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Experimental Models of Mycobacterial Replication and Killing in Macrophages

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Experimental models of mycobacterial replication and killing in macrophages

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by

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To the memory of my late father,
Pravit Laochumroonvorapong,
with gratitude.

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Abstract

This thesis investigates how human mononuclear phagocytes regulate the growth of mycobacteria. Previous studies carried out in our laboratory suggested that cell turnover [apoptosis and necrosis] within a granuloma may contribute to the control of mycobacterial infection. The experiments described here demonstrated that both apoptosis [Fas-mediated] and necrosis [perforin-induced] of infected human monocytes do not affect the survival of intracellular mycobacteria in an *in vitro* infection model. To determine the role of apoptosis and necrosis on mycobacterial infection *in vivo*, perforin gene-disrupted mice and Fas receptor-defective mice were experimentally infected with mycobacteria. The loss of either intact Fas pathway or perforin gene did not render mice more susceptible to mycobacterial infection, confirming the findings *in vitro*.

Further studies *in vitro* of the killing of intracellular mycobacteria indicated an oxidative [H_2O_2-induced] killing mechanism. This may be physiologically significant since release of H_2O_2 has been documented following phagocytosis of mycobacteria by human monocytes. *In vitro*, there was an inverse relationship between intracellular growth rate and susceptibility to H_2O_2. To determine whether different species of mycobacteria vary in their susceptibility to oxidative killing, *Mycobacterium tuberculosis* [H37Ra] and a clinical isolate of *M. avium-M.intracellulare* [MAI] were grown in cultured human monocytes, and the intracellular growth rate,

susceptibility to killing by H_2O_2, and cytokine induction were assessed. *M. tuberculosis* H37Ra replicated with a generation time of 48h while the isolate of MAI did not replicate in freshly explanted monocytes [generation time of 408h]. MAI was more sensitive to oxidative killing than H37Ra, although both mycobacterial species were equally sensitive to H_2O_2 treatment in cell-free culture media and in sonicated cell suspensions.

Maturation of human monocytes in culture for 8 days prior to infection with H37Ra resulted in more restricted intracellular growth and greater susceptibility to H_2O_2-induced killing of the bacilli. The patterns of cytokine production in response to H37Ra infection were different in freshly explanted and 8d monocytes, suggesting that the cytokines produced by infected monocytes may contribute to the regulation of intracellular mycobacterial growth.

PRIOR PUBLICATION OF WORK IN THIS THESIS

Primary publications

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P. Laochumroonvorapong, J. Wang, C.-C. Liu, W. Ye, A. Moreira, K.B. Elkon, V.H. Freedman, and G. Kaplan. Perforin, a cytotoxic molecule which mediates cell necrosis, is not required for the early control of mycobacterial infection in mice. Infect. Immun. 65: 127-132, 1997.

P. Laochumroonvorapong, S. Paul, L.G. Corral, V.H. Freedman, and G. Kaplan. Mycobacteria growth and sensitivity to H_2O_2 killing in human monocytes *in vitro*. Submitted, 1997.

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P. Laochumroonvorapong, A. Molloy, A. Moreira, E. Sarno, and G. Kaplan. An in vitro model of resistance in leprosy: Apoptosis, but not necrosis of mycobacteria-infected monocytes is coupled with killing of intracellular mycobacteria. The 29th U.S.-Japan Joint Research Conference on Tuberculosis and Leprosy, Kyoto, Japan, 1994.

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A. L. Moreira, L. Tsenova-Berkova, J. Wang, **P. Laochumroonvorapong**, R. North, and G. Kaplan. The effect of Thalidomide on the cellular immune response in experimental tuberculosis in mice. The 30th U.S.-Japan Joint Research Conference on Tuberculosis and Leprosy, Fort Collins, CO, 1995.

P. Laochumroonvorapong, J. Wang, C. Liu, K. Elkon, V. Freedman, and G. Kaplan. Perforin, a cytotoxic molecule which mediates cell necrosis, is not required for the early control of mycobacterial infection in mice. The 31st U.S.-Japan Joint Research Conference on Tuberculosis and Leprosy, Nagasaki, Japan, 1996.

I. Preface and Introduction

Preface

Since the identification of the tubercle bacillus in 1882, numerous investigators, in the course of many elegant studies, have contributed to our understanding of human mycobacterial infection. This chapter by no means attempts to review all the contributions made in the study of mycobacterial infection, but is rather meant to provide relevant background and information to help readers understand the objectives of the studies presented in this thesis.

Introduction

A. Mycobacteria

Mycobacteria are small, rod-shaped, non-spore-forming, aerobic bacilli which belong to the genus *Mycobacterium*, of the family *Mycobacteriaceae*, of the order *Actinomycetales*. The mycobacteria comprise a large family of organisms with a wide spectrum of virulence as defined by their ability to establish infection and cause active disease in humans. Several mycobacteria are important pathogens. Among these, *Mycobacterium tuberculosis* is the most virulent in humans, with an estimated one billion infected individuals worldwide (1). Approximately 10% of infected immunocompetent individuals develop active disease, most often in the lungs, over their lifetime. Before pasteurization was introduced, gastrointestinal tuberculosis,

caused by ingesting *M. bovis* in milk from infected cows, was common. In developing countries, *M. leprae* [the causative agent of leprosy] afflicts millions of people (2). Finally, *M. avium-M. intracellulare* [MAI], a common pathogen of birds and a contaminant of soil and water which does not usually cause symptoms in humans, has now emerged as a life-threatening opportunistic infection in immunocompromised patients, especially those suffering from AIDS (3,4).

B. Etiology of tuberculosis

Tuberculosis infection can be traced back to antiquity. A number of skeletons with abnormalities suggestive of Pott's disease [spinal tuberculosis] have been found to date to between 4000 and 1000 B.C. (5,6). The first uncontested evidence of pulmonary tuberculosis dated to about 1000 B.C. was found in a mummy of a 5 year old boy whose lungs remained intact in the thoracic cavity and showed typical signs of pulmonary tuberculosis infection (7). Historically, three main theories have been invoked to explain the cause of tuberculosis. In the fifth century B.C., Hippocratic physicians and Hippocrates himself believed that tuberculosis was caused by stress in people with an inherited susceptibility (8). During this time, a belief that tuberculosis was communicable began to emerge and the following remark is attributed to Aristotle [384 to 322 B.C.]: "With the phthisic [Greek name for tuberculosis] the reason is that the breath is bad and heavy... In approaching the

consumptive, one breathes this pernicious air. One takes the disease because there is in the air something disease-producing" (9). During the Roman Empire, the notion that tuberculosis was contagious was generally accepted. This view was also shared by Galen [129 to 200 A.D.], considered as the foremost physician of that era (10). By the seventeenth century, many famous pathologists were championing alternative views on the cause of tuberculosis, namely that the disease was constitutional, an abnormal form of a gland or a tumor (11).

During the second half of the nineteenth century, a French military surgeon named Jean-Antoine Villemin observed that healthy horses succumbed to the equine disease "glanders" [caused by *Actinobacillus mallei*] when housed in crowded military depots. Similarly, healthy young military personnel became consumptive when stationed in barracks, but not when deployed in the field. Convinced that tuberculosis must be contagious, Villemin injected infectious human sputum into healthy rabbits. In 1865, he reported that tuberculosis could be transmitted from man to rabbit by inoculation (12). With Robert Koch's discovery of the tubercle bacilli in 1882 (13), the causative agent of tuberculosis was finally identified. Later that year, acid-fast staining methods which facilitated the visualization of the bacterium under the microscope were developed by Ziehl, and subsequently modified by Nielsen (14). The same methods are still in use today.

C. Treatment of tuberculosis

Prior to the understanding of how the disease was transmitted, there was no successful therapeutic regimen for tuberculosis. During the seventeenth century, the ceremony of "Royal Touch" became popular in Europe for treating scrofula [a form of tuberculosis characterized by swelling of the lymphatic glands]. Patients traveled great distances to be touched by the French and English monarchs, who were believed to have received the healing powers from God (13). Later, by the mid 1800's, ideas had changed and the popular beliefs that fresh air, diet, rest and exercise were the means to combat the disease, thus leading to the sanatorium movement (15).

The movement began in Germany, then England, and spread to the United States under Edward Livingston Trudeau. It is noteworthy that during this time there was a steady decline in the number of cases of tuberculosis in the United States for reasons that were not clear (15). Isolation of infected individuals probably impacted on this decline. Despite this success, the prognosis of tuberculosis remained grim.

The search for anti-mycobacterial agents did not begin until the early 1900's. Inspired by Paul Ehrlich's discovery in 1910 of a substance active against syphilis, scientists began searching for chemical substances that killed tubercle bacilli (16). In 1943, a doctorate student named Albert Schatz attempted to identify an antibiotic which would be active against gram-negative bacteria and mycobacteria. After screening large numbers of

actinomyces isolates, Schatz isolated streptomycin from *Streptomyces griseus*. In January of 1944, Schatz reported that streptomycin completely inhibited the growth of *M. tuberculosis* even at small concentration (17). For the next ten months, Schatz devoted his time to the purification of the active ingredient and in November of that year, a woman with advanced pulmonary tuberculosis was given the first injection of streptomycin (18). Her condition gradually improved; she was discharged from the hospital in 1947. Then in 1952, the first use of isoniazid to treat tuberculosis was reported, a treatment which had far less serious side effects compared to streptomycin treatment (19), which caused vertigo in many patients. Patients responded well to isoniazid and reported increased appetite and significant weight gain. Most patients also cleared tubercle bacilli in their sputum. For the first time in human history, eradication of tuberculosis seemed possible.

D. Tuberculosis in the era of AIDS

The success of antibiotic therapy led many to believe that tuberculosis would soon be conquered. Indeed, from the 1950's on, the incidence of tuberculosis in the United States steadily declined. In 1985, this long-term trend was reversed and the incidence of tuberculosis increased for the first time since the mid 1950's (20). The major contributing factor was the AIDS epidemic which expedited the progression of primary tuberculosis infection to active disease. Another reason for this sharp rise in tuberculosis incidence

was the large number of immigrants coming to the United States from countries where tuberculosis was endemic. With these factors, compounded by deteriorating public health measures in many urban centers, tuberculosis once again became a significant public health threat in this country. The prospect became even worse with the emergence of strains resistant to drugs: certain multidrug-resistant tuberculosis [MDR-TB] which are resistant to all the drugs available have already claimed many lives (21). From an historical perspective, it appears that we have come almost to a full circle. Unless new strategies for combating infection can be developed in the near future, many patients today will soon be facing the grim prospects not so different from that faced by previous generations, of untreatable tuberculosis.

Another mycobacterium *M. avium-M. intracellulare* [MAI], a common contaminant of soil and water, which is usually nonvirulent in humans has recently emerged as a common opportunistic pathogen for immunocompromised AIDS patients (3,4). Although antimicrobial drugs against MAI are available (22,23), complete eradication of the organism is impossible in the immunosuppressed host, necessitating lifelong therapy. Thus, MAI constitutes a major life-threatening health problem for immunocompromised patients.

E. The immune response to mycobacterial infection

Our understanding of the immune response to mycobacterial infection

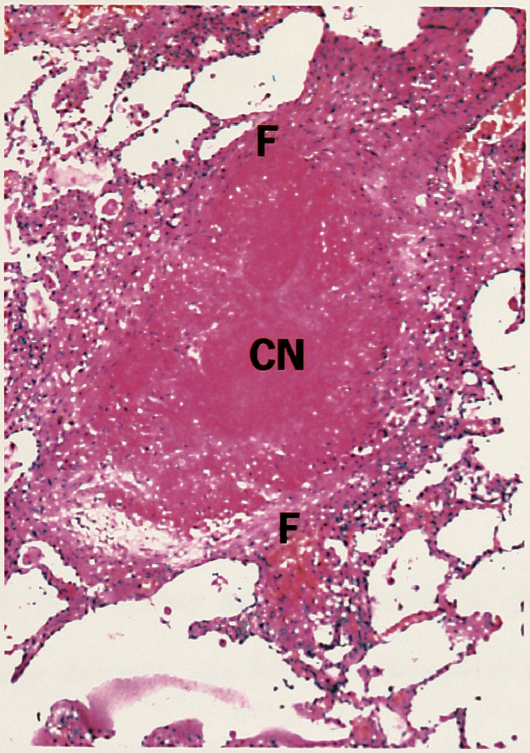
began in 1945 when Merrill Chase at the Rockefeller University demonstrated that tuberculin hypersensitivity in guinea pigs could be adoptively transferred into naive animals by living cells, but not by serum (24). This discovery provided the basis for our current understanding of delayed-type hypersensitivity and, in fact, cell-mediated immunity. Today, a purified protein derivative [PPD] test which consists of intradermal injection of tuberculin, a protein-lipopolysaccharide component of *M. tuberculosis*, is used to determine whether an individual has been exposed to and sensitized to the tubercle bacillus. Many studies carried out in the mouse have since confirmed the importance of cellular immunity in the protection and expression of resistance to mycobacteria infection.

Histopathological analysis of post-mortem tissues from patients that died of tuberculosis and experimentally infected animals has made possible the reconstruction of the physiological events leading to the establishment of *M. tuberculosis* infection and has thrown light on the nature of the cellular responses at the site of infection (25,26,27). We know that when a hitherto unexposed individual inhales aerosolized tuberculosis bacilli coughed out by an infected patient, infection is established in the lungs by a small number of organisms. At the primary site or sites of infection in the lungs, the bacteria are quickly phagocytosed by resident alveolar macrophages. In immunocompetent individuals, this is followed by the migration of other leukocytes to the site, eventually resulting in the formation of a granuloma

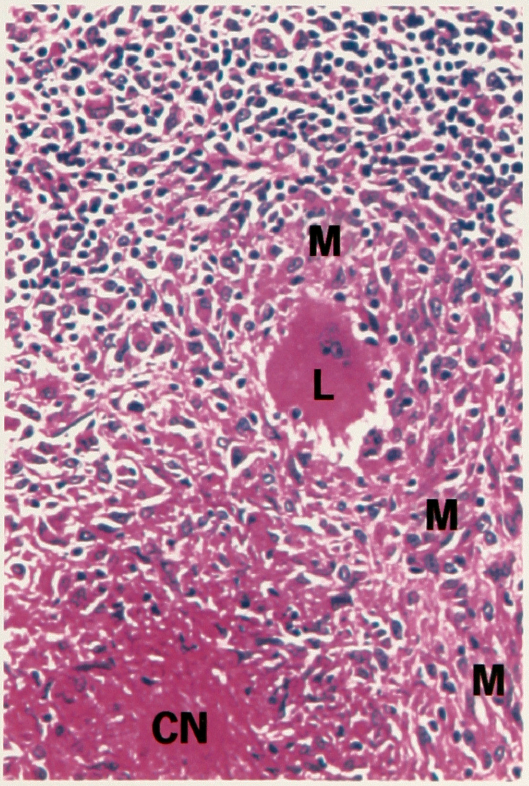
or a tubercle. The granuloma is characterized by the accumulation of activated lymphoid cells and myeloid cells at various stages of cytokine-induced differentiation [Figure 1-1]. The myeloid cells including recently-recruited blood monocytes, tissue macrophages, epithelioid cells, and multinucleated giant cells form the center of the granuloma, while a mantle of lymphocytes and fibroblasts appear to "wall off" the site of infection. Interaction between T cells, particularly $CD4^+$ and $CD8^+$ T cells, and infected macrophages within the granuloma are believed to be critical for the control of mycobacterial infection.

