium-M. intracellulare*-containing vacuoles which were originally tightly apposed to discrete bacilli (Fig. 5A and a) became swollen or irregular in appearance (Fig. 5B and b). Most impressively, some of the *M. avium-M. intracellulare* organisms in these altered vacuoles appeared to be degraded (Fig. 5B and b). These morphologic changes were seen only in the very few of the vacuoles containing H37Ra. H₂O₂-treated intracellular H37Ra remained predominantly intact (Fig. 5D and d). Electron microscopic examination of monocytes infected for 4 days with

Proposed Mechanism of Oxidative Killing of Mycobacteria in Human Monocytes

FIG. 3. The major pathways involved in the generation of ROI and reactive nitrogen intermediates (RNI) as well as the inhibitors used in our study to block their production. The agents used to block the generation of ROI and RNI are shown in blue, while their molecular targets are shown in yellow. Exogenously added H_2O_2 is shown in green. In our proposed mechanism of oxidative killing of mycobacteria within human monocytes, the putative host cell molecule which may be activated by H_2O_2 to mediate mycobacterial killing is shown in red. The figure is adapted from reference 35a and reproduced by permission of the publisher.

M. avium-M. intracellulare or H37Ra and treated for 6 h with a low dose of H_2O_2 revealed that the total numbers of morphologically degraded mycobacteria were similar. In *M. avium-M. intracellulare*-infected cultures, 29 of 84 organisms were found to be degraded in 34 different infected cells, while 33 of 362 H37Ra organisms were found to be morphologically damaged in 35 different infected cells. However, because the two mycobacterial species had replicated at different rates and had, by 4 days of culture, reached different total numbers of bacilli

per culture, the percentages of morphologically degraded bacilli present within phagocytic vacuoles were 34 and 9% for *M. avium-M. intracellulare* and H37Ra, respectively.

DISCUSSION

The ability of an intracellular pathogen to establish infection and cause disease depends on the capacity of the organism to survive and replicate despite the attempts of the host to elim-

FIG. 4. H₂O₂-induced killing of intracellular *M. avium-M. intracellulare*. A total of 3×10^5 monocytes were infected immediately after isolation and cultured for 4 days. On day 4 postinfection, H₂O₂ diluted in culture medium was added to intact monolayers at the final concentration of 10 mM. At the time points indicated, the exogenously added H₂O₂ was either washed away (A) or neutralized by the addition of 100 μ g of catalase per ml to the cultures (B) and the cultures were further incubated. At the end of the 6-h H₂O₂ treatment, cultures were harvested for CFU assay. The solid symbols in panels A and B denote the numbers of CFU at 6 h in the control untreated cultures of experiments A and B, respectively. Results are means $\pm$ standard errors of the means of triplicate cultures.

inate it. The success of the organism is therefore dependent on the balance between the replication of the pathogen and the killing of the pathogen by the host toxic defense mechanisms. We have examined the intracellular growth and killing of several different species of mycobacteria within human monocytes in vitro. These species are *M. tuberculosis* (strains H37Ra and H37Rv and the clinical isolate CDC551), BCG, and a clinical isolate of *M. avium-M. intracellulare* specifically selected for its relative inability to replicate within human monocytes in vitro (3). Our results indicate that mycobacteria which replicate efficiently and without any lag time, e.g., *M. tuberculosis*, have a survival advantage over a mycobacterium which replicates very slowly if at all, e.g., *M. avium-M. intracellulare*.

When infected monocytes were exposed to 10 mM H₂O₂, similar numbers (1.2×10^5 to 1.7×10^5) of intracellular H37Ra organisms and intracellular *M. avium-M. intracellulare* organisms were killed. This was also observed with the other mycobacteria tested. Because H37Ra replicated much faster than *M. avium-M. intracellulare* within the host phagocytes, the number of intracellular H37Ra organisms was higher than

TABLE 2. Killing of *M. avium-M. intracellulare* after treatment of infected monocytes with H₂O₂, actinomycin D, and CHX^a

