

# Apoptosis, but Not Necrosis, of Infected Monocytes Is Coupled with Killing of Intracellular *Bacillus Calmette-Guérin*

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## Summary

We have examined the effect of killing of host monocytes infected with bacillus Calmette-Guérin (BCG) on the viability of the intracellular mycobacteria. Peripheral blood monocytes were infected in vitro with a single bacillus per cell and maintained in culture for 6–8 d to allow the bacilli to replicate. Replicating viable BCG were found singly in perinuclear vacuoles bounded by tightly apposed lipid bilayers. Monocytes were then exposed to toxic mediators that induced killing of cells as evaluated by  $^{51}\text{Cr}$  release into the culture medium. Both hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (an inducer of cell necrosis) and adenosine triphosphate ( $\text{ATP}^{4-}$ ) (an inducer of cell apoptosis) treatment killed infected monocytes.  $\text{H}_2\text{O}_2$ -induced killing had no effect on BCG viability. ATP-induced cell death was accompanied by DNA fragmentation and nuclear condensation. Apoptosis was associated with a swelling of the phagocytic vacuoles which became multibacillary and with a reduction of BCG viability as enumerated by colony-forming units.

**T**uberculosis, once believed to be approaching eradication in developed countries, has reemerged in recent years as a potentially serious public health problem in the United States (1). Particularly threatening is the spread of multidrug-resistant strains (2), raising the spectre of untreatable disease and a return to the pre-antibiotic era, when tuberculosis was among the leading causes of death (3). It is becoming clear that our existing knowledge and current therapeutic and preventive strategies are inadequate to eliminate the disease. A comprehensive understanding of the pathogenesis of the disease is important in order to develop effective therapies and vaccines to combat tuberculosis.

Much has been learned about pathogenesis from studies of experimental *Mycobacterium tuberculosis* infections in animals. Disease progression has been studied in guinea pigs (4) and rabbits (5), which are highly susceptible to small infecting inocula. Resistance has been extensively studied in mice (6) because they routinely resolve infections initiated with massive intravenous inocula of virulent bacilli (7). A combination of in vivo and in vitro approaches has led to the definition of the mechanism of resistance: mycobacteria-responsive  $\text{CD4}^+$  T-lymphocytes are mobilized in response to infection (8), and activate infected macrophages to kill intracellular bacilli by locally secreting molecules such as  $\text{IFN-}\gamma$  (9, 10). Therefore, resistance in mice is acquired by the sensitization of  $\text{CD4}^+$  T-lymphocytes, and is expressed as the killing of bacilli by activated macrophages. However, none of the animal models fully reproduce the characteristics of the disease

in humans, in which persistent, asymptomatic infection is predominant, with  $\sim 10\%$  of infected individuals developing active disease at some point in their lives. The early onset of tuberculosis in pre-AIDS, HIV-infected individuals before other opportunistic infections, has provided circumstantial evidence for a central role for  $\text{CD4}^+$  T-lymphocytes in human resistance. However, mycobactericidal activity has never been detected in human macrophages infected in vitro (11, 12), not even in macrophages activated to kill other intracellular microbes such as *Toxoplasma gondii* (our unpublished results). In vivo during natural infection, early mycobacterial replication is restricted and some bacilli are probably killed at the time of development of cellular immunity, but the mechanism is not understood. At the present, animal models cannot explain how mycobacteria are killed in the immunocompetent human host.

The site of *M. tuberculosis* infection in an immunocompetent human host, and therefore the site where resistance is expressed and bacillary growth is restricted, is the pulmonary tubercle. This is an atypical, highly cellular granuloma, characterized by differentiated myeloid cells surrounded by lymphocytes (13). The anatomical architecture of the tubercle is such that cell products produced there would accumulate and be concentrated, including material released from dying cells and biologically active species secreted from activated monocytes and lymphocytes. The tubercle, in active disease, is distinguished from other granulomata by the extent of cell death that is observed there (14), which leads to liquefaction

and cavitation at the necrotic center. It is this necrosis which leads to the hallmark symptomatology of pulmonary tuberculosis, namely the destruction of lung tissue and bloody sputum. In severely immunocompromised patients, no granulomatous response or tissue necrosis is generated, and massive expansion of bacillary numbers is observed (15). Therefore, the control of tuberculous infection occurs in a granuloma and is intimately associated with the accumulation, activation, and death of mononuclear leukocytes.

Cells die by one of two known mechanisms, necrosis or apoptosis (16, 17). Necrosis, often referred to as accidental cell death, is induced when the plasma membrane of a cell is irreversibly damaged. Perforin, released from cytotoxic cells (18), and hydrogen peroxide ( $H_2O_2$ ) (19) produced by phagocytes during the phagocytic event, kill cells by destroying the integrity of their membranes, leading to osmotic dysregulation and necrosis. The other recognized mechanism of cell death is apoptosis, or programmed cell death. Apoptosis is distinguished from necrosis by biochemical and morphological criteria (20). Unlike necrosis, apoptosis is associated with rapid and profound changes in nuclear organization, and can be accompanied by fragmentation of the chromatin in the dying cell. Apoptosis is induced in different cell types by, among other signals, glucocorticoids (21), cytokines (22), and  $ATP^{4-}$  (23). Some CTL, in particular,  $CD4+$  T lymphocytes, have been reported to mediate their effect by inducing apoptosis in target cells (24). In the tubercle, both necrosis and apoptosis are observed, but apoptosis is believed to account for the bulk of the extensive cell death at that site (25).

To test the hypothesis that control of *M. tuberculosis* infection is associated with host-cell death, we have established an in vitro model of human mycobacterial infections. We have described previously how a population of MHC nonrestricted, antigen-independent, lymphokine-activated cytotoxic lymphocytes selectively recognizes and lyses monocytes chronically infected with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)<sup>1</sup>. Lysis of the host cell is predominantly through a necrotic mechanism by morphological criteria, and did not affect BCG viability (26). Here, we describe investigations to determine whether necrosis induced with  $H_2O_2$ , or apoptosis induced with  $ATP^{4-}$ , of cultured monocytes, chronically infected with BCG, is associated with any reduction in the viability of intracellular bacilli.

## Materials and Methods

### Reagents

**BCG.** The Pasteur Strain 1011 of BCG (Trudeau Institute, Saranac Lake, NY) was grown by serial 10-fold step-up cultures, each step-up grown for 7–10 d in spinning culture in endotoxin-free Proskauer-Beck medium modified by the addition of 0.05% Tween 80 to promote dispersion of the bacteria. Aliquots were stored in liquid nitrogen at  $3\text{--}10 \times 10^8$  CFU/ml. By the discrepancy be-

Tween 80 to promote dispersion of the bacteria. Aliquots were stored in liquid nitrogen at  $3\text{--}10 \times 10^8$  CFU/ml. By the discrepancy between the total particle count in a Petroff-Hauser chamber, and viable, CFU counts, frozen aliquots were shown to contain 30–50% of viable bacteria capable of growing on solid medium. Bacteria were endotoxin free and without reactivity in the Limulus Amoebocyte Lysate assay (Whittaker Bioproducts, Walkersville, MD). For infection of eukaryotic cells, an aliquot was thawed at  $37^\circ\text{C}$ , diluted 10-fold in culture medium, and sonicated to disperse clumps, with three 10-s pulses in a bath sonicator and cooling on ice between pulses.