Progression of *M. tuberculosis* infection is tightly coupled to the immune status of the host. Since only about 10% of infected individuals develop active disease at some points in their lives, it is clear that the majority of humans are capable of controlling tuberculosis infection. In immunocompetent humans, this delicate balance between the ability of the mycobacteria to cause active disease and the ability of the host immune response to control the infection is maintained for many years, usually in favor of the host. However, when the immune system of the host is compromised, the equilibrium is shifted in favor of the parasite and full blown disease develops. As the infection progresses, the granulomas within the infected lesions coalesce to form a confluent consolidation leading to liquefaction and cavitation due to necrosis at the center. When the lesions

Figure 1-1. The morphology of the human tubercle. [A] The granuloma contains densely packed differentiated myeloid cells surrounded by a mantle of lymphocytes and fibroblasts [F] which "wall off" the site of infection. This is an advanced tubercle with progressive central caseous necrosis [CN]. The zone of peripheral macrophages and lymphocytes is relatively thin. [B] At higher magnification, macrophages [M] and multinucleated giant cells, termed Langhans' giant cells [L] can be seen. [Taken from P.R. Wheater, H.G. Burkitt, A. Stevens, and J.S. Lowe, Basic Histopathology, 2nd Edition]



(a)



(b)

erode into bronchi, caseous materials may be coughed up, in association with the dissemination of *M. tuberculosis* to secondary sites of infection and progression of active disease. One of the hallmarks of active tuberculosis is bloody sputum which is a consequence of destruction of pulmonary tissue. At this stage, the disease can be transmitted when the infected individual coughs, releasing infectious tubercle bacilli in air-borne droplets.

F. T cells in mycobacterial infection

1. $CD4^+$ T cells

Several lines of evidence suggest that $CD4^+$ T cells are essential in establishing immune defense against mycobacterial infection *in vivo*. In mice experimentally infected with *M. bovis*, depletion of $CD4^+$ T cells rendered the animals unable to control mycobacterial growth (28). It has also been shown that adoptive transfer of $CD4^+$ T cells from sensitized mice could confer protection against *M. tuberculosis* infection in the recipient animals (29,30). Although $CD4^+$ T cells have been shown to have some cytotoxic activity against mycobacteria-pulsed and mycobacteria-infected macrophages (31,32), the main contribution of $CD4^+$ T cells to the protective anti-tuberculosis response probably involves the production of Th1 type cytokines including interleukin-2 [IL-2] and interferon-gamma [IFN-γ]. The importance of these and other cytokines in mediating defense against

mycobacterial infection has recently been confirmed in cytokine gene disrupted mice experimentally infected with mycobacteria (33,34). The absence of the protective cytokines reduces the animal's ability to control the infection, leading to early death of the infected animal.

In healthy humans, exposure to mycobacteria results in the development of circulating, sensitized $CD4^+$ T cells which can be confirmed either by assaying blastogenesis in response to mycobacterial antigens *in vitro* or by testing tuberculin sensitivity [PPD] *in vivo*. The responsiveness of $CD4^+$ T cells appears to correlate inversely with disease progression. Individuals who have controlled infection generate vigorous PPD reaction whereas patients with advanced disease have reduced or even absent PPD response. Moreover, the fact that HIV-infected individuals [whose $CD4^+$ T cells populations are selective depleted] are markedly susceptible to both primary and reactivated tuberculosis (35) is a strong circumstantial evidence that this cellular population plays a significant role in immunity to this infection. This qualitative observation is supported by quantitative data; it has been reported that the frequency of mycobacteremia increased from 4% in patients who had more than 200 $CD4^+$ T cells/μl to 49% in patients with 100 or fewer $CD4^+$ T cells/μl (36). Although it seems that $CD4^+$ T cells are essential in establishing resistance to mycobacterial infection, the mechanisms through which this occurs in humans are as yet unknown.

2. $CD8^+$ T cells

Recent evidence suggested that $CD8^+$ T cells may also contribute to the control of *M. tuberculosis* infection in mice. One study demonstrated that depletion of $CD8^+$ T cells in mice experimentally infected with *M. tuberculosis* increased the severity of tuberculosis infection (37) while another group reported variable results in a similar study (38). Previously, Flynn *et al.* showed that mice with a disruption in the β2-microglobulin [β2m] gene failed to develop functional $CD8^+$ T cells and succumbed to virulent *M. tuberculosis* infection much more rapidly than control mice (39). The authors interpreted these findings to indicate that MHC class I-restricted T cells [$CD8^+$] may be required for resistance to *M. tuberculosis* infection *in vivo*. However, how $CD8^+$ T cells contribute to the control of mycobacterial infection is not fully understood.

$CD8^+$ T cells, although capable of producing cytokines including IFN-γ (40), have been considered to be primarily responsible for cell-mediated cytotoxicity through MHC class I-restricted fashion. $CD8^+$ T cells are believed to destroy infected macrophages that fail to inhibit the growth of intracellular mycobacteria within a granuloma. The released organisms are rephagocytosed by recently migrated blood-derived monocytes which may be more capable of killing them. $CD8^+$ T cells kill by releasing cytoplasmic granules which contain a number of toxic molecules including perforin (41).

Upon contact with an antigen presenting target cell, degranulation occurs and perforin is released. Once released, perforin inserts itself into the target cell membrane, aggregates, and forms pores. This results in osmotic disregulation and target cell death by a necrotic process (41). In addition, $CD8^+$ T cells can mediate cytotoxic effects *via* the interaction between Fas ligands [Fas-L] on their cell surface and Fas receptors [Fas-R] expressed on the target cells (42,43,44). Unlike perforin which induces necrosis, Fas-mediated cell death results in apoptosis of the Fas-R expressing cells. Although $CD8^+$ T cells appear to be involved in murine mycobacterial infection, it is not clear whether the protective response conferred by $CD8^+$ T cells involves cytotoxic molecules such as perforin or the production of protective cytokines. In addition, there is no evidence to date that $CD8^+$ T cells play a central role in human tuberculosis infection.

3. $CD4^-$ $CD8^-$ T cells

Both $CD4^-CD8^-TCR\alpha\beta^+$ and $CD4^-CD8^-TCR\gamma\delta^+$ T cells have been isolated from the blood of normal sensitized donors. These cells recognize mycobacterial antigens presented by monocytes in a non-MHC class I or class II-restricted fashion (45). When activated, they exhibit cytotoxic activity against mycobacteria-pulsed or mycobacteria-infected monocytes (46). It has been proposed that $CD4^-CD8^-TCR\gamma\delta^+$ T cells play a role in the initial immune

response to primary *M. tuberculosis* infection. In the murine model, several studies reported an accumulation and/or expansion of $CD4^-CD8^-TCR\gamma\delta^+$ T cells in the lungs and draining lymph nodes of mice after primary infection with *M. tuberculosis*(47,48). In humans, $CD4^-CD8^-TCR\gamma\delta^+$ T cells have been shown to proliferate in response to *M. tuberculosis* (49,50), suggesting that these cells possess an innate ability to recognize and respond to mycobacterial antigens. The responsiveness of $CD4^-CD8^-TCR\gamma\delta^+$ T cells to *M. tuberculosis* appears to correlate inversely with disease progression. Barnes *et al.* reported that $CD4^-CD8^-TCR\gamma\delta^+$ reactive T cells obtained from patients with protective immune responses to infection proliferated vigorously in response to *M. tuberculosis* than the same population of cells isolated from patients with advanced disease (51). Taken together, these findings implicate both $CD4^-CD8^-TCR\alpha\beta^+$ and $CD4^-CD8^-TCR\gamma\delta^+$ T cells in immunity to tuberculosis infection. However, direct evidence regarding the role of these populations in resistance awaits future investigations.

G. The role of mononuclear phagocytes in mycobacterial infection

Histopathological examination of *M. tuberculosis*-infected tissues reveals that mononuclear phagocyte is the cell-type that is predominantly parasitized by the organism and that replication mainly occurs within these cells. As well as playing a central, passive role in the life-cycle of the

intracellular mycobacteria, it seems likely that mononuclear phagocytes which have long been recognized as potent microbicidal effectors also play a central, active role in the expression of resistance against tuberculosis. In mice, resistance to mycobacterial infection depends on the cross-talk between T lymphocytes and the infected macrophages. Mycobacteria-reactive T cells, in particular $CD4^+$ T cells, produces lymphokines which activate the macrophage's microbicidal mechanism to kill intracellular bacteria. *In vitro*, IFN-γ treatment of murine mycobacteria-infected macrophages has been shown to induce killing of the intracellular bacilli (52). In humans, mycobacteria-reactive, lymphokine-producing T cells are readily detectable in normal sensitized donors (53,54). However, lymphokine-mediated activation of mycobacteria-infected human monocytes has never been shown to induce killing of the intracellular bacteria (55,56,57,58), and the importance of lymphokines to the protective response in humans remains to be demonstrated.

H. Cytokines produced by mononuclear phagocytes

Following phagocytosis of *M. tuberculosis* bacilli, macrophages produce a range of cytokines including tumor necrosis factor-alpha [TNF-α], interleukin-1 [IL-1], interleukin-6 [IL-6], interleukin-12 [IL-12], interleukin-10 [IL-10], and transforming growth factor-beta [TGF-β]. The first four cytokines

are pro-inflammatory while the last two are anti-inflammatory cytokines. Based on experimental and clinical observations, these cytokines have been proposed as having immunomodulatory effects and thus influencing the clinical manifestations of tuberculosis infection.

TNF-α is a potent cytokine contributing to both protection and immunopathology. Neutralization of TNF-α in mice experimentally infected with *M. bovis* BCG interferes with the development of granulomata, leading to accelerated replication of the organism (59). In humans, high levels of TNF-α can be detected in serum of patients with active infection. PBMCs obtained from these patients also release more TNF-α than cells from treated asymptomatic patients or cells from normal healthy individuals (60). High concentration of TNF-α has been shown to induce fever by acting directly on the hypothalamus and long-term administration of TNF-α to mice induces wasting (61). Thus, it appears that different levels of TNF-α result in different outcomes. For the protective response, local release of TNF-α at the site of infection is necessary for the generation of granuloma. However, excessive production of the cytokine can result in leakage of TNF-α into the circulation which contributes to the systemic manifestations of tuberculosis [fever, weight loss, etc.].

Interleukin 1 [IL-1] is an endogenous pyrogen and may be responsible

for the characteristic fever often associated with tuberculosis. However, the role of IL-1 in the immune response to *M. tuberculosis* infection remains uncertain. *In vitro*, IL-1 triggers mononuclear phagocytes to produce other pro-inflammatory cytokines such as TNF-α and IL-6, and also stimulates T cell proliferation (62). However, certain investigators have suggested that IL-1 may suppress the immune response to tuberculosis infection in patients (63). Thus, it is not yet clear how IL-1 influences the course of tuberculosis infection.

The other two pro-inflammatory cytokines are IL-6 and IL-12. As a potent B-cell growth and differentiation factor that induce immunoglobulin production by activated B cells, IL-6 has been postulated to be responsible for the characteristic hyperglobulinemia associated with tuberculosis. Addition of IL-6 was shown to reduce the binding of TNF-α to murine macrophages *in vitro* (64) and to enhance the growth of MAI within human monocytes (65). In contrast, IL-12 is believed to be involved in T-cell expansion during the early phase of mycobacterial infection. With a suboptimal amount of IL-2 [a T-cell growth factor], IL-12 may augment T cell proliferation in response to mycobacterial antigen. Neutralizing anti-IL-12 antibodies have been reported to inhibit *M. tuberculosis*-induced lymphocyte proliferation (66). Future investigations are required to understand how IL-6 and IL-12 affect the course of tuberculosis infection.

Both interleukin 10 [IL-10] and transforming growth factor-beta [TGF-β] are anti-inflammatory cytokines. Both have been reported to inhibit cytokine synthesis by both murine and human macrophages (67,68,69), to down-regulate class II MHC expression on macrophages (70,71), and to suppress T-cell proliferation (70,72,73). These cytokines [IL-10 and TGF-β] have been shown to reverse the antimicrobial activities of murine and human macrophages (74,75,76). Neutralization of either IL-10 or TGF-β in mice experimentally infected with MAI resulted in a significant reduction in the bacterial burden of the infected animals (77,78). Therefore, these anti-inflammatory cytokines appear to inhibit the immune response to mycobacterial infection, and may facilitate the survival of mycobacteria.

I. How mycobacteria evade the host immune response

Without antibiotics, immunocompetent individuals cannot usually sterilize infection with *M. tuberculosis,* suggesting that the organism somehow evades the host immune response. Our laboratory has shown that when various mycobacteria including *M. tuberculosis* and MAI are phagocytosed by human monocytes, the bacilli survive and/or replicate within a unique phagocytic vacuole (79,57). Usually, when bacteria are taken up by mononuclear phagocytes, the organisms initially reside within phagosomes containing early endosomal markers. As the phagosomes

undergo a maturation process by fusing with host-cell membrane-bound compartments, the endosomal markers are gradually replaced by late endosomal markers and eventually by lysosomal markers. Following fusion with lysosomes, bacteria within the phagosomes are exposed to a variety of hydrolytic enzymes of the lysosomes which attacks the organisms. Many studies have suggested that fusion between the phagosome containing mycobacteria and the lysosome does not occur within infected mononuclear phagocytes (80,81,82). It has been reported that *M. tuberculosis* were capable of retarding the normal maturation process of the phagosome since early endosomal, but not late endosomal or lysosomal, markers were present on the membrane of the vacuole containing *M. tuberculosis* (83). The ability to stop this maturation process is thought to be responsible for the lack of fusion between lysosome and vacuoles containing mycobacteria. Other investigators reported that the proton ATPase pump which is required for the acidification of the phagolysosome was absent from the vacuole containing MAI (84). Whether the vacuole containing *M. tuberculosis* exhibits similar exclusion of the proton ATPase pump is not known. In addition to the ability to regulate fusion with lysosomes, mycobacteria may also dictate their own intracellular environment in other ways. Chronically BCG-infected macrophages have been reported to present antigen poorly (85). This finding suggests that the vacuoles in which mycobacteria reside may be sequestered from the normal intracellular, intervacuolar trafficking which results in the presentation of

antigen on MHC class II molecules. These observations, taken together, help explain how mycobacteria adapt to the inhospitable environment within microbicidal host macrophages. Nevertheless, the mechanism[s] by which these pathogens evade the host immune response remains difficult to define.