Treatment	Concn	CFU (10 ⁴) ^b in:	
		Intact monolayers	Sonicated cultures
None (control)		18.0 $\pm$ 0.9	24.1 $\pm$ 1.0
CHX	20 μ g/ml	20.1 $\pm$ 1.0	24.0 $\pm$ 1.3
Act-D ^c	5 μ g/ml	17.2 $\pm$ 0.9	21.2 $\pm$ 1.1
H ₂ O ₂	10 mM	10.8 $\pm$ 0.7	21.0 $\pm$ 1.0
H ₂ O ₂ + CHX	10 mM + 20 μ g/ml	8.9 $\pm$ 0.7	18.4 $\pm$ 0.9
H ₂ O ₂ + Act-D	10 mM + 5 μ g/ml	6.7 $\pm$ 0.6	17.1 $\pm$ 0.9

^a Monocytes infected for 4 days were incubated for 6 h at 37°C and 5% CO₂ and treated as described in Materials and Methods.

^b Results are means of triplicate cultures $\pm$ one standard error.

^c Act-D, actinomycin D.

TABLE 3. Killing of *M. avium-M. intracellulare* after treatment of infected monocytes with H₂O₂ and antioxidants^a

Treatment ^b	Concn	CFU (10 ⁴) in:	
		Intact monolayers	Sonicated cultures
None (control)		20.0 $\pm$ 1.1	21.9 $\pm$ 1.4
H ₂ O ₂	10 mM	9.4 $\pm$ 0.7	18.1 $\pm$ 1.6
H ₂ O ₂ + catalase	10 mM + 5–10 μ g/ml	15.2 $\pm$ 0.9	17.7 $\pm$ 1.4
H ₂ O ₂ + SOD	10 mM + 5–10 μ g/ml	8.4 $\pm$ 0.8	15.9 $\pm$ 0.3
H ₂ O ₂ + DMTU	10 mM + 5 mM	6.5 $\pm$ 0.4	23.6 $\pm$ 1.4
H ₂ O ₂ + L-histidine	10 mM + 10 mg/ml	11.5 $\pm$ 0.6	18.7 $\pm$ 1.2

^a Monocytes infected for 4 days were pretreated with each antioxidant for 1 h before the addition of H₂O₂ and then incubated for 6 h at 37°C and 5% CO₂. Results are means of two to four experiments $\pm$ one standard error; each experiment was done in triplicate.

^b The addition of catalase, SOD, DMTU, and L-histidine alone at the concentrations shown to the intact monolayers and the sonicated cultures did not result in CFU that were significantly different from those of the control untreated cultures.

the number of intracellular *M. avium-M. intracellulare* organisms at the time of exposure to H₂O₂ despite analogous infecting inocula. Thus, because of the higher growth rate of H37Ra, a greater absolute number of intracellular organisms escaped killing following exposure to this toxic molecule. However, even when 10 times the starting number of *M. avium-M. intracellulare* organisms were used to infect monocytes, the same percentage of organisms were killed by 10 mM H₂O₂. It therefore appears that for a particular strain of mycobacteria, a specific percentage of infecting organisms is killed in a monocyte culture rather than a particular number of organisms. When we tested the sensitivities of extracellular *M. avium-M. intracellulare* and extracellular H37Ra as well as those of the other mycobacteria to exogenous H₂O₂, all mycobacteria were found to be equally sensitive to the toxic effects of the molecule. It is of interest to note that exposure of mycobacterial species residing in the phagocytic vacuoles of infected monocytes to a specific amount of exogenous H₂O₂ leads to killing of bacteria that is more efficient than the killing observed outside of the monocyte. There appears to be an inverse relationship between the intracellular growth rate of mycobacteria and the susceptibility of the organism to H₂O₂-induced killing. A similar relationship between growth rate and susceptibility to H₂O₂ treatment has also been reported for *Listeria monocytogenes* (1). In that paper, log-phase *L. monocytogenes* was shown to be less susceptible to killing by H₂O₂ than lag-phase bacteria.