**Media.** Human monocytes were washed and cultured in media containing RPMI 1640 (GIBCO BRL, Gaithersburg, MD) supplemented with  $AB^+$  serum produced from donors or pooled  $AB^+$  human serum obtained commercially (Biocell Ltd., Carson, CA). All cells were cultured in medium with 20% serum (R-20), and washed after culture in medium with 1% serum (R-1).

**Other Reagents.** Nucleotides ( $ATP^{4-}$  and  $GTP^{4-}$ ) (Boehringer Mannheim Biochemicals, Indianapolis, IN),  $H_2O_2$  and Gliotoxin (Sigma Chemical Co., St. Louis, MO), and recombinant IFN- $\gamma$  (Genzyme, Cambridge, MA) were obtained commercially.

### Cell Preparations

**PBMC.** Peripheral blood was obtained from donors by venipuncture, collected into heparinized syringes, and processed within 60 min of drawing. PBMC were isolated as described (27). Briefly, whole blood was diluted with 0.5 volume of RPMI 1640, layered on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and centrifuged at  $500 g$  for 20 min at  $25^\circ\text{C}$ . Interface cells were washed three times at  $4^\circ\text{C}$  including a low-speed centrifugation step at  $100 g$  to remove contaminating platelets. Cells were resuspended in R-1 and counted in a hemacytometer.

**Monocytes.**  $50\text{--}75 \times 10^6$  PBMC were directly adhered to a 100-mm tissue culture plate and washed with warm R-1, to remove nonadherent cells, after 60 min at  $37^\circ\text{C}$ . Adherent cells were cultured overnight at about  $2 \times 10^6$ /ml R-20. Cells were detached by replacing the culture medium with  $Ca^{2+}$ -,  $Mg^{2+}$ -free PBS, supplemented with 0.5 mM EDTA for 20 min at  $4^\circ\text{C}$ . Detached cells were pelleted, resuspended in 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 R-20 and cells plated on 13-mm Thermanox coverslips (Lux, Naperville, IL) in wells of 24-well plates, or Falcon Primaria tissue culture (Becton Dickinson Labware, Lincoln Park, NJ) 96- or 24-well plates so that the final density was  $1\text{--}2 \times 10^5$  cells/cm<sup>2</sup>, a density which gives rise to an even monolayer after 3–4 d of culture. Plates were incubated at  $37^\circ\text{C}$ , 5%  $CO_2$ . Cells isolated by either protocol were 90–95% monocytes by the criterion of CD14 expression. For infection, monocytes were exposed immediately after plating, or after 7 d of culture, to a single-cell suspension of BCG containing about one viable bacillus per cell. Since all bacilli ( $>98\%$ ) were phagocytosed, it was unnecessary to wash monolayers after infection to remove extracellular organisms. In all experiments, uninfected and infected monocytes were cultured and usually assayed in parallel.

### Procedures

**$ATP^{4-}$ - and  $H_2O_2$ -induced Cytotoxicity.** Monolayers of infected and uninfected monocytes were cultured for 6–8 d in flat bottom, Primaria 96-well tissue culture plates. Some cultures were treated with 100 U human IFN- $\gamma$ /ml for 3 d before assay. Adherent cells were loaded with 0.5  $\mu\text{Ci}$  sodium [<sup>51</sup>Cr]chromate (New England Nuclear, Boston, MA) for 16–18 h in situ at  $37^\circ\text{C}$ , washed twice

<sup>1</sup> Abbreviation used in this paper: BCG, bacillus Calmette-Guérin.

with warm R-1, incubated for 60 min in R-20, and washed twice more before the addition of 0.1 ml of culture medium and/or solutions of ATP<sup>4-</sup>, GTP<sup>4-</sup>, or H<sub>2</sub>O<sub>2</sub> reconstituted in R-20. Depending on the assay, as described in the text, nucleotide solutions were administered as a 30-min pulse and washed away, or were present throughout the assay. Plates were incubated for 6–18 h at 37°C in 5% CO<sub>2</sub>. Supernatants were removed and replaced with 0.1 ml 1% Triton X-100 which was harvested after 1–4 h. Supernatants and lysates were counted in a gamma counter (Packard Instrument Company, Downers Grove, IL) and <sup>51</sup>Cr release was calculated as (cpm in supernatant)/(cpm in supernatant + cpm in lysate). Spontaneous <sup>51</sup>Cr release was calculated as cpm in supernatants of control wells, and total <sup>51</sup>Cr release was calculated as total cpm in supernatants and lysates of control wells. Specific <sup>51</sup>Cr release was calculated as 100 × [(experimental <sup>51</sup>Cr release – spontaneous <sup>51</sup>Cr release)/(total <sup>51</sup>Cr release – spontaneous <sup>51</sup>Cr release)].

**CFU Assay.** Monolayers of ~2.5 × 10<sup>5</sup> infected monocytes in 0.5 ml of culture medium were cultured for 6–8 d in wells of flat bottom, Primaria 24-well tissue culture plates. Some cultures were treated with 100 U human IFN-γ/ml for 3 d before assay. For assay, an additional 0.5 ml of culture medium and/or solutions of ATP<sup>4-</sup>, GTP<sup>4-</sup>, or H<sub>2</sub>O<sub>2</sub> reconstituted in culture medium were added. Plates were incubated for 6 h at 37°C in 5% CO<sub>2</sub>. Culture medium was removed, replaced with 1 ml of 0.008% digitonin (Sigma Chemical Co.) in PBS and incubated at 37°C for 10 min. Culture medium and cell lysate were pooled and sonicated with five half-second pulses at low output power to disperse clumps of bacilli. This amount of sonication had been previously determined to be optimal for dispersing bacteria without causing any loss of viability. Serial 10-fold dilutions were made with vigorous 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 and kept at 37°C. After 2–3 wk, drops containing 5–50 colonies arising from single mycobacteria were counted with the aid of a dissecting microscope. For each culture, six replicate drops were counted and the mean calculated. At least three replicate cultures were assayed for each data point.

**In Situ Nick Translation.** Monolayers of monocytes on coverslips were fixed in methanol/acetone (3:1) and washed. 60 μl of a reaction mixture of 6 μl 10X nick translation buffer (0.5 M Tris-HCl, pH 8, 0.1 M MgSO<sub>4</sub>, 1 mM dithiothreitol, 500 μg/ml BSA), 1.2 μl digoxigenin-labeled nucleotides (Dig DNA labeling mixture, Boehringer Mannheim), 5 U DNA Polymerase I (Boehringer Mannheim) was added to each coverslip which was incubated at 37°C for 60 min. The reaction was stopped by washing in 50 mM EDTA in Tris-buffered saline (TBS) and washed four times with TBS. The coverslips were incubated with 20 μg/ml FITC-labeled anti-digoxigenin Fab (Boehringer Mannheim) in TBS with 3% fetal bovine serum for 40 min at room temperature. The coverslips were washed three times, mounted in glycerol, and examined with epifluorescence microscopy. Parallel control coverslips were treated identically, except that DNA polymerase I was omitted from the reaction mixture.