J. The use of animal models to study mycobacterial infection

In an attempt to understand the disease process following tuberculosis infection in humans, early studies employed several species of animals such as mouse, guinea pig, and rabbit as experimental infection models. Mice have been a popular model to study tuberculosis infection because of the relatively low cost, easy handling, and the recent explosion of readily available murine immunological reagents. Like humans, mice generate a strong immune response to infection by mycobacteria. However, mice are in general more resistant to mycobacterial infection and have been shown to control and even clear 10^6 virulent mycobacteria delivered by the intravenous route (86,87). This innate ability to resolve mycobacterial infection renders mice the ideal model for studying immunity and resistance to these organisms.

Unlike mice, guinea pigs are very susceptible to human tubercle bacilli; a few viable bacteria can produce a progressive disease which culminates in death of the animals. The physiology of the guinea pig's pulmonary tract is quite similar to that of humans (88). The guinea pig model was used for the development and early testing of BCG vaccine (89). However, the major

disadvantage of using guinea pigs for mycobacterial studies is the lack of useful reagents for the monitoring of the cells and soluble mediators of the immune response which limits the scope of investigations that can be undertaken.

Rabbits provide a good model to study resistance and susceptibility. On the basis of their response to infection with the virulent Ravenel strain of *M. bovis*, strains of rabbits are classified as either resistant or susceptible. The resistant animals develop cavitary tuberculosis closely mimicking the disease pattern found in adult immunocompetent humans while the susceptible rabbits develop hematogenously [blood-borne] spread tuberculosis, a manifestation often found in infants and immunocompromised individuals (27,90,91,92). Rabbits are not susceptible to human tubercle bacilli. Therefore, direct comparisons between rabbits infected the Ravenel strain of *M. bovis* and human tuberculosis disease may not be appropriate. Although none of these animal models exactly duplicates the disease pathogenesis of human tuberculosis infection, studies of experimental infections of these animals have generated much of our present knowledge of the course of mycobacterial infection *in vivo*.

K. Host species-specific determination of mycobacterial virulence

The virulence of a mycobacterium is often defined as the ability of the organism to establish infection and cause active disease in animals from

which the organism was isolated. In mice, virulence is associated with the unrestricted growth of the organisms in infected organs (87). In a mouse, H37Rv [a virulent *M. tuberculosis* strain] causes a progressive lethal infection whereas infection with H37Ra [an avirulent *M. tuberculosis* strain] is readily controlled by the animal (93). *In vitro*, H37Rv replicates significantly faster than H37Ra in mouse macrophages (94). Recently, our laboratory reported that in contrast to the observation made in the murine model, these two strains of *M. tuberculosis* replicated with similar doubling times in human monocytes cultured *in vitro* (79). This suggests that virulence factors defined in the context of the murine model may not necessarily be relevant to human models or to human tuberculosis.

Activation of murine macrophages by cytokines *in vitro* has been shown to induce the cells to restrict the growth of or even kill intracellular mycobacteria including *M. tuberculosis* and MAI (52,95). In contrast, human monocytes are much less capable of restricting the growth of BCG, H37Ra, or H37Rv, and cytokine-induced activation of human monocytes fails to inhibit the growth of these intracellular mycobacteria (57,96). Thus far, treatment of the human monocytes with TNF-α alone or in combination with IL-2 have been reported to reduce the survival of MAI (95). Some studies suggested that *M. tuberculosis* may be killed in human macrophages activated by IFN-γ and vitamin D_3 (97,58). However, activation of murine macrophages by IFN-γ

and vitamin D_3 did not induce killing of intracellular *M. tuberculosis*. (98). These *in vitro* observations suggest that human and murine mononuclear phagocytes may be fundamentally different. Therefore, attempts to understand how human monocytes/macrophages regulate the growth of intracellular mycobacteria must begin with the establishment of an *in vitro* mycobacterial infection model of human mononuclear phagocytes.

L. Requirement for an *in vitro* model of mycobacterial infection of human mononuclear phagocytes

Histological examination of mycobacteria-infected tissues reveals that mycobacteria are phagocytosed by human mononuclear phagocytes, survive and replicate intracellularly. Therefore, the first requirement of an *in vitro* mycobacterial infection model of human mononuclear phagocytes is that the system must support the intracellular survival and growth of the mycobacterium. First, an appropriate source of human mononuclear phagocytes [alveolar macrophages or blood-derived monocytes] must be considered. In human pulmonary tuberculosis infection, *M. tuberculosis* bacilli are first phagocytosed by alveolar macrophages. However, subsequent extravasation of blood-derived monocytes and their maturation to macrophages is the driving process in the development of a granuloma and associated resistance at the site of the infection. To establish an *in vitro* infection model, blood-derived monocytes can be readily obtained from

volunteer donors and *in vitro* these can be induced to differentiated into cells that closely resemble macrophages both morphologically and functionally. In contrast, alveolar macrophages can only be obtained after a bronchopulmonary lavage [an invasive procedure]. Therefore, with respect to both the physiological relevance and the availability of the cells, blood-derived monocytes are more suitable for the *in vitro* model.

Once the model mononuclear phagocyte has been decided, other parameters must also be optimized for successful development of the model. In the murine model, virulent mycobacteria begin replicating without any delay in murine macrophages, leading to death of the infected host cells (94). Therefore, studying the host-parasite interaction is only possible as long as the infected host cells remain intact and viable. *In vivo*, tuberculosis is a chronic infection and therefore a relevant experimental model should be based on chronically infected macrophages i.e. with most of the intracellular bacteria having been generated intracellularly [as *in vivo*] rather than having been grown *in vitro* and recently phagocytosed. Following infection with mycobacteria, the growth of the organism within human monocytes must be carefully monitored, and the model must allow a reasonable time-frame for experiments to be carried out. To develop such an infection model, the multiplicity of infection [MOI] is an important factor. On one hand, the MOI must be high enough to produce an evenly infected culture i.e. most cells infected and infected with approximately the same number of bacilli. On the

other hand, the MOI must be low enough so that, immediately after infection, each cell contains number of bacilli low enough to allow for several rounds of intracellular replication [experimental window] before the physical burden of replicated mycobacteria begins to disrupt the cell culture. Examination of the infected culture by acid-fast staining will help determine whether the chosen MOI produces an infected culture optimal for experimental studies.

M. Current understanding of how human monocytes kill mycobacteria

In the immune competent human, *M. tuberculosis* infection once established is not readily resolved, but rather the organisms appear to evade host defense mechanisms and establish a chronic infection. As long as the host immune response is sufficient, the organisms are contained within small, completely isolated granulomas. Clinically, the infected individuals have a chronic, asymptomatic infection which may but will probably not progress to active disease at some point in the future.

In vitro, the antibacterial activity of mononuclear phagocytes has long been associated with the ability of the macrophage to generate an oxidative burst. Macrophages have been shown to release reactive oxygen intermediates [ROI] like H_2O_2 and superoxide anion during phagocytosis of intracellular pathogens, including mycobacteria such as MAI and BCG (99,100,101). This activity is potentiated if the macrophages are primed with either the lymphoid cytokine IFN-γ or the monocyte cytokine TNF-α: both

human and murine macrophages primed with these cytokines have been shown to release increased amounts of ROI during phagocytosis of bacteria and to better restrict the growth and survival of intracellular pathogens as compared with unprimed cells (102,103). The production of ROI by phagocytes has been correlated with restricting the growth and/or killing of a variety of intracellular pathogens including *Candida albicans*, *Listeria monocytogenes*, *Toxoplasma gondii*, and *Leishmania* (104,105,106,107,108, 109,103).

Previous studies have suggested that oxygen and reactive oxygen intermediates contribute to the control of mycobacteria. For instance, growth of *M. tuberculosis* in culture media is markedly inhibited under high oxygen tension (110), and reactive oxygen intermediates [ROI] generated by peroxidase or catalase-H_2O_2-halide have been shown to kill *M. leprae*, *M. tuberculosis*, and MAI (111,112,113). Human monocytes activated by IFN-γ (114) or by mycobacterial cell wall products (115) have been shown to be more oxidatively active and better capable of killing *M. leprae*. Thus, these early studies suggested that ROI may be important in the control of mycobacterial infection.

Since the late 1980s, the discovery of reactive nitrogen intermediates [RNI] as important anti-microbial molecules shifted the attention away from ROI (116,117). In 1992, Chan *et al.* reported that RNI produced by murine macrophages were capable of killing virulent *M. tuberculosis* (52). Many

murine studies confirmed the role of RNI in the control of mycobacterial infection (118,119). However, the existence of similar bactericidal pathways in human monocytes/macrophages has been difficult to demonstrate. A number of studies failed to report NO synthesis in human macrophages stimulated with either bacterial products or lymphokines (120,121,122,123). This may be explained by the inability of human mononuclear phagocytes to synthesize tetrahydrobiopterin, an essential cofactor of NO synthase (124,125,126). In addition, induction of the NO synthase has not been clearly observed in human peripheral blood monocytes or peritoneal macrophages (127). However, one study recently reported the expression of NO synthase in alveolar macrophages obtained from active tuberculosis patients (128). Whether nitric oxide is involved in the protective response to *M. tuberculosis* infection in humans remains to be elucidated.

Some studies previously suggested that both ROI and RNI may be involved in the generation of toxic anti-microbial molecules. For instance, peroxynitrite anion, a product of nitric oxide and superoxide anion, has been shown to kill *E. coli* (129), but whether this molecule is also toxic to *M. tuberculosis* is not yet known. In addition, treatment of activated macrophages with catalase has been shown to inhibit the phagocyte's ability to generate RNI (130). Therefore, ROI, RNI, or both may be involved in the killing of intracellular mycobacteria by human mononuclear phagocytes.

N. The role of cell death in mycobacterial infection

1. Cell death: a normal physiological process

Cell death is a normal physiological process and most multicellular organisms have developed molecular machinery necessary to implement it. It is of no great surprise that a multicellular organism would benefit from cell death. For example, during metamorphosis in insects and amphibians the larval tissues must clear to make room for those of the adult. In mammals, cell death is evident very early on in organ development, namely in remodeling of the limb buds, cartilage and bones. In the immune system, self-reactive T and B cells are removed to prevent autoimmune disease. Mammals have also evolved mechanisms to protect themselves from viral infection: the host's cytotoxic T cells can recognize and kill virally infected cells *via* a perforin-dependent mechanism (131). Manipulation of host mechanisms concerned with cell death has also been implicated in the survival of other pathogens including *Shigella flexneri*, *Bordetella pertussis*, and *Leishmania donovani* (132,133,134). Because cell death is a normal part of so many different systems, it can be perceived as a dynamic equilibrium which must be properly regulated to maximize the benefit to the individual. When this process is disrupted, disease is usually the outcome (131).

Cells die by one of two known mechanisms: necrosis and apoptosis. Necrosis is a passive process where death is induced by physical alteration, and tends to affect many cells in a given location. Necrosis is induced when

the plasma membrane of a cell is irreversibly damaged [i.e. perforin-induced pore formation], leading to osmotic disregulation. The accumulation of cellular debris resulting from necrosis is accompanied by an inflammatory infiltrate. In contrast, apoptosis is an active process. Induction of apoptosis requires a death signal either by ligation of a membrane receptor or a cytoplasmic receptor binding to an appropriate ligand. This initial stimulus triggers the generation of secondary messengers which activates the host cell's killing machinery to execute its own death. Apoptosis is distinguished from necrosis by both morphological and biochemical criteria. The morphological sequence of apoptosis follows a specific pattern of nuclear condensation, surface blebbing, cytoplasmic contraction; and cellular components are packaged into membrane-bound apoptotic bodies before budding from the cell (135). The hallmark of apoptosis is DNA fragmentation. The degraded DNA of apoptotic cells, when analyzed by gel electrophoresis, forms a characteristic ladder. Unlike necrosis, apoptosis is associated with death of isolated cells and no inflammatory infiltrate. The dead cells at the site are phagocytosed by professional and adjacent nonprofessional phagocytic cells.

At present, the mechanism of apoptosis is not clearly understood. The initial stimulus can include events as diverse as exposure to glucocorticoids, irradiation, growth-factor withdrawal, hormone withdrawal, T cell-mediated signal [FasR-FasL] among others (136,137,44,138). The intracellular molecules/events found to be involved in apoptosis include interleukin-1-

beta-converting enzyme, its related proteases, protein tyrosine kinases, *myc* oncogene, p53 molecule, and activation of a sphingomyelinase, to name a few (44,136,139,140). To further complicate the matter, many studies have suggested that the signaling pathway may vary depending on cell types and their activation and differentiation status (141,142). At this point in time, investigation of the mechanisms that underlie apoptosis represents a major research focus.

2. Evidence that cell death may be involved in the regulation of mycobacterial survival

In response to *M. tuberculosis* infection, an immunocompetent host orchestrates a complex cellular immune response eventually resulting in the formation of a granuloma at the site of infection. The granuloma is characterized by the accumulation of differentiated myeloid cells including blood monocytes, tissue macrophages, epithelioid cells and multinucleated giant cells, surrounded by a mantle of lymphocytes, mainly $CD4^+$ T cells. Many studies have confirmed the importance of both $CD4^+$ and $CD8^+$ T cells in the control of mycobacterial infection (28,30,29,37,39). $CD4^+$ T cells are primarily responsible for the production of Th 1 type cytokines such as IFN-γ and IL-2 which are essential for the recruitment of leukocytes and the successful containment of the infection. On the other hand, $CD8^+$ T cells are believed to mediate cytotoxic effects on infected macrophages which are

unable to restrict the growth of intracellular mycobacteria. The mycobacteria released from dead phagocytes are probably phagocytosed by newly recruited mononuclear phagocytes, which may be better able at restricting the growth of mycobacteria.

In vitro studies carried out in our laboratory indicate that human macrophages, even when activated by GM-CSF, IFN-γ, TNF-α, or a combination of TNF-α and IFN-γ, are not capable of killing intracellular BCG, a member of the *M. tuberculosis* complex (57). Thus, activation of infected human monocytes/macrophages by cytokines alone may not be sufficient to render the phagocyte capable of killing these intracellular pathogens. Additional signals mediated by the complex interactions between T cells and infected macrophages may be necessary to activate the phagocytes to kill intracellular mycobacteria.