This in vitro model may mirror the situation during chronic mycobacterial infection in vivo, since it has already been shown that human macrophages release H₂O₂ and superoxide anion during phagocytosis of intracellular pathogens, including mycobacteria (31, 33). When a mycobacterium entering the lung survives the reactive oxygen burst during phagocytosis and establishes infection within a lung macrophage, the organism is more likely to successfully maintain the infection if it can replicate efficiently before any successive reactive oxygen bursts from neighboring monocytes occur during phagocytosis. If, consistent with our results in vitro, when viable mycobacteria within phagocytes of the lung granuloma are exposed to H₂O₂, a specific percentage of mycobacteria are killed and the surviving mycobacteria do not replicate efficiently, then the next oxidative burst will further reduce the number of organisms, ultimately resulting in elimination of the infection. On the other hand, if the surviving mycobacteria replicate efficiently enough to overcome the killing capacity of successive

FIG. 5. Electron micrographs of mycobacterially infected monocytes. Freshly explanted monocytes were infected with one viable bacillus per cell and cultured for 4 days. (A and a) *M. avium-M. intracellulare*-infected monocytes, untreated; (B and b) *M. avium-M. intracellulare*-infected monocytes treated with 2 mM H_2O_2 for 6 h; (C and c) H37Ra-infected monocytes, untreated; (D and d) H37Ra-infected monocytes treated with 5 mM H_2O_2 . N, cell nucleus. Magnifications: $\times 5,700$ (A), $\times 33,600$ (a), $\times 8,625$ (B), $\times 17,250$ (b), $\times 9,750$ (C), $\times 24,375$ (c), $\times 2,250$ (D), $\times 27,600$ (d).

oxidative bursts within a granuloma, a chronic mycobacterial infection will be established and maintained.

How exogenous H_2O_2 treatment of infected cells results in the killing of intracellular mycobacteria is not understood. However, our studies suggest that ROI other than peroxides are not the mediators of killing in this system. In the experiments reported here, we observed that the presence of H_2O_2 during the first 30 min of the treatment period was both necessary and sufficient to induce killing of intracellular mycobacteria. After 30 min, if H_2O_2 was washed away or catalase was added to the culture supernatants, the percent killing of *M. avium-M. intracellulare* was unaffected. Since H_2O_2 diffuses freely through cell membranes, it is conceivable that treatment of the infected phagocytes with exogenous H_2O_2 may lead to the accumulation of a sufficiently toxic concentration of H_2O_2 within the phagosomes containing mycobacteria (Fig. 3). The observations reported here support the already established finding that the production of ROI by phagocytes contributes to the growth restriction and killing of a variety of intracellular pathogens including *Candida albicans*, *L. monocytogenes*, *Toxoplasma gondii*, and *Leishmania* spp. (21, 22, 29, 30, 32, 33, 36). Indeed, the virulence of *M. tuberculosis* clinical isolates has been reported to correlate directly with sensitivity of the bacilli to exogenously added H_2O_2 (15, 25). In addition, isoniazid-resistant strains of *M. bovis* shown to lack catalase activity have been found to be less capable of survival (less virulent) in infected rodents (42).

In the present studies, H_2O_2 treatment of the infected phagocytes may induce a secondary effector molecule in the monocytes, which is not inhibited by exogenously added cata-

lase, either because the effector molecule is not accessible to extracellular catalase or because it is not sensitive to degradation by the enzyme. It is possible that the effector molecule may exist in an inactive form, and during H_2O_2 treatment, the reaction of the exogenously added H_2O_2 with the effector molecule results in the generation of a secondary reactive effector molecule such as a lipid peroxide (Fig. 3). Indeed, organic hydroperoxides have been shown to be toxic to mycobacteria (38). Lipid peroxides produced by reaction with ROI are resistant to antioxidants (28). In our studies, antioxidants such as SOD, DMTU, and L-histidine did not affect the H_2O_2 -induced killing of intracellular *M. avium-M. intracellulare* (Fig. 3 and Table 2).

A correlation between the degrees of antimycobacterial activity of various cell fractions and the amount of nonesterified fatty acids has been reported (17). In that study, lysosome extracts from guinea pig liver cells, after immune activation, did not inhibit growth of BCG, while lysosomal membranes and cell extracts exhibited some growth-inhibiting activity. The strongest growth-inhibiting activity was localized to the cell membrane fractions (17). Since mycobacteria are resident in vacuoles derived from the plasma membranes of the mononuclear phagocytes, H_2O_2 -induced lipid peroxides produced following exposure to exogenous H_2O_2 may be present within the vacuolar membrane. It is thus possible that reactive lipid peroxides on the vacuoles containing mycobacteria may be responsible for the killing of intracellular mycobacteria following H_2O_2 treatment. We are currently investigating whether such organic peroxides are generated when mycobacterially infected monocytes are treated with H_2O_2 .

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