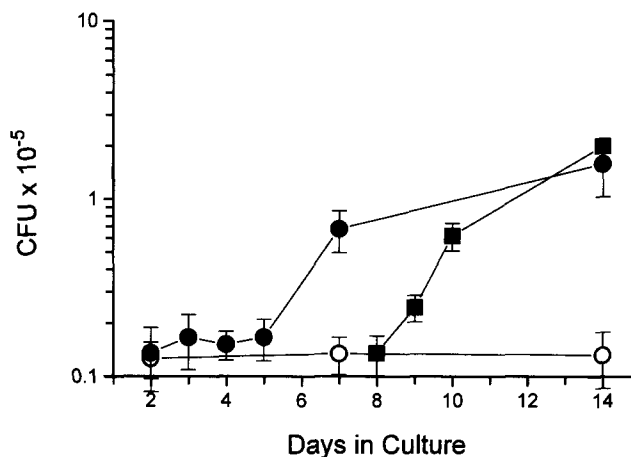
**DNA Fragmentation Assay.** Monocytes harvested as described above were cultured for 7 d in 100 × 20 mm Primaria tissue culture plates. 50–75 × 10<sup>6</sup> macrophages were used for each condition. ATP<sup>4-</sup> reconstituted in R-20 was added to cultured cells at the final concentration of 2.5 mM and incubated for 4 h at 37°C in 5% CO<sub>2</sub>. Both treated and untreated control cells were detached from the plates using a rubber policeman, and centrifuged at 100 g for 10 min. The pellet was lysed with 0.6 ml hypotonic lysing buffer (10 mM Tris, 1 mM EDTA, pH 7.5) containing 0.2% Triton X-100, and the lysates were centrifuged at 13,000 g for 10

min to separate intact from fragmented chromatin. The pellet was discarded and the supernatant was incubated at 50°C for 1 h with 100 μg/ml Proteinase K, extracted twice with phenol/chloroform, and precipitated overnight at –20°C in 50% isopropanol and 0.5 M NaCl. The precipitates were pelleted by centrifugation at 13,000 g for 10 min, air-dried, and resuspended in 10 mM Tris, 1 mM EDTA, pH 7.4. The DNA solution was subjected to conventional electrophoresis: 1.5% agarose for 2 h at 60 V. DNA was visualized with ethidium bromide.

**Electron Microscopy.** Monolayers of monocytes on coverslips were fixed in 2% glutaraldehyde/2% paraformaldehyde/0.1 M cacodylate buffer for 60 min at 4°C. The monolayers were postfixed in OsO<sub>4</sub> for 6 h at 4°C, stained for 2 h with 0.25% uranyl acetate, dehydrated in increments with alcohol, and embedded in Epon blocks. Sections were stained with uranyl acetate and lead citrate and examined with a Jeol JEM 100CX transmission electron microscope. Photographs were taken on Kodak electron imaging film.

## Results

**Freshly Isolated Monocytes Have some Capacity to Inhibit BCG Replication, but Aged Monocytes Are Permissive.** Monolayers of purified monocytes were infected with one viable bacillus per cell, immediately after isolation from blood and introduction into tissue culture. Alternatively cells were maintained for 7 d of in vitro culture and then infected. At the time points indicated, numbers of viable bacilli (CFU) were determined (Fig. 1). When monocytes were infected immediately after isolation, BCG replicated slowly at first, and more quickly after 3 d. The intracellular doubling time, initially 60–100 h between days 1 and 3, fell to ~40 h between days 4 and 6. When monocytes were cultured for 7 d before infection, no lag in the rate of growth was seen and BCG replicated at the optimal rate as soon as they were phagocytosed.

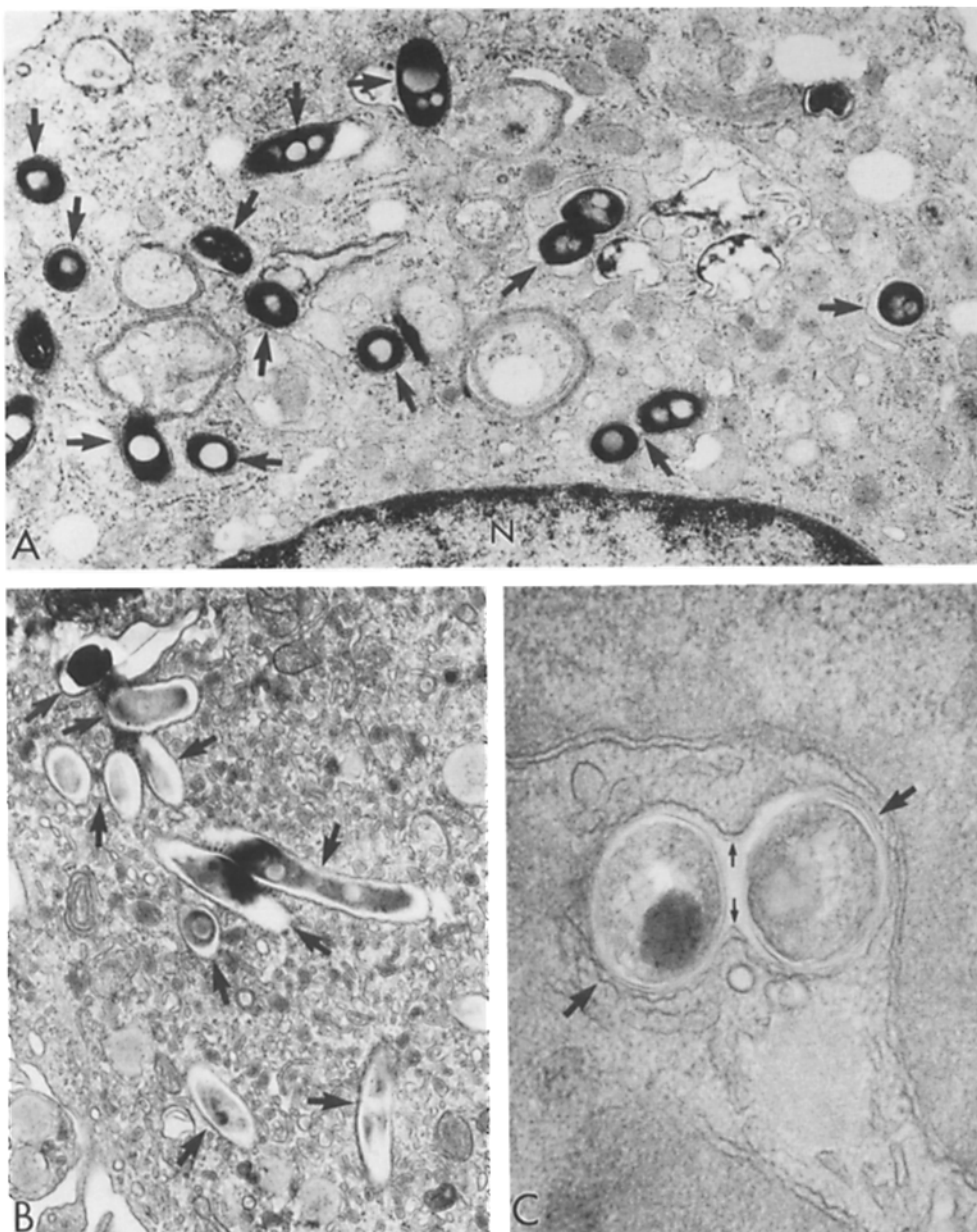


**Figure 1.** BCG growth in cultured monocytes. On day 1 after the establishment of monocyte cultures, 2–10 × 10<sup>3</sup> viable BCG were added to 0.2 ml culture medium containing no cells (○) or 5 × 10<sup>4</sup> freshly isolated monocytes (●). Alternatively, 2–10 × 10<sup>3</sup> viable bacilli were added to 5 × 10<sup>4</sup> 7-d cultured monocytes (■). At the time points indicated, viable bacilli were estimated. Monocytes, but not culture medium alone, supported the growth of BCG. Aged monocytes supported more rapid growth than young monocytes. Results expressed as CFU/culture as means ± SD of octuplicate cultures.