Like *M. tuberculosis* infection, the control of another mycobacterium, *M. leprae*, is also dependent on the host cellular immune response. *M. leprae* is the causative agent of leprosy, a disease in which the immune response to the infecting mycobacterium determines the clinical and histopathological manifestations. Histopathological studies of *M. leprae*-infected tissues indicate that cell turnover in the granuloma may be important in the control of mycobacterial infection (143,144). Our laboratory has demonstrated by light and electron microscopy (145,146) that tuberculoid leprosy lesions contain epithelioid and multinucleated giant cells, surrounded by a mantle of

lymphocytes. A significant proportion of the mononuclear macrophages in these lesions appear damaged or dead. The foci of cell death are interspersed with large numbers of viable epithelioid cells and lymphocytes, suggesting a dynamic site with high cellular turnover in which continuous death and disintegration of older myeloid cells and the influx of young, freshly recruited mononuclear cells from the circulation are continuous. Examination by acid-fast staining reveals few, if any, bacilli in tuberculoid leprosy lesions. Unlike the foci of infection seen in tuberculoid leprosy, lepromatous leprosy lesions, which are far more widespread, accounting for most of the area of the skin in the most extreme cases, do not have well-organized granuloma, and many acid-fast bacilli are observed in the cytoplasm of macrophages. Cell turnover is not high in lepromatous lesions. Thus, the number and integrity of intracellular bacilli appears inversely related to the turnover of macrophages, and the intensity of the T cell response.

O. Attempts to understand how mycobacteria are killed within human monocytes

When mycobacteria are phagocytosed by human mononuclear phagocytes, the bacilli survive within a phagosome and replicate intracellularly. Up until now, human monocytes have not been shown to efficiently inhibit the multiplication of *M. tuberculosis* or MAI, much less to kill them *in vitro*.

The observations that mycobacteria appear not to be killed by human mononuclear phagocytes and that cell death is a major feature within the granuloma [the site at which mycobacterial infection is contained] has led our laboratory as well as others to investigate the effect of infected host cell death on the survival of intracellular mycobacteria. Several studies reported that mycobacteria-reactive T cells, including $CD4^+$, $CD8^+$, $CD4^-CD8^-TCR\gamma\delta^+$ T cells, were capable of destroying mycobacteria-infected target cells. However, in none of these studies, had a reduction in bacillary viability ever been observed (31,32,46). Similarly, our laboratory has shown that lymphokine activated killer [LAK] cells are also capable of lysing BCG-infected macrophages, but this cytotoxic activity had no effect on the viability of the bacilli (147). Taken together, these studies have shown that killer cells, whose cytotoxic functions are predominantly mediated by perforin, fail to kill mycobacteria within infected macrophages. These findings suggest that perhaps perforin may not be central to the control of mycobacterial infection.

Besides perforin-based cytotoxicity, killer cells have also been shown to kill by inducing apoptosis in target cells. We have previously reported that ATP^{4-}-induced apoptosis of BCG-infected macrophages and that the apoptotic event was accompanied by a reduction in the viability of the intracellular bacilli. This finding suggests that apoptotic death of the infected host cell may be linked to the death of intracellular bacilli. However, ATP^{4-} is unlikely to be an important mediator of apoptosis *in vivo* as the millimolar

concentration required are unlikely to be attained in real physiological circumstances. Cell-mediated apoptotic cytotoxicity has been shown to depend on the interaction between the Fas ligand [Fas-L] on killer cells and the Fas receptor [Fas-R] on the surface of target cells (42,43,44). How Fas-mediated apoptosis of infected cells affects the survival of mycobacteria has not been studied.

P. Objectives

This thesis begins with an investigation of whether the death of mycobacteria-infected human monocytes *in vitro* leads to killing of intracellular mycobacteria. Using an *in vitro* infection model, I focused on potential physiological mediators of cell death [perforin, Fas-R, and H_2O_2] to determine whether induction of cell death by any of these mediators induced concomitant killing of intracellular mycobacteria. In addition, mice with a targeted disruption in either Fas-R or perforin genes were experimentally infected with mycobacteria to assess the contribution of some of these death-inducing molecules to the control of mycobacterial infection *in vivo*. *In vitro*, I examined the growth and susceptibility to killing of various species of mycobacteria in cultured human monocytes, and cytokine production by the infected monocytes. The findings and their implications will be discussed in the context of our understanding of mycobacterial infection in humans.

II. Materials and Methods

In vitro studies

A. Reagents

1. Blood donors

Peripheral blood was obtained from donors by venipuncture, collected into heparinized syringes, and processed within 60 min of drawing. Informed consent was obtained from all donors participated in the studies. Leukocyte-enriched buffy coats were obtained from the New York Blood Services Center West [New York, NY].

2. Antibodies and recombinant cytokines

Polyclonal antibodies to human TNF-α, human IL-6, human IL-12 [Endogen, Boston, MA], murine monoclonal antibody [IgM] to human Fas receptor [Kamiya Biomedical Company, Thousand Oaks, CA], and FITC-conjugated goat $F[ab']_2$ fragment of IgG anti-mouse IgM [μ chain] [Organon Teknika Corp., West Chester, PA] were obtained commercially. Anti-APO-1 hybridoma supernatant was a kind gift from Dr. P. H. Krammer [Heidelberg, Germany] (148)

Recombinant human interferon-gamma [rhIFN-γ] [Genzyme, Cambridge, MA] and recombinant human IL-10 [rhIL-10] [Endogen, Boston,

MA] were obtained commercially.

3. Mycobacteria

Four mycobacterial strains were used for these studies: a patient isolate of *Mycobacterium avium-Mycobacterium intracellulare* [MAI] [a kind gift from Dr. Sharon Mannheimer, Cornell University Medical College, NY]; a mouse virulent MAI [mMAI], strain 25291 [American Type Culture Collection, Rockville, MD]; *Mycobacterium bovis* Bacillus Calmette-Guerin [BCG], Pasteur Strain 1011 [Trudeau Institute, Saranac Lake, NY]; and *Mycobacterium tuberculosis* [H37Ra], Pasteur Strain 201 [Trudeau Institute, Saranac Lake, NY].

MAI, BCG and H37Ra were grown to mid-log phase in 100 ml of lipopolysaccharide [LPS]-free Proskauer-Beck medium containing 0.05% Tween 80. mMAI were grown to mid-log phase in LPS-free 7H9 containing 0.05% Tween 80. Aliquots of bacillary suspensions in growth medium were stored in liquid nitrogen, and when thawed, shown to contain ~2 x 10^8 colony forming units [CFU]/ml capable of growing on 7H10 solid medium.

All bacteria were lipopolysaccharide [LPS] free and without reactivity in the Limulus Amoebocyte Lysate assay [Whittaker bioproducts, Walkersville, MD].

4. Media

Human mononuclear leukocytes were washed and cultured in RPMI 1640 medium [GIBCO BRL, Gaithersburg, MD] supplemented with pooled human AB+ serum obtained commercially [Biocell Ltd., Carson, CA]. Cells were washed in 1% human serum [R-1], and cultured in 20% human serum [R-20].

5. Other reagents

Catalase, superoxide dismutase, L-histidine, 1,3-dimethyl-2-thiourea, H_2O_2, cycloheximide, actinomycin-D, deferoxamine myselate, ethylenediaminetetraacetic acid [EDTA], ethylglycoltetraacetic acid [EGTA], aminoguanidine, and N^G-monomethyl-L-arginine [L-NMMA] were obtained commercially [Sigma Chemical Co., St. Louis, MO]; radioactive isotopes: sodium [^{51}Cr]chromate and L-[^{35}S]methionine protein labeling mix were similarly obtained [New England Nuclear, Boston, MA].

B. Cell preparations

1. Peripheral blood mononuclear cells [PBMC]

PBMC were isolated as follows. Whole blood and buffy coats were diluted with 0.5 volume and 1.0 volume of RPMI 1640 medium respectively, layered on 15 ml Ficoll-Hypaque [Pharmacia, Uppsala, Sweden] in a 50 ml conical tube, and centrifuged at 500 g for 30 min at 25^{o}C. Interface cells were

washed three times at 4°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.

2. Enriched monocytes

Fluorescence-activated cell sorting [FACS] analysis demonstrated that approximately 10-15% of PBMC isolated were monocytes. Two procedures were used to prepare monocyte enriched cultures.

[1] *Tissue culture plastic adherence.* Depending on the assays, PBMC density was adjusted to 10^7/ml R-1 and 100 µl of the cell suspension was plated on 13-mm Thermanox coverslip [Lux, Naperville, IL] in 24 well plates [Becton Dickinson Labware, Lincoln Park, NJ], or adjusted to 6 x 10^6/ml R-1 and 500 µl and 100 µl of the cell suspension were plated on Falcon Primaria tissue culture 24- and 96-well plates [Becton Dickinson Labware, Lincoln Park, NJ] respectively. Nonadherent cells were washed away with R-1 after 1 h incubation at 37°C so that the final density was 1 x 10^5 cells/coverslip, 6 x 10^4 cells/well [for 96-well plates] and 3 x 10^5 cells/well [for 24-well plates]; this cell density gave rise to an even monolayer after 4 d of culture.

[2] *Monocyte enrichment before tissue culture plastic adherence.* In assays requiring a large number of monocytes, monocytes were enriched by incubation of PBMC with neuraminidase-treated [*Vibrio cholera*

neuraminidase; Calbiochem-Behring Corp., La Jolla, CA] sheep erythrocytes [Cocalico Biologicals, Inc., Reamstown, PA] for 1 h in R-1 on ice. Separation of the mononuclear phagocytes from the rosetted population was performed by a 20 min centrifugation over a Ficoll-Hypaque gradient. Non-rosetted cells [E^- cells] were allowed to adhere directly to 100-mm tissue culture plates [Becton Dickinson Labware, Lincoln Park, NJ] and 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 Ca^{2+}-, Mg^{2+}-free PBS for 20 min at 4°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 monocytes were cultured in teflon beakers [Savillix, Minnetonka, MN] and incubated at 37°C, 5% CO_2. Cells obtained from either protocol were 95% monocytes as assessed by FACS analysis of CD14 expression.

3. Infection of monocytes with mycobacteria

An aliquot of mycobacteria was thawed at 37°C, diluted 10-fold in culture medium, and dispersed in a bath sonicator [Laboratory Supplies Co., Hickville, NY] with three 10 second pulses and cooling on ice between pulses. Sonicated single bacillary suspensions of mycobacteria were added to freshly isolated adherent monocytes or to monocytes that had been cultured for 5 or 8

d at a multiplicity of infection of one viable bacillus per cell, and incubated in R-20 medium for 0-14 d. At 6 h post infection, 30-50 % of monocytes were infected with a mean of 1.4 MAI, 1.5 mMAI, 2.7 BCG and 2.5 H37Ra per infected cell as determined by direct counting of acid fast bacilli [see below].

C. Procedures

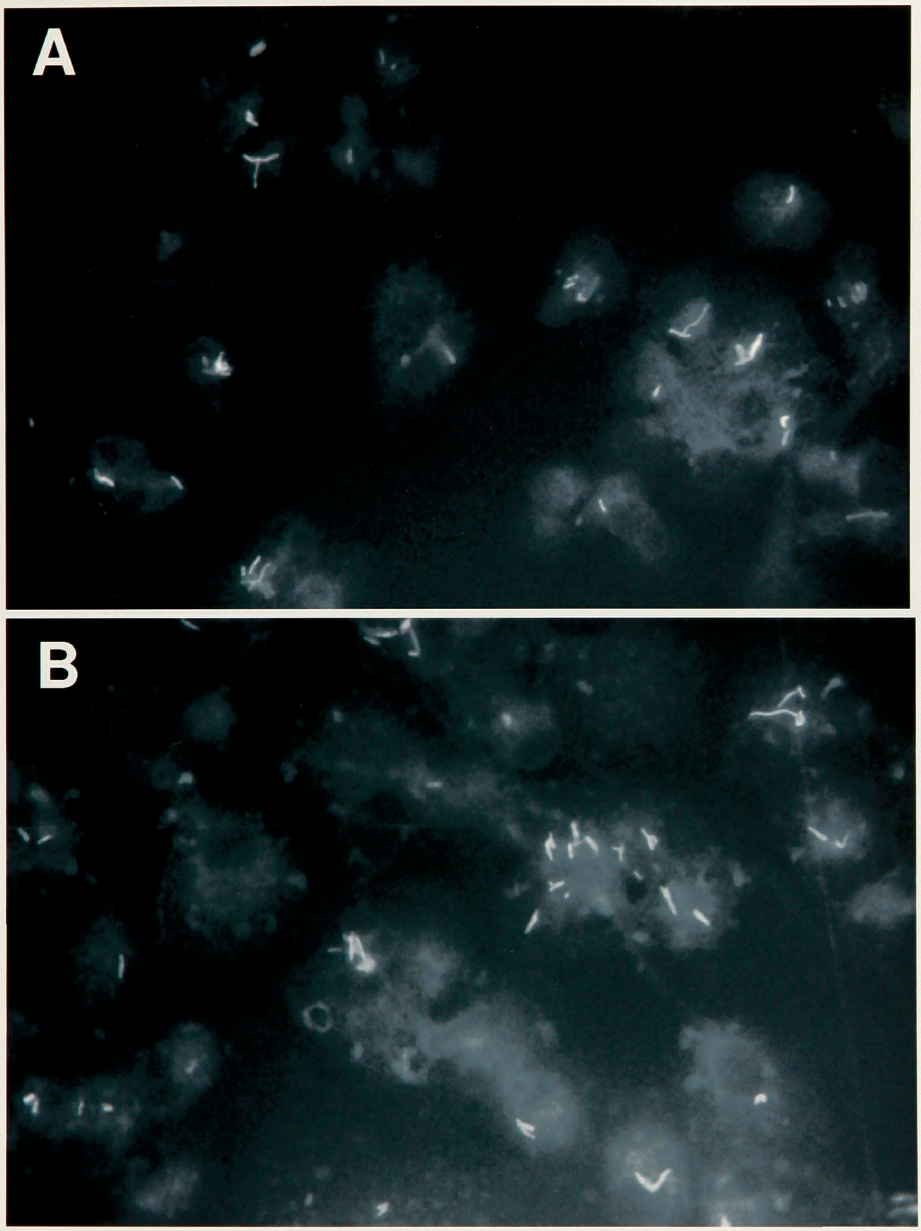
1. Acid Fast Staining

The monolayers of infected monocytes on 13-mm Thermanox coverslips 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. Briefly, one drop of auramine/rhodamine stain was added to each coverslip. After 15 min, the coverslips were rinsed in water and shaken in acid alcohol [70% ethanol and 0.5% HCl]. After drying, the coverslip with the cells facing up was mounted in glycerol under a glass coverslip on a glass microscope slide. The bacteria were visualized by fluorescent acid fast staining, counted and photographed under a phase/fluorescence microscope using a Nikon BG-12 excitation and OG-1 barrier filter for epifluorescence [Figure 2-1].

2. Purification of perforin

Perforin was purified from murine CTL line CTLL-R8. Using a high salt extraction method, perforin was extracted from granule-enriched

Figure 2-1. Acid-fast staining of mycobacteria. [A and B] Human monocytes were infected with a patient isolate of MAI. The cultured coverslips were fixed and stained as described.