Replication was obligately intracellular, since no replication was observed in cultures without human cells. In a series of experiments, optimal intracellular, doubling times ranged from 22 to 50 h. These observations suggested that freshly explanted monocytes could partially restrict the replication of BCG and that, as monocytes differentiated into macrophages in vitro, they lost the small capacity they initially had to inhibit mycobacterial replication.

*The Morphology of the BCG Vacuole.* High power examination of cells by light microscopy revealed that acid fast bacilli were located around the nucleus in the body of the cell (data not shown). Structures with two bacilli joined at one extremity were apparent. These V-shaped forms were only seen after replication of bacilli, and may represent dividing bacilli.

The ultrastructure of the vacuole was investigated by electron microscopy (Fig. 2). Bacilli were perinuclear, confirming the observations in light microscopy. Electron-dense bacilli were surrounded by an electron-translucent zone, which was in turn bounded by a tightly apposed lipid bilayer. All bacilli were surrounded by similar structures. Electron micrographs revealed that unphagocytosed bacilli were uniformly electron dense (data not shown), suggesting that the lipid bilayer was specific to the bacillus-monocyte interaction, and probably derived from the host cell. Given the low multiplicity of infection (one bacillus per cell) and the fact that 50–70% of the phagocytes were infected with a single organism, the bacilli seen after 7 d in most cells arose from the single infecting organism. These entered a phagosome, a vacuole derived from



**Figure 2.** Transmission electron micrographs of chronically infected monocytes. Two low power (A,  $\times 17,500$  and B,  $\times 16,000$ ) and a high power (C,  $\times 66,000$ ) micrographs showing BCG, surrounded by an electron translucent zone, within tight lipid bilayer-bounded vacuoles inside monocytes (arrows). In C the vacuole can be seen to divide with the division of bacilli (small arrows). Thus, each bacillus is ultimately found within a separate vacuole. (N) Cell nucleus.

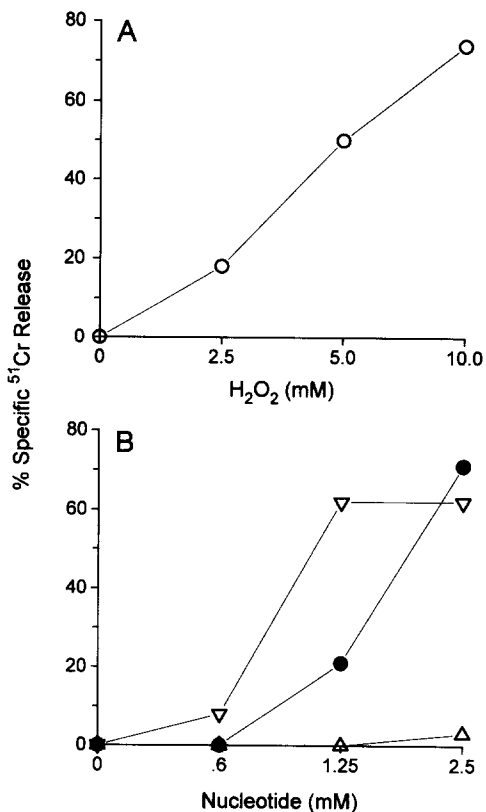
the plasma membrane of the host cell. The fact that all daughter bacilli were surrounded by a tightly apposed membrane, suggested that vacuolar membranes had divided along with the dividing bacillus. The structure shown (Fig. 2 C) may represent a vacuole in the process of dividing. It was not clear whether or not vacuoles pinched off as do the bacilli, i.e., whether the system consisted of discrete vacuoles, or of one or a few continuous threadlike vacuoles. In these studies, no escape of any bacilli from the vacuole into the cytoplasm was observed. This is in contrast to observations made in similar systems investigating cultured macrophages infected with virulent tubercle bacilli (28, 29).

*H<sub>2</sub>O<sub>2</sub> and ATP<sup>4-</sup>, but Not GTP<sup>4-</sup>, Killed Aged, Infected Monocytes.* Infected monocytes, exposed to solutions of H<sub>2</sub>O<sub>2</sub>, died in a dose-dependent fashion (Fig. 3 A). H<sub>2</sub>O<sub>2</sub> was present throughout the 18-h assay and the dose-response curve for H<sub>2</sub>O<sub>2</sub>-induced cell death was similar for different donors. Infected monocytes, treated with a pulse of ATP<sup>4-</sup>

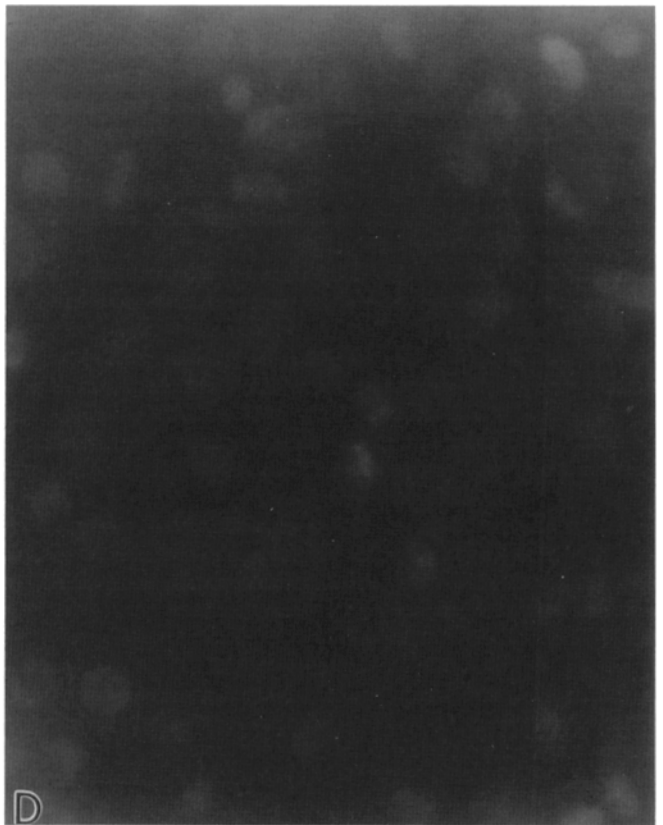
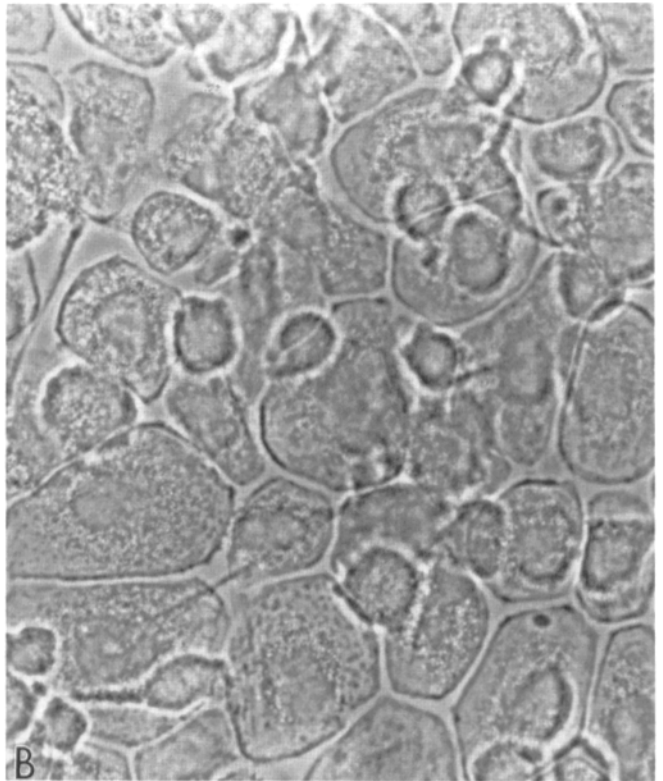
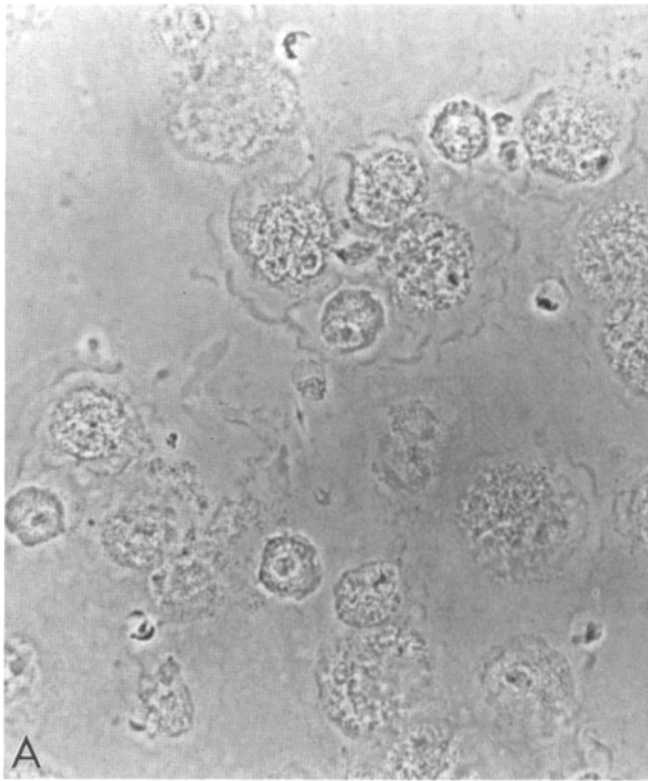
for 30 min, also died in a dose-dependent fashion (Fig. 3 B). No specific <sup>51</sup>Cr-release was detected at the end of the 30-min pulse of ATP<sup>4-</sup>, but cells died over the following 4–20 h. Therefore, during the pulse, a signal was delivered, after which the presence of ATP<sup>4-</sup> was no longer necessary for cell death to eventually occur. The dose-response curve for ATP<sup>4-</sup>-induced cell death varied considerably between donors, and between assays. In 18-h assays, the concentration of ATP<sup>4-</sup>-inducing 50% <sup>51</sup>Cr-release varied between 0.5 and 2.5 mM. 20 h after the exposure of cultured monocytes to ATP<sup>4-</sup>, at least 30% of the intracellular radiolabel was still associated with cell debris. However, morphological examination of ATP<sup>4-</sup>-treated cultures revealed few (<10%) undamaged cells. Parallel treatment with GTP<sup>4-</sup> had no effect on cell viability. The dose-response curves of parallel assays with infected and uninfected cells were identical (data not shown), suggesting that the presence of replicating bacilli neither predisposed towards, nor protected against, either H<sub>2</sub>O<sub>2</sub>- or ATP<sup>4-</sup>-induced cell death. ATP<sup>4-</sup>-induced cell death has been reported to be favored by preexposure of target macrophages to IFN- $\gamma$  (30). This condition is probably represented in the granuloma; therefore, the effect of pretreatment with IFN- $\gamma$  was investigated. This treatment altered slightly the dose-response curve (Fig. 3 B). However, the kinetics of cell death was not affected (data not shown). Other treatments, including serum starvation and gliotoxin (1–10  $\mu$ M), reported to induce apoptosis in other systems (31), failed to result in the death of 7-d cultured human monocytes (data not shown).

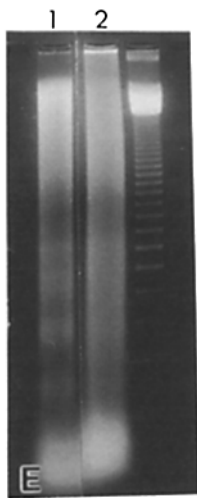
*ATP<sup>4-</sup> Induces DNA Fragmentation in Cultured Monocytes.* DNA fragmentation is a hallmark of apoptotic cell death (17, 32). ATP<sup>4-</sup>-induced death of infected monocytes was accompanied by fragmentation of cellular chromatin (Fig. 4). Phase contrast microscopy of control and ATP<sup>4-</sup>-treated cells revealed a reduction in cell density, and alterations in morphology induced by ATP<sup>4-</sup> treatment. Cells which remained attached were rounded and small. In particular, pyknotic nuclei were apparent. Pyknotic nuclei are dense, shrunken residual bodies of condensed chromatin. When free ends of DNA were enzymatically identified by fluorescent labeling, pyknotic nuclei induced by ATP<sup>4-</sup> treatment were intensely fluorescent (Fig. 4 C), suggesting that the chromatin inside had been fragmented. No fluorescent signal was obtained without DNA polymerase (Fig. 4 D). Fragmentation of DNA into discrete, nucleosome-sized fragments was detected by agarose gel electrophoresis (Fig. 4 E). Cell death was not blocked by a combination of emetine and cycloheximide which blocked over 95% of incorporation of [<sup>35</sup>S]methionine into TCA-precipitable protein. However, these results suggested that ATP<sup>4-</sup> induced apoptosis in cultured monocytes.

*H<sub>2</sub>O<sub>2</sub>-induced Cell Death Is Accompanied by Morphological Changes Typical of Necrosis.* The morphology of dying cells was investigated by transmission electron microscopy. H<sub>2</sub>O<sub>2</sub>-induced cell death (Fig. 5 A) was characterized by the loss of integrity of internal organelles and the cytoplasmic membrane, with less obvious early gross alteration in nuclear organization. These morphological changes were typical of necrotic cell death.