A
B

fractions as described (149). The purification procedure required successive steps of liquid chromatography. The granule extract containing solubilized perforin was applied sequentially on DEAE-Sepharose, Q Sepharose, and Mono Q columns [all from Pharmacia, Uppsala, Sweden] connected to a FPLC system [Pharmacia]. Columns were equilibrated with a buffer solution containing 20 mM Tris-HCl, pH 7.4, 1 mM EGTA. Applied samples were resolved with linear NaCl gradients for ion-exchange columns; perforin was eluted at 400-500 mM NaCl from DEAE-Sepharose, at 300 mM from Q Sepharose, and at 300 mM from Mono Q. All chromatographic steps were done at 4°C. To monitor for perforin activity in various column fractions, a hemolytic microassay was performed as described (150). Protein determinations were carried out in microtiter plates using a procedure adapted from Bradford's assay as described (150).

3. *Perforin-, anti-APO-1- and H_2O_2-induced cytotoxicity*

Monolayers of infected and uninfected monocytes were cultured for 4 d in flat bottom, Primaria 96-well tissue culture plates. Some cultures were treated with 100 U/ml human IFN-γ immediately after removal of nonadherent cells. Adherent cells were loaded with 0.5 μCi sodium [^{51}Cr]chromate for 16-18 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 or

solutions of anti-APO-1, or cycloheximide or both, or varying amounts of purified perforin, or varying dilutions of H_2O_2 in R-20. Plates were incubated for either 6 or 18 h at 37°C, 5% CO_2. Supernatants were removed and the cells were lysed by incubation in 0.1 ml 1% Triton X-100 for 1 h. Supernatants and cell lysates were counted separately in a gamma counter [Packard Instrument Company, Downers Grove, IL]. Spontaneous ^{51}Cr release was calculated as cpm in supernatants of control wells, and total ^{51}Cr release was calculated as total cpm in supernatants and lysates of control wells. Specific ^{51}Cr release was calculated as 100 x {[experimental ^{51}Cr release - spontaneous ^{51}Cr release]/[total ^{51}Cr release - spontaneous ^{51}Cr release]}.

4. Flow cytometric analysis of expression of Fas receptor

Immunofluorescence analysis of Fas receptor expression on human monocytes was performed as follows. Briefly, 3 x 10^5 monocytes purified by adherence were incubated with 10 μg/ml mouse anti-human Fas receptor monoclonal antibody [IgM] on ice for 45 min, washed with ice-cold 2% fetal bovine serum [FBS] in PBS twice before a 20 min incubation on ice with [1:500] FITC-conjugated goat $F[ab']_2$ fragment of IgG anti-mouse IgM [μ chain]. 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

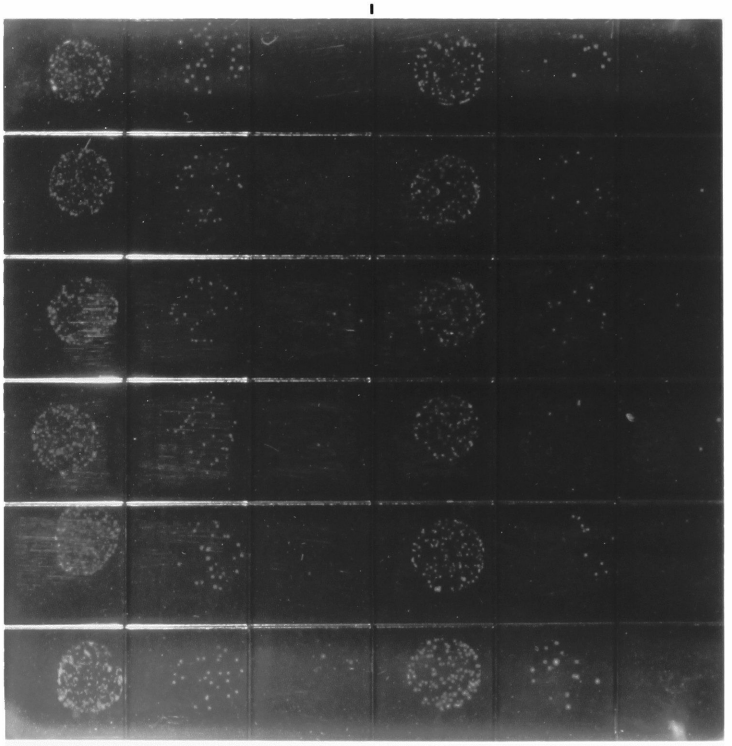
in PBS, and analyzed by a FACScan [Becton Dickinson, Mountain View, CA].

5. Colony forming units [CFU] assay

Monolayers of 3 x 10^5 infected monocytes in 0.5 ml of R-20 medium were cultured for 2-14 d in wells of flat bottom, Primaria 24-well tissue culture plates under varying conditions as described below. Depending on the nature of the experiments, after removal of nonadherent cells some cultures were treated with 100 U/ml rhIFN-γ, 2-10 ng/ml rhIL-10, 20-40 μg/ml anti-TNF-α antibody, 20-100 μg/ml anti-IL-6 antibody, or 20 μg/ml anti-IL-12 antibody. After the incubation period [6 or 18 h depending on the experiment], 0.5 ml of PBS containing 0.016% digitonin [Sigma Chemical Co.] and 0.25% Tween 80 was added to each well and incubated at 37°C for 10 min. The organisms were processed for the CFU assay as follows: The cultures were sonicated with six half-second pulses at low output power to disperse clumps of bacilli. This amount of sonication had been shown to be optimal for dispersing bacteria without causing any loss of viability. Serial 10-fold dilutions were made, and 10 μl of bacillary suspensions were plated on Middlebrook and Cohn 7H10 agar plates. After 7-21 d, dilutions containing 10-100 colonies arising from single mycobacteria were counted with the aid of a dissecting microscope. An example of mycobacterial colonies on a Middlebrook and Cohn 7H10 agar plate is shown in Figure 2-2. For each culture, six replicate

Figure 2-2. Mycobacterial colonies on a Middlebrook and Cohn 7H10 agar plate.

MAI Infection of Monocytes



7. DNA agarose gel electrophoresis

Monocytes harvested as described above were cultured for 4 d in teflon beakers, and 12 x 10^6 monocytes were used for each treatment. Varying dilutions of H_2O_2 in R-20 were added to monocytes to give a final concentration of 2, 5, 10 and 20 mM. Anti-APO-1 hybridoma supernatant and cycloheximide in R-20 were added to monocytes to give final concentrations of 0.1μg/ml and 1 μg/ml respectively. Both treated and untreated monocytes were incubated for 6 h. DNA was harvested as follows: Both treated and untreated control cells were detached from the plates using a rubber policeman, and centrifuged at 100 g for 10 min. The cell 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 electrophoresis: 1.5% agarose for 2 h at 60 V. DNA was visualized following staining with ethidium bromide.

8. Electron microscopy

Monolayers of monocytes cultured on plastic coverslips were fixed in 2% glutaraldehyde/0.1 M sucrose/0.1 M cacodylate buffer for 60 min at 4°C. The monolayers were postfixed in OsO_4 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.

9. Cytokine determination

TNF-α, IL-6, IL-12, and IL-10 concentrations in the culture supernatants were determined by commercial ELISA kits [Endogen, Boston, MA] according to the manufacturer's instruction. The concentration of TGF-β was determined similarly by commercial ELISA kits [R&D Systems, Minneapolis, MN]. Each data point was calculated from the mean of triplicate cultures.

In vivo studies

A. Mice

The original perforin-deficient [$P^{-/-}$] mice were 129/C57BL/6, H^{2b}/H^{2b} (151). At the Rockefeller University Animal Facility, these were backcrossed to C57BL/6 mice to obtain a more homogenous genetic background. Control

littermates were also from our breeding facilities; some additional control C57BL/6 animals were purchased from Jackson Laboratory [Bar Harbor, ME]. CBA/lpr^{cg}, H2^{k} Fas-receptor defective mice and control littermates [CBA$^{+/+}$] were kindly provided by Dr. Keith B. Elkon. CBA/lpr^{cg} mice have a point mutation in the receptor rendering it non-functional. All mice were 2-3 months old at the beginning of the study.

B. Mycobacteria

Mycobacterium bovis Bacillus Calmette-Guerin [BCG], Pasteur Strain 1011 [Trudeau Institute, Saranac Lake, NY] was grown in lipopolysaccharide [LPS]-free Proskauer-Beck medium containing 0.05% Tween 80. Aliquots were stored in liquid nitrogen, and when thawed, were shown to contain ~2 x 10^8 colony forming units [CFU]/ml capable of growing on 7H10 solid medium. *Mycobacterium tuberculosis*, strain Erdman was provided by the Trudeau Institute mycobacteria culture collection [Saranac Lake, NY].

C. Experimental design

Control littermates and mice with a disruption in the perforin gene [P$^{-/-}$] were injected intravenously in a lateral tail vein with 4 x 10^5 BCG or 2 x 10^5 *M. tuberculosis* strain Erdman per mouse. Control mice and Fas-receptor defective mice [CBA/lpr^{cg}] were injected intravenously with 4 x 10^5 BCG per mouse. At 1, 3, 5, and 13 weeks post injection, the infected P$^{-/-}$, Fas-receptor

defective and their respective control littermates were sacrificed. Whole organs as well as portions of the organs used to determine the number of viable mycobacteria in the CFU assay were weighed. Portions of infected organs were also fixed in 10% formalin [Fisher Scientific], sectioned, stained, and examined histologically. In addition, portions of spleens of both uninfected and infected mice were collected and immediately frozen at -70°C for determination of cytokine mRNA levels by RT-PCR.

D. Procedures

1. Colony forming units [CFU] assay

Bacterial load in the lungs, livers and spleens of infected mice were determined as follows: A weighed portion of each infected organ was homogenized in 1 ml of sterile PBS in a Glas-Col tube [Glas-Col, Terre Haute, IN]. Serial 10-fold dilutions of organ homogenates were prepared in PBS, and suspensions were plated onto Middlebrook 7H10 agar plates [Difco laboratories, Detroit, MI]. Plates were incubated at 37°C for 2-3 weeks. Bacterial colonies were counted with the aid of a dissecting microscope. For each culture, six replicate samples were counted and the results were expressed as the mean. The counts were adjusted per whole organ and expressed as the number of CFU per infected organ.

2. Histologic evaluation

Organs were fixed in 10% buffered formalin for 24 h, paraffin-embedded and processed for histology. Sections were stained with hematoxylin-eosin for histologic examination and with Ziehl-Nielsen for evaluation of acid fast bacilli [AFB] by light microscopy. To determine the relative numbers of granuloma in infected $P^{-/-}$, Fas-receptor defective and control littermates, 10 random fields of infected livers were examined at 25X magnification under a light microscope and the numbers of granulomata counted.

3. In situ staining of apoptotic cells

Organs were fixed in formalin overnight, paraffin-embedded, and 6 μm thick sections were placed on silanized glass slides [Oncor, Gaithersburg, MD]. The staining procedures were described in the ApopTag Peroxidase [Oncor], and modified as follows: the anti-digoxigenin-peroxidase was diluted 1:3 [v/v] in phosphate-buffer and stained with diaminobenzidine substrate solution containing hydrogen peroxide for 1 min.

4. Reverse transcription-polymerase chain reaction [RT-PCR] for cytokine mRNA

Total RNA was prepared from the spleens of both uninfected and infected mice using the RNAzol B solution [Tel-Test, Inc., Friendswood, TX] according to the manufacturer's instruction. Complementary DNA [cDNA]

was synthesized from 1 μg of the isolated RNA using Moloney murine leukemia virus reverse transcriptase [Perkin-Elmer, Norwalk, CT]. PCR was performed using the paired 5' and 3' primers for specific cytokine by the following protocol: 1 cycle of 3 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. A negative control reaction in which no RNA template was added was included in each experiment. In each PCR reaction, a trace amount of ^{32}P-dCTP [2 x 10^5 cpm/mixture] was included to label the PCR product. The PCR products were subjected to electrophoreses on 1% agarose gels containing ethidium bromide and visualized by UV illumination. Following photodocumentation, the agarose gels were fixed in 10% tricholoacetic acid [TCA] for 15 min at room temperature, washed twice with H_2O to remove TCA, and dried under vacuum. The dried gels were exposed to autoradiographic film [XAR-5; Kodak]. Cytokine mRNA levels were quantitated using a Phosphorimager [Molecular Dynamics, Sunnyvale, CA] and results were first normalized to the amount of β-actin mRNA in the same sample. The results are expressed as a fold increase over baseline amount of cytokine mRNA expressed in the uninfected control tissues.

III. The role perforin-induced necrotic host cell death in the control of intracellular mycobacterial viability

A. Introduction

Mycobacteria including *M. tuberculosis*, BCG and MAI parasitize human mononuclear phagocytes and replicate intracellularly. Our laboratory has long been interested in the fate of intracellular mycobacteria following host cell death. Cells die by one of two known mechanisms: necrosis and apoptosis. Necrosis is induced when the plasma membrane of a cell is irreversibly damaged leading to osmotic disregulation. 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 distinguished from necrosis on the basis of biochemical and morphological criteria.

In an attempt to understand how necrotic cell death regulated the survival of intracellular mycobacteria, Molloy *et al.* set up an *in vitro* human monocyte infection model and demonstrated that human lymphokine activated killer [LAK] cells were able to induce necrosis of BCG-infected macrophages *in vitro* (147). Although the infected host cells were killed by this cytotoxic process, the viability of the intracellular BCG was not affected (147).

Cell death mediated by LAK cells is perforin-dependent (41). Perforin is

also produced by other killer lymphocytes including $CD8^+$T cells, $CD4^+$T cells, $CD4^-CD8^-\gamma\delta^+$ T cells, and NK cells. The molecule is synthesized in the cells and stored in the cytoplasmic granules of these cells (41). Upon contact with an antigen-presenting target cell, degranulation of the cytoplasmic granules occurs and perforin is released into the extracellular space. Once released, perforin inserts itself into the target cell membrane, aggregates and causes the formation of pores. This results in osmotic disregulation and target cell death by a necrotic process (41).

To directly examine the role of perforin-induced necrosis in the regulation of mycobacterial survival, purified perforin was used to treat mycobacteria-infected monocytes *in vitro* and the viability of the mycobacteria was evaluated by colony forming units [CFU] assay. The contribution of perforin-mediated target cell death in the control of mycobacterial infection *in vivo*, was studied in mice with a disruption in the perforin gene [$P^{-/-}$]. Animals were infected intravenously with *Mycobacterium bovis* BCG [a nonvirulent organism] or *Mycobacterium tuberculosis* strain Erdman [an organism virulent in mice], and the course of the infection was followed. At 1, 3, 5, and 13 weeks post infection, the number of viable mycobacteria in lungs, spleens, and livers of mice were determined by colony forming units [CFU] assay, the infected tissues were examined histologically, and cytokine mRNA levels in the spleens were measured.