**Figure 3.** H<sub>2</sub>O<sub>2</sub> and ATP<sup>4-</sup> induce death of monocytes. 10<sup>5</sup> <sup>51</sup>Cr-labeled chronically infected monocytes were incubated under standard conditions (normal culture medium, 5% CO<sub>2</sub>) for 16 h in the presence of (A) 2.5–10 mM H<sub>2</sub>O<sub>2</sub> (O) and (B) after exposure to a 30-min pulse of 0.6–2.5 mM GTP ( $\Delta$ ) or ATP ( $\bullet$ ). Assays of ATP<sup>4-</sup>-induced death were also performed upon cells pretreated for 3 d with 100 U IFN- $\gamma$ /ml ( $\nabla$ ). Cells were treated with solutions of nucleotides in culture medium for 30 min, then washed and returned into normal culture medium for the duration of the assay. H<sub>2</sub>O<sub>2</sub> and ATP<sup>4-</sup>, but not GTP, were toxic to monocytes. Results are means of triplicate cultures.

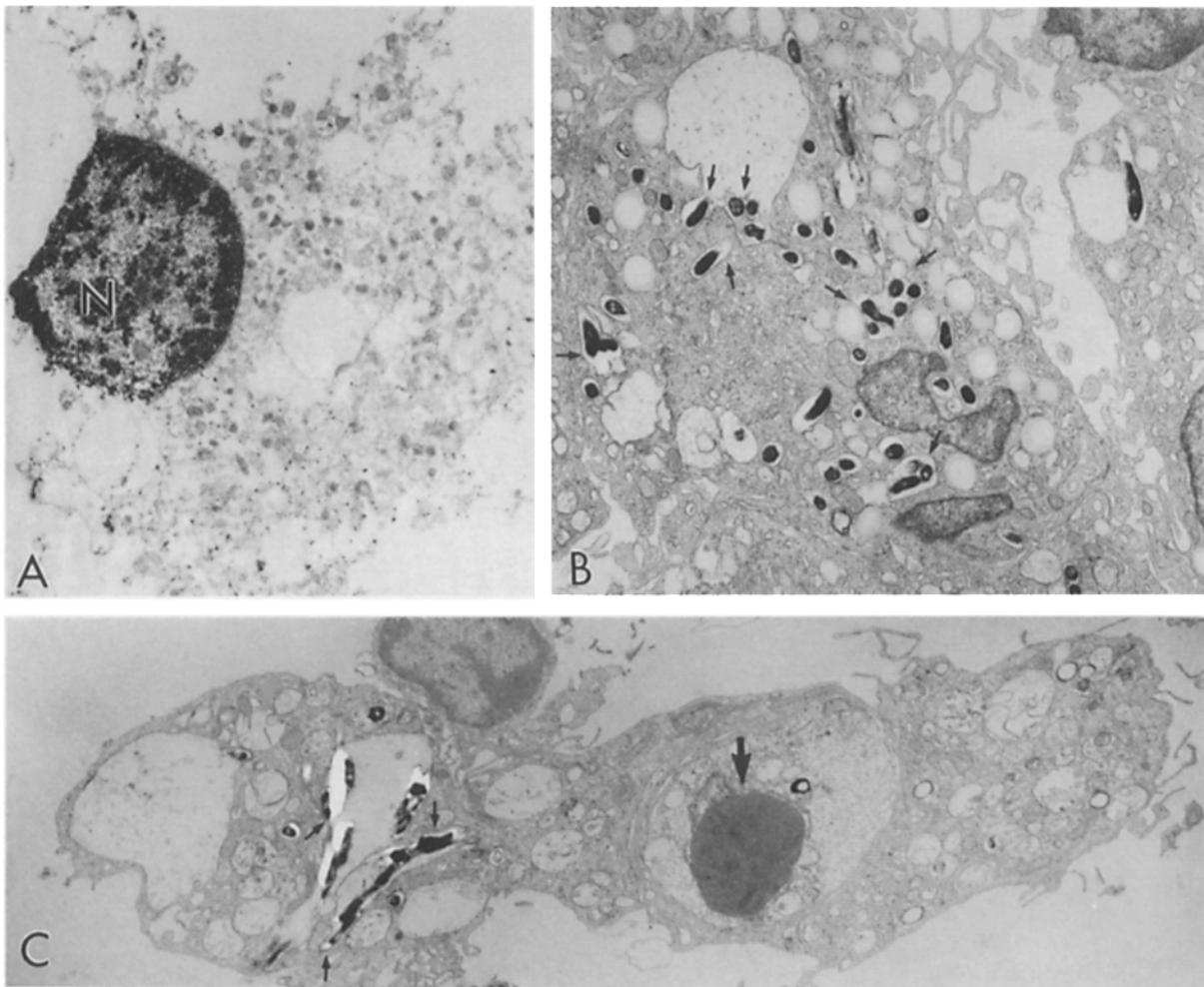




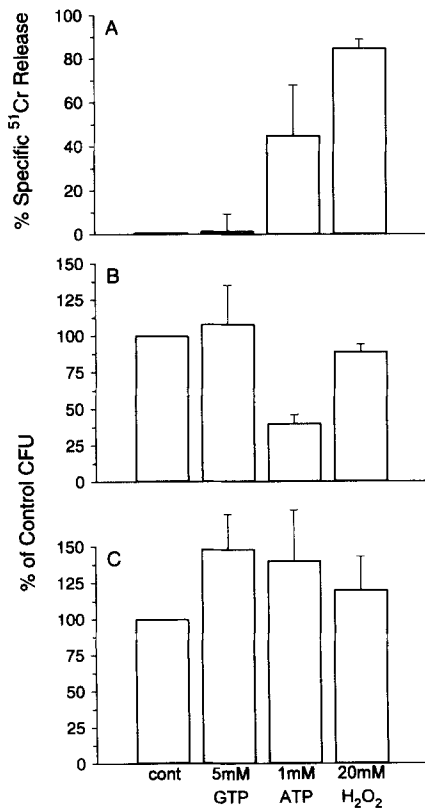
**Figure 4.** ATP-induced cell death is accompanied by fragmentation of chromatin.  $5 \times 10^5$  monocytes on coverslips were incubated for 18 h with medium (B and D) or with ATP<sup>4-</sup> (A and C). Free ends of DNA were labeled with FITC. Corresponding fields were photographed for phase (A and B) and fluorescence (C and D) optics. Free ends of DNA were detected in ATP<sup>4-</sup>-treated cells (arrows) and not in controls. DNA electrophoresis (E),  $5-7.5 \times 10^7$  monocytes were incubated for 4 h with medium (lane 2) or with ATP<sup>4-</sup> (lane 1). The characteristic ladder pattern of fragmented DNA is seen in lane 1. The numbers on the left denote the size (basepair) of a DNA marker.

ATP<sup>4-</sup>-induced Apoptosis of Infected Monocytes Is Accompanied by Changes in BCG Vacuolar Organization. ATP<sup>4-</sup>-induced cell death was accompanied by morphological changes typical of apoptosis. Within 1 h, and more prominently after 6 h of ATP<sup>4-</sup> treatment, morphological characteristics of apoptosis were apparent, including shrinkage of cytoplasm, nuclear condensation, and phagocytosis of apoptotic cells by neighboring cells (Fig. 5, B and C). Of interest in these dying cells were alterations in vacuolar apparatus. Vacuoles which originally were tightly apposed to the bacteria and contained discrete bacilli (Fig. 2) had now swollen and contained several, often morphologically damaged bacilli in a single vacuole.

ATP<sup>4-</sup>-induced Apoptosis, but Not H<sub>2</sub>O<sub>2</sub>-induced Necrosis Is Coupled with Killing of Intracellular BCG. To study the effects of different mechanisms of cell death on bacillary viability, parallel cytotoxicity (Fig. 6 A) and CFU assays (Fig. 6, B and C) were performed. When infected cells were incubated for 6 h with 20 mM H<sub>2</sub>O<sub>2</sub> more than 80% of intracellular



**Figure 5.** The morphology of dying monocytes examined by transmission electron microscopy. Monocytes were incubated with 10 mM H<sub>2</sub>O<sub>2</sub> for 4 h (A). Cell swelling, loss of plasma-membrane integrity, and major changes of the organelles were observed. Monocytes were incubated with 5 mM ATP<sup>4-</sup> for 60 min (B) and 6 h (C). Nuclear condensation was observed in cells treated with ATP<sup>4-</sup>. ATP<sup>4-</sup> treatment was also associated with swelling of vacuoles and possible fusion (small arrows) of hitherto tight, isolated BCG-containing vacuoles. (N) Cell Nucleus. (A)  $\times 6,600$ ; (B)  $\times 6,300$ ; and (C)  $\times 2,000$ .



**Figure 6.** Host-cell death and BCG viability.  $2.5 \times 10^5$  monocytes were infected immediately after isolation and cultured for 7 d.  $^{51}\text{Cr}$ -release (A) and BCG growth (B and C) were evaluated. Cells were labeled with  $^{51}\text{Cr}$  and treated with medium alone (cont), 5 mM GTP, 1 mM ATP, and 20 mM  $\text{H}_2\text{O}_2$ . After 6 h, specific  $^{51}\text{Cr}$  release was determined (A). In the same experiments, parallel  $^{51}\text{Cr}$  unlabeled cultures were treated with the same agents. Solutions were added to intact monolayers (B), or to monolayers that had first been sonicated (C). Cultures were harvested for CFU assay after 6 h. As seen in B, ATP- but not  $\text{H}_2\text{O}_2$ -induced cell death resulted in a reduction in bacillary viability. In four experiments, CFU ( $\times 10^{-4}$ ) were reduced relative to the GTP/control as follows: from 24 to 7 (24%); 62 to 28 (42%); 64 to 20 (31%); and 25 to 11 (45%). This reduction was coupled with the apoptotic event since no reduction in viability was observed in cells lysed before ATP $^{4-}$  treatment as shown in C. Results are means  $\pm$  SEM of the same four experiments (A and B), and means  $\pm$  SD of triplicate cultures of one of the above experiments.

$^{51}\text{Cr}$  was released.  $\text{H}_2\text{O}_2$ -induced host cell death had no effect on bacillary viability as detected by comparison of CFU from treated and untreated cultures. Exposure to 1 mM ATP $^{4-}$  resulted in the release of  $\sim 45\%$  of intracellular  $^{51}\text{Cr}$  over 6 h (Fig. 6 A). ATP $^{4-}$ -induced cell death was coupled to a threefold reduction in bacillary numbers (Fig. 6 B). If infected monocytes were killed by sonication before the beginning of the assay, the presence of ATP $^{4-}$  had no effect on bacillary viability (Fig. 6 C). Recoveries of CFU were only decreased in populations allowed to undergo apoptosis, suggesting that the reduction in bacillary viability was directly coupled with the apoptotic event. Parallel treatment with 5 mM GTP $^{4-}$  had no effect on either monocyte viability or recovery of viable bacilli.

When conditions representative of those in the granuloma,

**Table 1.** The Effect of IFN- $\gamma$  Pretreatment on ATP $^{4-}$ -induced Bacillary Killing

	CFU $\times 10^{-4}$ *	
	Control	+ IFN- $\gamma$
Culture medium	66.0 $\pm$ 5.2 $^\dagger$	55.8 $\pm$ 6.2 $^\S$
1 mM ATP $^{4-}$	34.4 $\pm$ 9.6	29.3 $\pm$ 10.1

\* Results are means  $\pm$  SEM for sextuplicate estimates of parallel cultures.  
 $^\dagger$  7-d infected monocytes were incubated for 6 h at 37°C, 5%  $\text{CO}_2$ , with and without ATP $^{4-}$ .

$^\S$  Parallel cultures were incubated with 100 U IFN- $\gamma$ /ml for 3 d before assay.

namely the presence of IFN- $\gamma$ , were investigated, although an effect was seen on the dose-response of cell death in response to ATP $^{4-}$  (Fig. 3), no significant effect on the extent of bacillary inhibition was observed (Table 1) in a parallel assay.

## Discussion

Apoptosis, but not necrosis, of chronically infected cells resulted in a 60–70% loss in viability of intracellular bacilli. Since the intracellular doubling time of the bacilli was between 20 and 40 h, the threefold reduction in bacillary numbers observed over 6 h suggested that active killing, rather than stasis, of the intracellular organisms was induced. Because reductions in bacillary numbers were only seen if cells were allowed to undergo apoptosis, rather than lysed before treatment with ATP, killing was specifically associated with the apoptotic event. In another in vitro system, infection with *Shigella flexneri* was observed to induce apoptosis of infected cells (33), but the effect on bacterial viability was difficult to assay in that system. The mechanism by which apoptosis is linked to the killing of intracellular bacteria is currently under investigation in our laboratory. An observation of major interest is that the apoptotic death of the infected monocyte was accompanied by profound changes in the architecture of the vacuolar system of the intracellular mycobacteria (Fig. 5). Vacuoles, previously discrete, became multibacillary. It has been suggested that the resistance of mycobacteria to macrophage microbicidal pathways is intimately associated with a capacity of the parasite to dictate its own intracellular environment, in particular, to inhibit fusion of its own vacuole with other membrane-bound host-cell compartments, including lysosomes (34, 35). It has been shown that chronically infected monocytes present antigens derived from intracellular mycobacteria poorly (36), suggesting that the vacuole, in which BCG resides, is indeed outside the normal intracellular, intervacuolar trafficking. Also, recently, vacuolar acidification has been shown to be inhibited in *Mycobacterium avium* containing phagosomes (37). Assuming that observations made on one mycobacterium species are relevant to another and that control of acidification and fusion are sub-



verted during apoptosis might explain why intracellular mycobacteria, previously not killed, are suddenly killed. For example, apoptosis of host cell could be accompanied by a breakdown in inhibition of acidification of the vacuoles and/or by the induction of fusion of BCG-containing vacuoles with toxin-containing host vacuoles, like lysosomes. Alternatively, since apoptosis is accompanied by fragmentation of nuclear chromatin (Fig. 4), the integrity of the genetic material of bacilli could be compromised.