B. Results

1. The effect of purified perforin on the viability of intracellular mycobacteria in vitro. After isolation, monocytes were cultured for 5 d before infection with either BCG or *M. tuberculosis* H37Ra at the multiplicity of infection of one bacillus per cell. Infected monolayers appeared intact and remained adherent for the duration of the experiment. On day 5 post infection, purified perforin isolated from mouse cytotoxic T lymphocyte cell line [CTLL-R8] was added to some infected cultures at the final concentration of 2 hemolytic units per µl. [This concentration of purified perforin had previously been shown in a cytotoxicity assay to induce 80-90% specific release of intracellular ^{51}Cr within 4 h (152).] Some cultures were sonicated to disrupt all infected monocytes before the addition of perforin. Cultures were incubated for a further 6 h, and CFU assays were performed on both untreated control and treated cultures. Our results revealed no significant reduction in the numbers of CFU in either BCG-infected or H37Ra-infected cultures treated with purified perforin relative to untreated control cultures [Table 3-1]. Perforin had no direct effect on the bacillary viability since infected cultures sonicated before the addition of perforin contained similar numbers of viable organisms comparable to those observed in sonicated control cultures not treated with perforin. This finding that necrotic host cell death is not coupled with any killing of intracellular bacilli is consistent with our laboratory's previous *in vitro* findings with LAK-induced killing of infected macrophages.

Table 3-1. Bacillus viability after treatment of infected macrophages with purified perforin

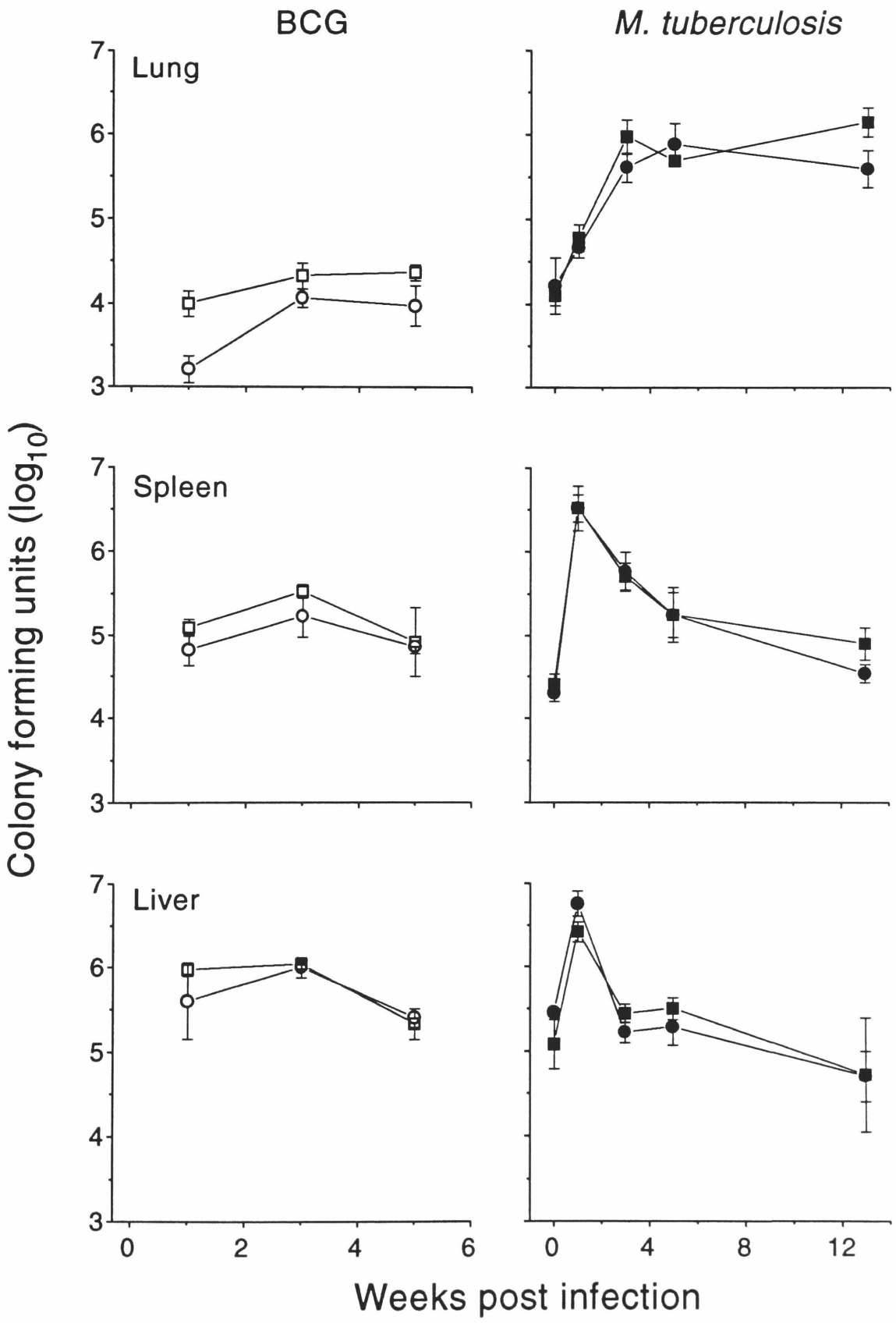
Culture	Treatment	Colony forming units@ [10^{-3}]	
		Intact monolayers	Sonicated cultures
BCG-infected macrophages	None [control]	8.1 ± 0.5	10.6 ± 0.5
	Purified perforin	9.1 ± 0.6	10.6 ± 1.0
H37Ra-infected macrophages	None [control]	12.3 ± 0.7	15.8 ± 0.5
	Purified perforin	13.1 ± 0.9	16.2 ± 1.0

@ Five-day-old monocytes were incubated at 37°C, 5% CO_2, infected with either BCG of H37Ra for 5 d, and treated as above for 6 h before cultures were harvested for CFU. Results are means ± SEM of triplicate cultures.

2. The course of mycobacterial infection of $P^{-/-}$ mice. To evaluate the contribution of perforin-mediated necrosis in the control of mycobacterial infection *in vivo*, $P^{-/-}$ mice and control [$P^{+/+}$] littermates were infected intravenously with either BCG or *M. tuberculosis* strain Erdman. Following BCG infection, there was no significant increase in the numbers of viable organisms in lung, spleen, or liver during the early course of infection in the control mice [Figure 3-1]. In the $P^{-/-}$ mice, the number of CFU recovered from the lungs of mice at 1 week post infection was almost one log lower than the CFU obtained from the lungs of control littermates [Figure 3-1]. At 3 and 5 weeks post infection, the CFU from infected lungs were also lower in $P^{-/-}$ mice; however, the difference was less pronounced. In BCG-infected livers and spleens, the numbers of CFU obtained from $P^{-/-}$ mice were the same as the number obtained from control mice at 1, 3, and 5 weeks post infection. A slight decrease in the numbers of CFU was observed in both infected spleens and livers at 5 weeks post infection in all mice.

Unlike BCG infection, *M. tuberculosis* infection of $P^{-/-}$ and control mice resulted in a significant increase in the numbers of CFU in the infected lungs by 1 and 3 weeks post infection. In infected spleens and livers, there was an initial increase followed by a decrease in the numbers of CFU [Figure 3-1]. The numbers of viable mycobacteria recovered from infected organs of $P^{-/-}$ and control mice were not significantly different in the two strains of mice at all time points and in all tissues studied. These findings suggest that

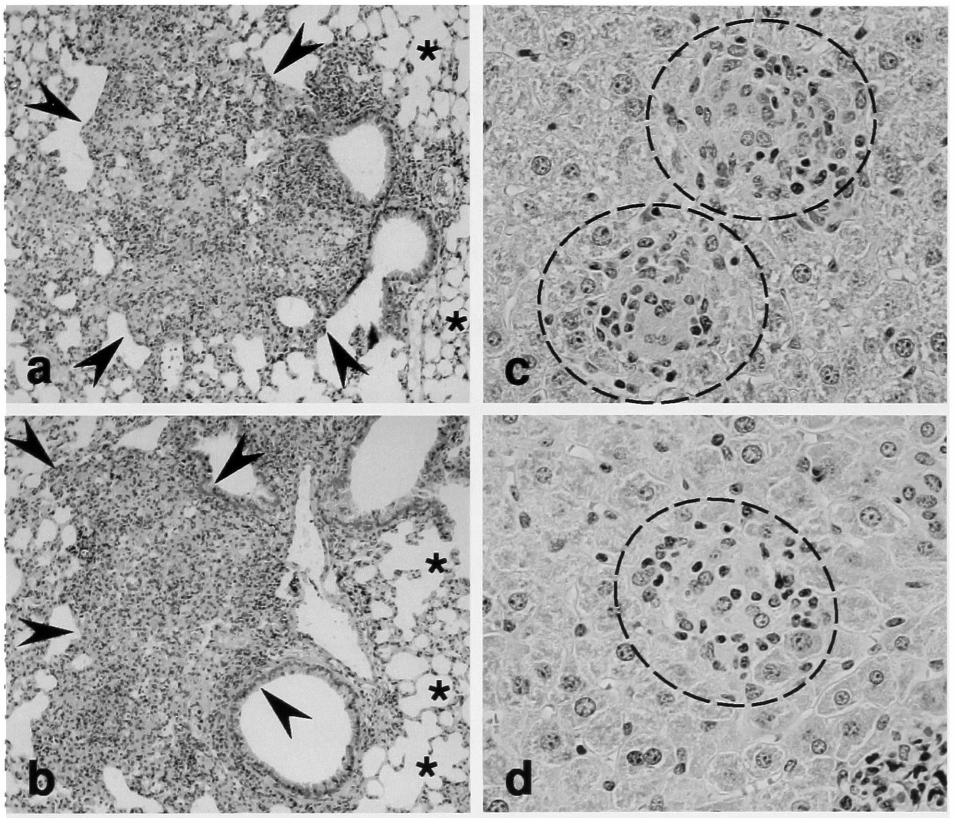
Figure 3-1. Mycobacterial load expressed as colony forming units [CFU] per organ in $P^{-/-}$ mice [circle] and control $P^{+/+}$ mice [square]. Mice were infected intravenously with either BCG [open symbols] or *M. tuberculosis* Erdman [closed symbols]. 3-5 mice were sacrificed for CFU analysis per time point. Results are means $\pm$ SD of 2 experiments.


BCG
M. tuberculosis
Lung
Spleen
Liver
Colony forming units (log_{10})
Weeks post infection
7
6
5
4
3
0
2
4
6
8
12

M. tuberculosis was better able to replicate in the lungs of both $P^{-/-}$ and control mice than BCG. Furthermore, $P^{-/-}$ mice were as capable of controlling the infection by BCG and *M. tuberculosis* strain Erdman as the control littermates as evaluated by CFU assay [Figure 3-1]. These results indicate that perforin does not appear to play a protective role in the early control of either avirulent [BCG] or virulent [*M. tuberculosis*] mycobacterial infection *in vivo*.

3. Histologic evaluation of infected organs. The lungs and livers of both $P^{-/-}$ and control mice infected with either BCG or *M. tuberculosis* were examined. No significant morphologic difference was observed between $P^{-/-}$ and control mice at any time point [Figure 3-2]. In both $P^{-/-}$ and control mice infected with BCG, granulomas were rarely seen in the lungs. In contrast, the lungs of both $P^{-/-}$ and control mice infected with *M. tuberculosis* contained many large granulomata [Figures 3-2a and 3-2b, arrow heads] surrounded by normal lung tissue [Figures 3-2a and 3-2b,*]. However, in the livers of control mice, both BCG and *M. tuberculosis* infection resulted in granuloma formation as early as one week post infection [Figure 3-2d]. The same morphology was observed in $P^{-/-}$ livers following either infection [Figure 3-2c]. In addition, the number of granuloma following BCG infection was examined. Both $P^{-/-}$ and $P^{+/+}$ mice demonstrated no significant difference in the number of granuloma following BCG infection [Figure 3-3]. Thus, granuloma formation induced in

Figure 3-2. Histology of mycobacteria-infected tissues. Lungs of $P^{-/-}$ [a] and control $P^{+/+}$ mice [b] at 3 weeks post *M. tuberculosis* Erdman infection. Arrow heads demarcate the granulomas which are surrounded by normal lung tissue [*]. Livers of $P^{-/-}$ [c] and control $P^{+/+}$ mice [d] at 3 weeks post BCG infection. Granulomas are observed within the enclosed ellipses. Magnification: [a-b] x 25; [c-d] x 100.

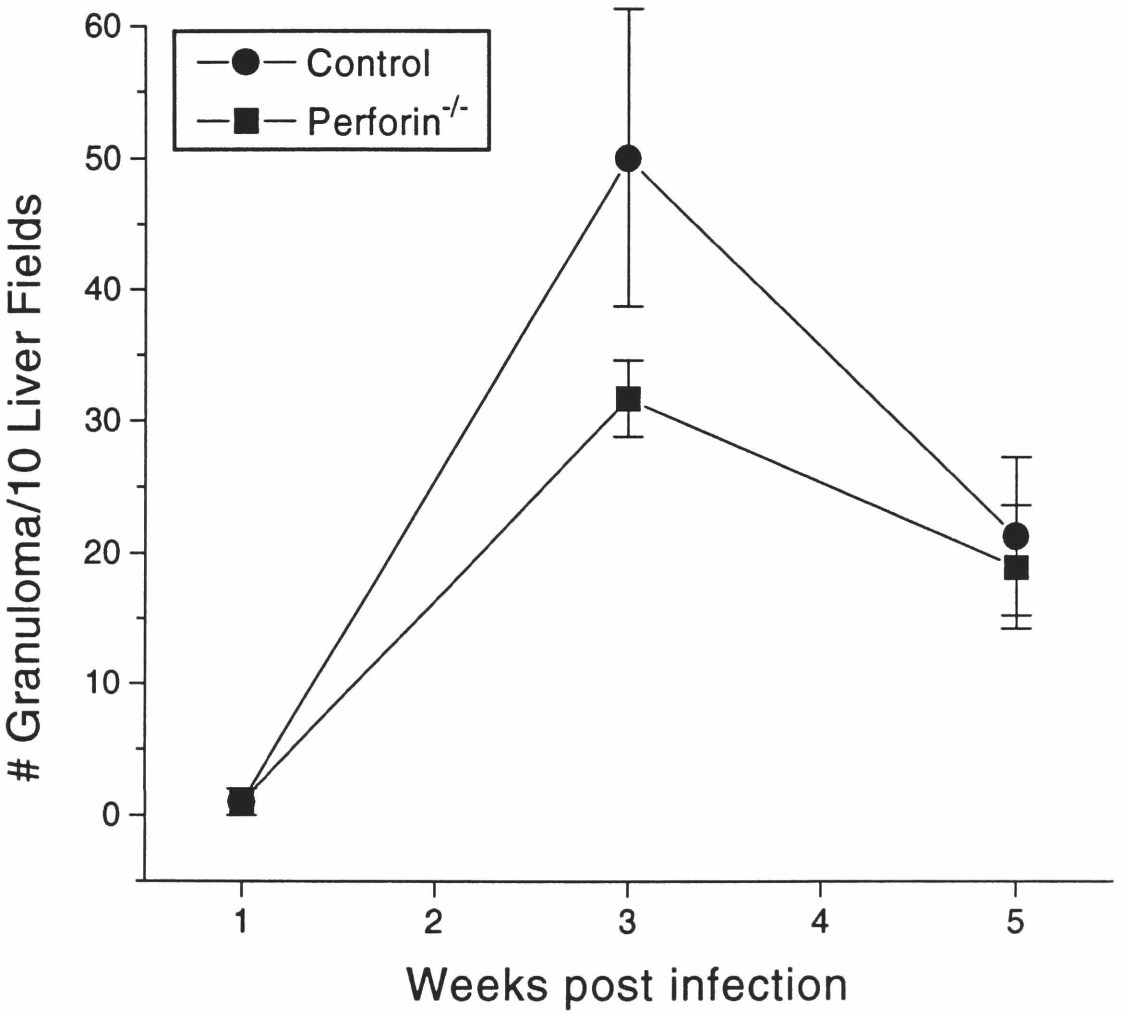

a
b
c
d

response to mycobacterial infection was not affected in either lungs or livers by the disruption in the perforin gene in $P^{-/-}$ mice. Together with the CFU findings, these results suggest that the absence of an intact perforin gene did not render $P^{-/-}$ mice more susceptible to either avirulent or virulent mycobacterial infection.