A limited nonsignificant effect on the extent of apoptosis, and on the effect of apoptosis on BCG viability was seen if assays were performed after pretreatment with IFN- $\gamma$ . The rationale for the investigation of this condition in this system was that IFN- $\gamma$  has been observed to potentiate apoptosis of macrophages in other systems (30), and the cytokine might be expected to be present in the tubercle, in vivo. In our hands, not only was there little effect of IFN- $\gamma$  on apoptosis, but neither was there observed any effect on intracellular BCG growth even in the presence of other macrophage-activating factors. No effect on growth was seen if monocytes were treated before infection, or at the same time as infection, with moderate to high concentrations of IFN- $\gamma$ , TNF- $\alpha$ , GM-CSF, or a combination of IFN- $\gamma$  with TNF- $\alpha$  (our unpublished results). In other systems, monocytes treated with IFN- $\gamma$  have been reported to be more permissive for the growth of some intracellular mycobacteria (11, 12, 38). This may have been an indirect effect due to enhanced adherence of IFN- $\gamma$ -treated monocytes. In our hands, fewer cells were lost in IFN- $\gamma$ -treated cultures, compared with control, untreated cultures, and therefore recoveries of CFU were slightly higher. When CFU per nucleus were quantitated, no effects of IFN- $\gamma$ , or of any of the other cytokines assayed, were observed.

In vitro, apoptosis of infected monocytes was induced with ATP $^{4-}$ . This apoptotic response of human monocytes to ATP $^{4-}$  is physiological in that ATP $^{4-}$  is produced in vivo and this interaction is mediated by a signaling event initiated at an ATP $^{4-}$  receptor normally found on the cell surface (39, 40). Whether the millimolar concentrations used in vitro would be achieved in vivo in the tubercle is not known. ATP $^{4-}$  is released from many cells upon dying, and has been shown to be released from cytotoxic lymphocytes that induce apoptotic death in target cells. High concentrations of biologically active species, conceivably including ATP $^{4-}$ , might be attained in the synapselike cleft formed between a cytotoxic cell and its target. Alternatively, ATP $^{4-}$  may bind to the apoptosis-inducing receptor on monocytes with low affinity and may not be the physiological ligand for the receptor. The true physiological ligand may have a higher affinity, and induce apoptosis at a lower concentration.

Apoptosis is a normal, physiological response observed in a variety of metazoan systems, including the developing embryo and the developing immune system, where sacrifice of certain cells is ultimately of benefit to the organism. Therefore, it is not surprising that an apoptotic mechanism may have evolved to eliminate individual parasitized cells and inhibit the parasite simultaneously. In this respect, the apoptotic pathway has been implicated in resistance to viral infec-

tions (41), and perturbation of the apoptotic pathway is implicated in the pathogenesis of both viral infections (42) and malignancies (43).

Modest control of BCG was observed in the in vitro system. Young, freshly isolated monocytes exerted a minor inhibitory effect on the growth of intracellular BCG (Fig. 1), and a threefold reduction in bacillary viability was coupled with the apoptotic death of populations of infected cells (Fig. 6). These effects appear modest compared with physiological cytotoxic responses to other pathogens. However, they are striking when one considers the generation time of the bacilli as compared with other microorganisms. After infection of a naive animal, the numbers of *L. monocytogenes* begin to fall a few days after infection and decrease by about 2 log over 4 d, or by about 0.5 log per day (44). In contrast, in BCG-infected mice, it takes about 10 d after bacillary numbers begin to decline, to observe a decrease of only one log, corresponding to about 0.1 log per day (45). Based on the rate of killing, in vivo, resistance to BCG is less efficient than resistance to *L. monocytogenes*. However, the rate of killing does not measure the efficiency of resistance. Regardless of the rate of killing, if over 50% of bacilli are killed each generation, the host is resistant and will ultimately overcome the infection. If fewer than 50% per generation are killed, the numbers of the pathogen will increase and the host will succumb. Therefore, a better measure of "efficiency" of resistance quantitates the percentage of organisms killed in each doubling time. *L. monocytogenes* has a generation time of about 4 h, and BCG one of about 24 h. A killing of 57% of *L. monocytogenes* per generation results in a 2-log reduction over 4 d (or 24 generations), and the same efficiency of killing of BCG results in a 1-log reduction over 10 d (10 generations). These figures correspond to those measured in resistant mice. BCG and *L. monocytogenes* are controlled with approximately the same efficiency in resistant animals, although the killing of the slow-growing pathogen is much slower than that of the fast-growing pathogen. As a result, it is not necessary to kill a slow-growing pathogen as rapidly as a faster growing one in order to achieve the same level of resistance, and relatively modest levels of killing of slow-growing pathogens might underlie resistance. The difference between virulent and avirulent mycobacteria in the mouse is reflected in, or perhaps determined by, their differential doubling times in vivo, and this difference is apparent immediately after infection, before resistance is acquired (8). In guinea pigs (46) as well as in mice (47), the bacillary load by the time resistance begins to be expressed is a crucial factor in determining the ultimate outcome of the infection.

On the basis of the observations described here, a scenario can be proposed whereby control of mycobacterial infection is manifested in a cyclical pattern. Apoptosis linked with killing of bacilli is followed by phagocytosis of released viable bacilli by blood-derived monocytes migrating into the tubercle. These recently recruited monocytes are less permissive for intracellular growth. After the bacilli begin to replicate, the infected cell undergoes apoptosis, reinitiating the cycle. Whether this could account for the reduction in viability of  $\sim 55\%$  every

24 h seen in vivo, is a matter of speculation, since such dynamics cannot be meaningfully investigated in vitro, and controlled experiments on the role of apoptosis in vivo are difficult to design. However, the scenario is plausible since both apoptosis and the influx of monocytes are prominent features of the tubercle.

The evidence that CD4<sup>+</sup> T cells play a role in acquired resistance to human tuberculosis is circumstantial, but compelling. In this scenario, CD4<sup>+</sup> T cells could play a dual role. First, they and the cytokines they release are central in the construction of the tubercle, where apoptosis may be favored, and the detrimental effects of massive cell death may be contained. Second, CD4<sup>+</sup> T cells could be the effectors in inducing apoptosis of infected macrophages. Cytotoxic CD4<sup>+</sup> T cell clones have been shown to induce apoptosis in target cells (24). Whether mycobacteria-reactive CD4<sup>+</sup>

T cells can induce apoptosis in chronically infected human monocytes, and whether T cell-induced apoptosis is coupled with any inhibition of intracellular bacilli, are questions under investigation. De Libero et al. (48) have shown that under conditions in which *M. bovis*-infected murine macrophages were killed by cytotoxic CD8<sup>+</sup> T lymphocytes in vitro there was a significant growth inhibition of the bacilli which was IFN- $\gamma$  independent. In addition, studies using gene-targeted disruption in the mouse suggest a role for  $\beta_2$ -microglobulin positive cells such as CD8<sup>+</sup> T cells in protection against mycobacterial infection (49). Thus, MHC class I-restricted cytotoxic cells could also be involved. Histological and immunohistological analysis of tissue from sites of mycobacterial infection, presently underway, will reveal the extent of apoptosis occurring in vivo.

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We thank Wilhemine Hellman and Jean Barker for help with the electron microscopy, Judy Adams for help with the graphics, and Marguerite Nulty for typing the manuscript.

These studies were supported in part by U. S. Public Health Service grants AI-22616 and AI-07012.

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Received for publication 4 April 1994 and in revised form 13 June 1994.

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