4. Systemic cytokine activation in $P^{-/-}$ mice. In order to better understand the immune response to mycobacterial infection in the two strains of mice, we investigated systemic leukocyte cytokine activation. RT-PCR analysis of cytokine mRNA levels in the spleen revealed that even at baseline before infection, $P^{-/-}$ mice had 3.4-5.0 times higher levels of IL-10 mRNA, IL-12p35 subunit mRNA, IL-6 mRNA, and IFN-γ mRNA in their spleens relative to the uninfected control littermates [Figure 3-4]. Baseline [week 0] levels of IL-12p40 subunit and IL-1β were higher [although not statistically significantly higher] in $P^{-/-}$ mice than in the $P^{+/+}$ control animals. Baseline levels of IL-15 mRNA were similar in both types of mice. Thus, it appeared that perforin gene disruption was associated with a compensatory immunologic activation of leukocytes and the expression of increased levels of leukocyte cytokine mRNAs, even in the absence of experimental infection.

Following both BCG and *M. tuberculosis* infection, cytokine genes were upregulated in both control $P^{+/+}$ and $P^{-/-}$ mice. There were no

Figure 3-3. The numbers of granulomata in livers of $P^{-/-}$ mice [squares] and control $P^{+/+}$ mice [circles] infected with BCG. Ten random fields of infected livers were examined for each infected animal, and the numbers of granulomata counted. Results are means $\pm$ SEM for 4 infected animals per each test group.

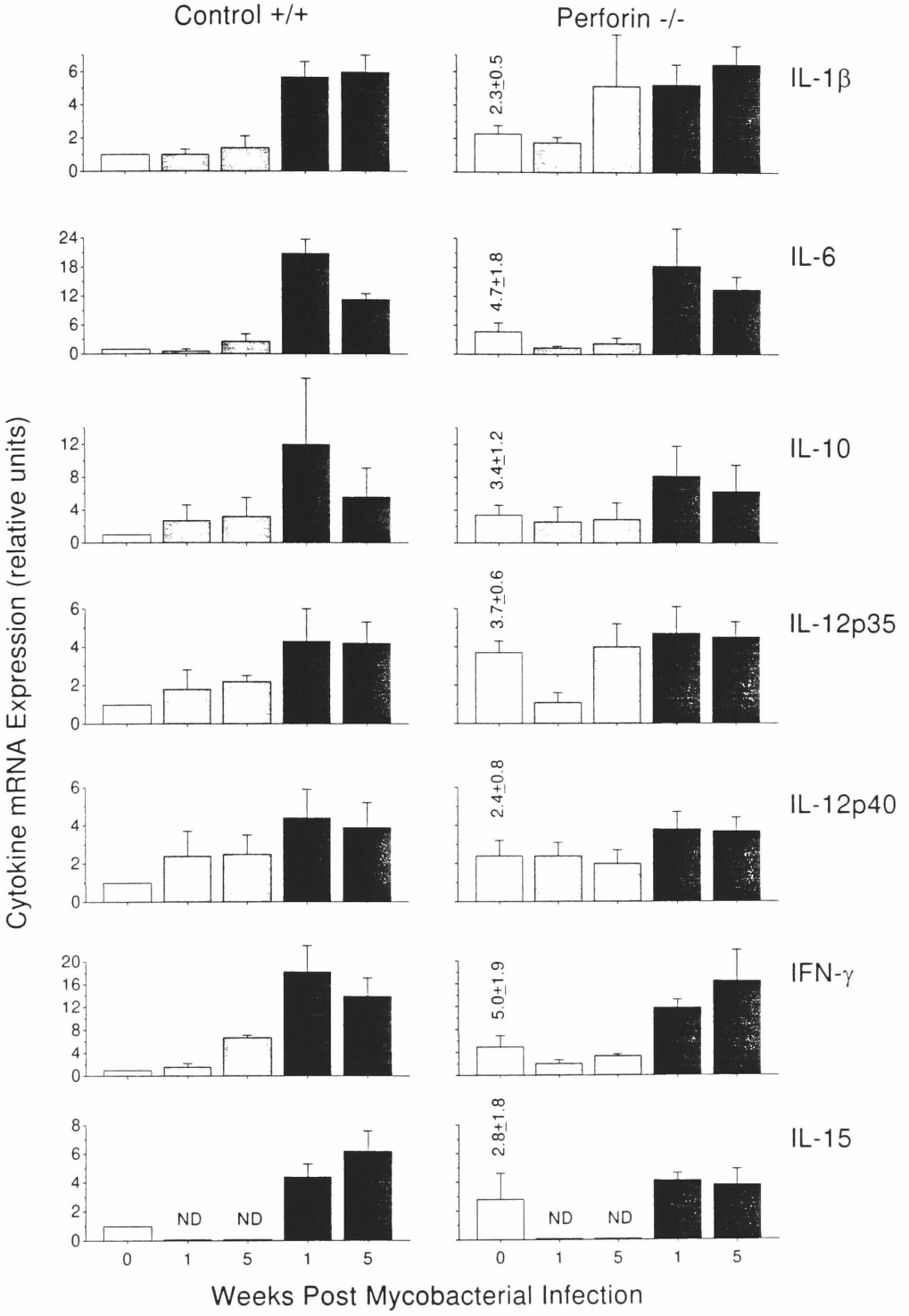

Control
Perforin-/-
Granuloma/10 Liver Fields
0
10
20
30
40
50
60
1
2
3
4
5
Weeks post infection

significant differences in cytokine levels between $P^{+/+}$ control and $P^{-/-}$ mice by 5 weeks post-mycobacterial infection [Figure 3-4]. Thus, activation of cytokine genes in response to mycobacterial infection occurs in both control and knockout mice, blurring the baseline cytokine activation observed in the uninfected gene-disrupted mice. However, virulent *M. tuberculosis* infection induced a cytokine response of greater magnitude than the one observed with avirulent BCG.

C. Discussion

In response to *M. tuberculosis* infection, the infected host mounts a cellular immune response in the context of a granuloma. $CD4^+$ as well as $CD8^+$ T cells have been shown to be important in this resistance mechanism to mycobacterial infection *in vivo* (30,39). However, the precise role of each of these cells in the control of the infection has not been fully elucidated. The main contribution of $CD4^+$ T cells to the protective anti-tuberculosis response may involve the production of Th1 type cytokines including IL-2 and IFN-γ. $CD4^+$ T cells have also been shown to have some cytotoxic activity against mycobacteria-pulsed and mycobacteria-infected macrophages (31,32). $CD8^+$ T cells are also capable of producing cytokines including IFN-γ (40), but have been considered to be primarily responsible for cell-mediated cytotoxicity. $CD8^+$ T cells mediate cytotoxicity by releasing cytoplasmic granules which

Figure 3-4. Cytokine mRNA levels in the spleens of mice infected with BCG [gray bars] or with *M. tuberculosis* Erdman [black bars]. Spleens obtained from uninfected mice [open bars] and infected mice [shaded bars] were assayed by RT-PCR at 0, 1 week and 5 week post infection. Cytokine mRNA levels were quantitated as described in Materials and Methods. Results were first normalized to amount of β-actin mRNA in each lane, and then expressed as fold increase over the amount of cytokine message expressed by the uninfected $P^{+/+}$ control mice at time 0. N.D.-not done. The numbers above the time 0 cytokine levels of $P^{-/-}$ mice are the fold increase over time 0 cytokine levels of control $P^{+/+}$ mice normalized to 1. The results are means ± SEM for 3 infected and 6 uninfected mice used per each test group from 2 experiments.


Control +/+
Perforin -/-
IL-1β
IL-6
IL-10
IL-12p35
IL-12p40
IFN-γ
IL-15
2.3±0.5
4.7±1.8
3.4±1.2
3.7±0.6
2.4±0.8
5.0±1.9
2.8±1.8
ND
ND
Cytokine mRNA Expression (relative units)
Weeks Post Mycobacterial Infection

contain a number of toxic molecules including granzymes and perforin (41). In addition, $CD8^+$ T cells can mediate cytotoxic effects *via* the interaction between Fas ligands on their cell surface and Fas receptors expressed on the target cells (42,43,44).

Previously, Flynn *et al.* showed that mice with a disruption in the β2-microglobulin [β2m] gene failed to develop functional $CD8^+$ T cells and succumbed to virulent *M. tuberculosis* infection much more rapidly than control mice (39). The authors interpreted these findings to indicate that MHC class I-restricted T cells [$CD8^+$] may be required for resistance to *M. tuberculosis* infection *in vivo*. However, how $CD8^+$ T cells contribute to the control of mycobacterial infection is not fully understood.

Perforin-mediated cytotoxicity has been implicated in *in vivo* clearance of some intracellular bacteria and virus. For instance, mice with a targeted disruption in the perforin gene [$P^{-/-}$ mice] infected with *Listeria monocytogenes* were found to clear both primary and secondary infections more slowly than control mice (153). Also, clearance of a non-cytopathic lymphocytic choriomeningitis virus [LCMV] is mediated through the perforin-dependent mechanism without any measurable involvement of the Fas ligand-Fas receptor [FasL-FasR] pathway (154).

In this study, our findings indicate perforin-induced necrosis did not appear to play a protective role in the control of mycobacterial infection *in vivo*. This observation is consistent with Molloy's *in vitro* observation.

From the *in vitro* study using purified perforin, and the *in vivo* study using perforin knockout mice experimentally infected with mycobacteria, these results establish that perforin-mediated cytotoxicity neither kills mycobacteria directly nor is it necessary for the early control of both virulent and avirulent mycobacterial infection. It is worth noting that although granzymes [serine proteases] present within the granules containing perforin molecules are capable of inducing cytotoxicity, several studies have shown that this cytotoxic pathway is dependent on the presence of perforin at sub-lytic doses for its activity (155,156,157). Due to the absence of perforin in the perforin-deficient mice used in our study (151), the contribution of granzymes to the control of mycobacterial infection seems unlikely.

If cytotoxic cells are indeed necessary for the protective immune response to mycobacterial challenge *in vivo*, the results presented here suggest that the response is mediated by alternative cytotoxic molecules and/or the production of protective cytokines. Since it has been shown that cytotoxic T cells derived from perforin knockout mice retain the Fas-mediated cytotoxic [FasL-FasR] pathway, it is possible that Fas-mediated killing may be involved in the control of mycobacterial infection *in vivo*. The contribution of FasL-FasR pathway to the control of mycobacterial infection both *in vitro* and *in vivo* will be discussed in the following chapter.

Finally, gene-targeted disruption of the perforin gene results in activation of many cytokine genes in $P^{-/-}$ animals [Figure 3-4]. It is

conceivable that these newly activated genes represent the redundancy of the immune response which may be necessary for the development of the animals in the absence of perforin gene. Whether a compensatory activation of cytokine genes may partially rescue the function of the disrupted perforin gene is not known. However, the fact that $P^{-/-}$ mice appear to have a compensatory activation of cytokine genes raise an important issue with respect to the use of gene-disrupted mice in *in vivo* studies. For example, in experimental models of microbial infection, it is difficult to evaluate how the expression of genes which frequently remain silent in the normal immune response may affect the course of infection. Since these abnormally activated genes may compensate for essential functions normally performed by the product of the disrupted gene, results obtained from these knock-out animals must be carefully interpreted.

IV. The role of Fas-induced apoptotic host cell death in the control of intracellular mycobacterial survival

A. Introduction

Studies carried out on the lesions of leprosy patients have suggested that cell turnover within the granuloma of mycobacteria-infected tissues may be important in the control of mycobacterial infection (143,144). In the previous chapter, we showed that perforin-induced necrosis of mycobacteria-infected macrophages did not affect the viability of intracellular mycobacteria *in vitro*. In addition, the absence of an intact perforin gene did not render $P^{-/-}$ mice more susceptible to both virulent and avirulent mycobacterial infection *in vivo*. Thus, perforin-induced necrosis does not appear to have any significant, direct effect on mycobacterial survival. Because apoptosis has been documented in the granulomata of mycobacteria-infected tissues (143,144), apoptosis of infected mononuclear phagocytes may be involved in the regulation of mycobacterial survival in the granuloma.

We have previously reported that induction of apoptosis of BCG-infected monocytes with ATP^{4-} was associated with killing of the intracellular mycobacteria (57). To further examine the role of apoptotic mechanism in survival and death of intracellular mycobacteria, a physiologic pathway of apoptosis was investigated. Fas receptor, which belongs to the nerve growth factor receptor/tumor necrosis factor receptor [NGFR/TNFR]

family, is constitutively expressed in many normal tissues and cells including peripheral blood monocytes. Fas receptor is inducible in other cell types (158,159,160). Treatment with mAb directed against the Fas receptor triggers apoptosis of Fas receptor-expressing cells both *in vitro* (158) and *in vivo* (161). This suggests that Fas ligand may play a role in the regulation of apoptosis in a wide range of tissues.

A long term *in vitro* infection model of MAI-infected human monocytes was established. MAI is a facultative intracellular organism that is phagocytosed by and multiplies in human macrophages (162). MAI is nonpathogenic in healthy individuals but an opportunistic pathogen for AIDS patients. This suggests that there is a protective mechanism in the immune competent human host that can clear MAI infection. How human monocytes/macrophages control the growth and/or kill intracellular MAI is as yet unknown (163).

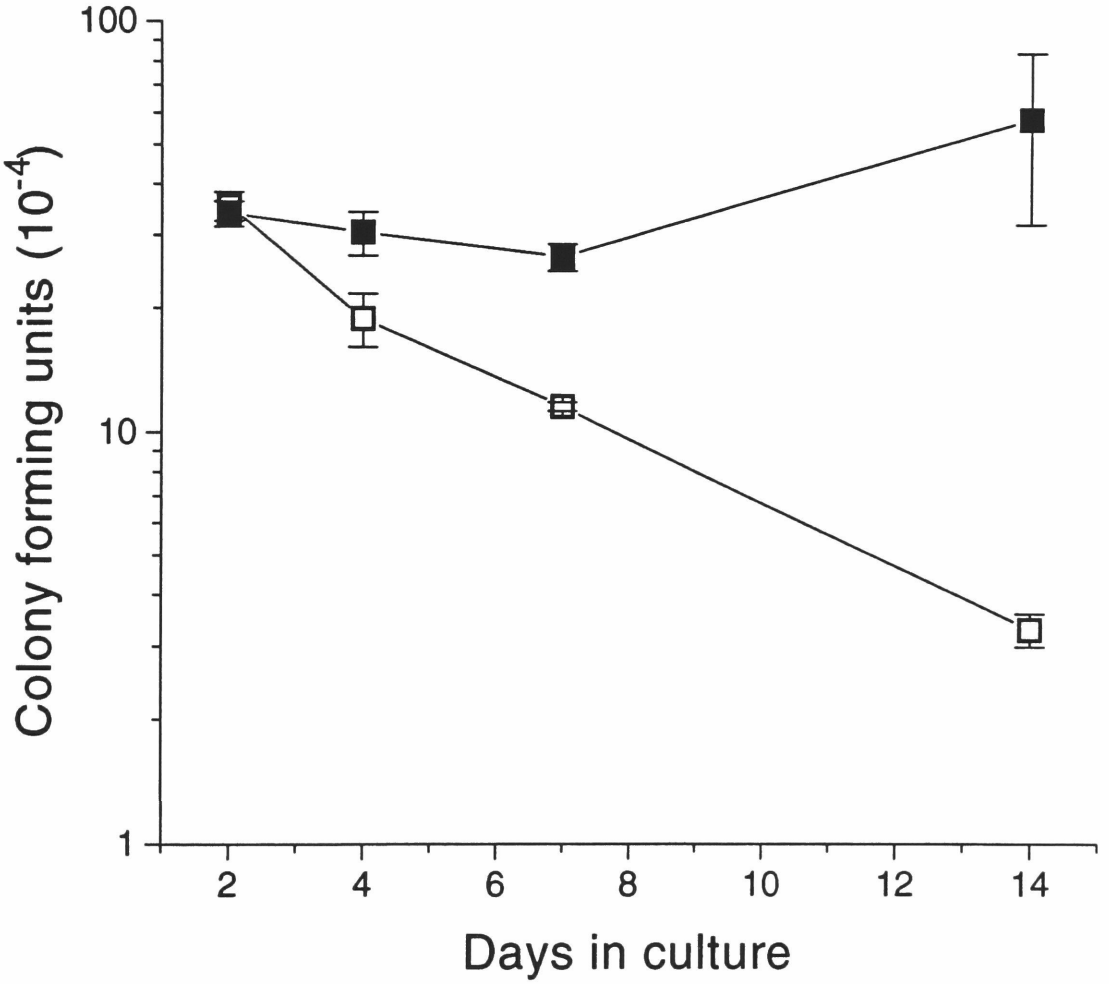
In this study, MAI-infected human monocytes were treated with a monoclonal antibody to Fas receptor [APO-1/CD95], and CFU assay was performed to determine whether apoptosis of the host cell *in vitro* affects the survival of intracellular MAI. To evaluate whether Fas-mediated apoptosis plays a role in the control of mycobacterial infection *in vivo*, Fas receptor defective mice [CBA/lpr^{cg}] and control [CBA$^{+/+}$] mice were infected intravenously with *M. bovis* BCG. [CBA/lpr^{cg} mice carry a point mutation in the receptor rendering it non-functional.] At 1, 3, and 5 weeks post infection,

the number of viable mycobacteria in lungs, spleens, and livers of mice were determined by CFU assay, the infected tissues were examined histologically, and cytokine mRNA levels in the spleens of these mice were measured.

B. Results

1. Growth of MAI in human monocytes. Immediately after isolation, monolayers of adherent monocytes were infected at a multiplicity of approximately one viable bacillus per cell. At 2 d post infection, 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, the numbers of viable bacilli were determined by CFU assay [Figure 4-1]. No significant change in bacillary numbers was observed during the first 7 d in culture. However, over the next 7 d the mean number of viable intracellular MAI [CFU] increased somewhat [Figure 4-1]. Infected monocyte monolayers remained confluent and viable throughout the 14 d assay. The total number of viable infected monocytes did not change significantly during the 14 d culture as determined by direct microscopic examination. Parallel cultures of R-20 medium alone were inoculated with the same number of MAI as cultures containing monocytes. The number of viable bacilli in culture medium alone fell for the 2 week duration of the experiment. Thus, in the absence of human monocytes, MAI did not survive. This result indicates

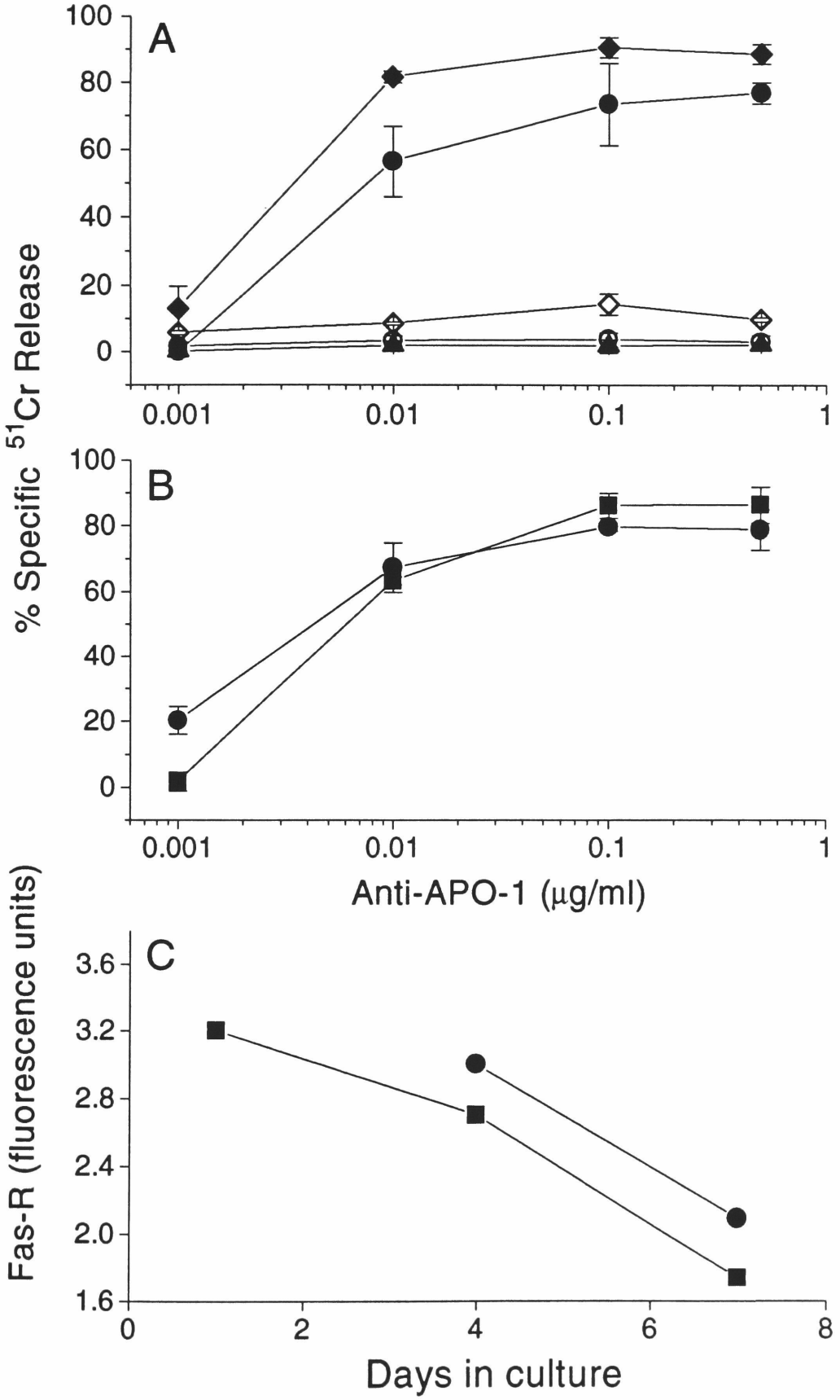
Figure 4-1. MAI growth in cultured monocytes. 3×10^5 viable MAI were added to wells containing 0.5 ml culture medium alone [open] or 0.5 ml culture medium containing 3×10^5 freshly isolated adherent monocytes [closed]. At the time points indicated, colony forming units [CFU] were assayed. Results are expressed as CFU/well and are means $\pm$ SD of 2 experiments, each done in triplicate.


100
10
1
Colony forming units (10^{-4})
2
4
6
8
10
12
14
Days in culture

that this patient isolate of MAI is an obligate intracellular organism that survives and may even replicate slowly in human monocytes.

2. *The effect of anti-APO-1 treatment on 4 d cultured monocytes.* To determine whether ligation of the Fas receptor induces monocyte death, monocytes were treated with anti-APO-1 antibody. Fas-induced cell death was seen only when cells were treated with anti-APO-1 in the presence of cycloheximide [CHX] [Figure 4-2A]. This is consistent with a previous report that Fas-induced death was enhanced when protein synthesis was blocked (164). Induction of monocyte death required 18 h of coculture with the antibody [Figure 4-2A]. When cultured monocytes were pretreated with IFN-γ, the killing of cultured monocytes induced by anti-APO-1 + CHX treatment was slightly enhanced [Figure 4-2A]. IFN-γ has been shown to upregulate Fas receptor expression in human colon carcinoma HT-29 cells (158); using FACS analysis, we 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 [Figure 4-2C]. When parallel assays were carried out with uninfected and MAI-infected cells, the dose-response to Fas-induced killing was similar, indicating that the presence of intracellular MAI neither protected nor predisposed host monocytes to death induced by anti-APO-1 + CHX [Figure 4-2B].

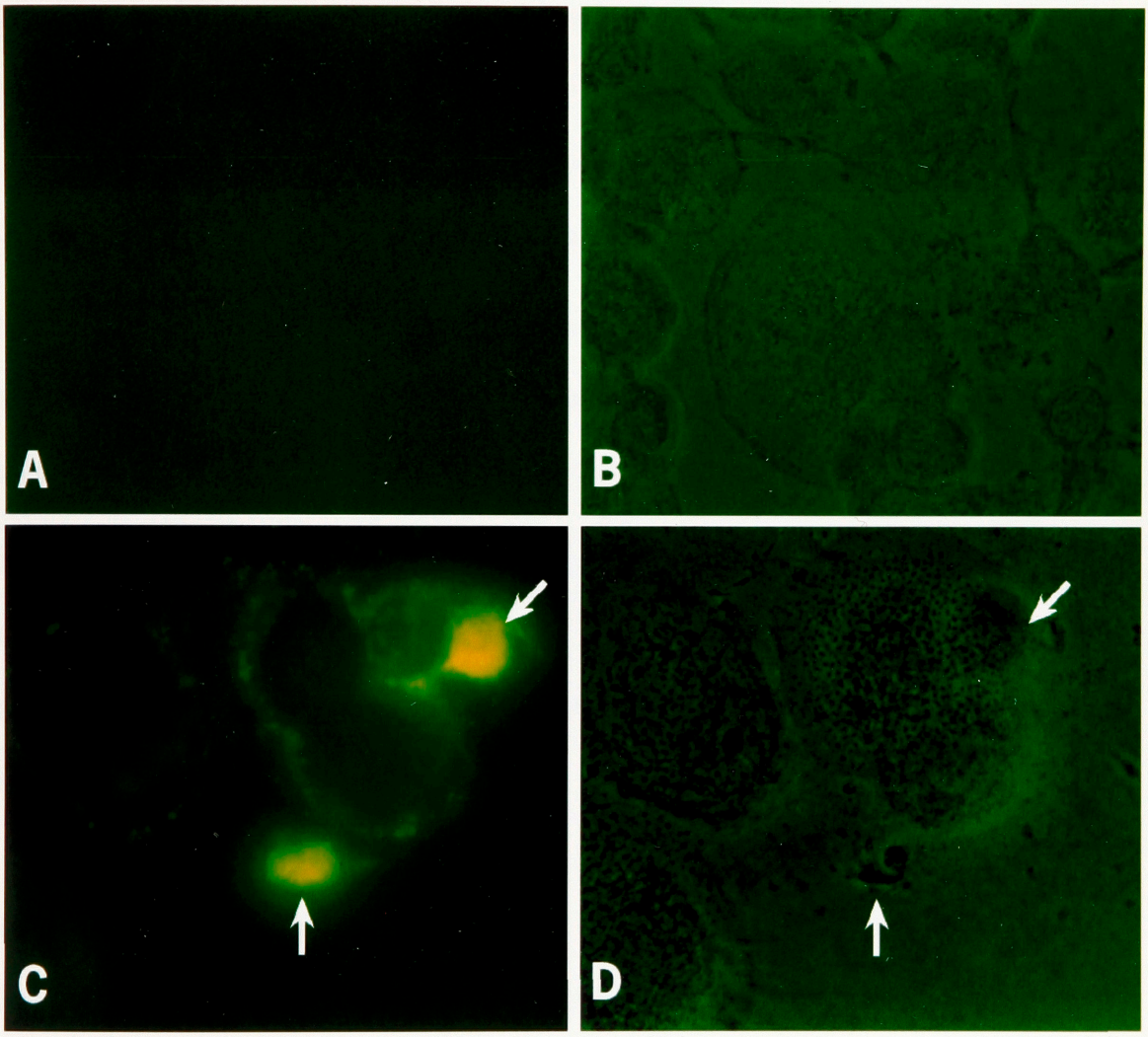
Figure 4-2. Effect of anti-APO-1 + CHX on cultured monocytes. [A] Anti-APO-1 + 1 μg/ml CHX treatment of monocytes for 18 h without pretreatment [closed circles], or pretreated with IFN-γ [closed diamonds]. p values for 0.001, 0.01, 0.1, and 0.5 μg/ml anti-APO-1 + CHX vs anti-APO-1 + CHX + IFN-γ are 0.03, 0.02, 0.08, and 0.009 respectively. In some cultures, cells were treated for 6 h with anti-APO-1 + CHX and IFN-γ pretreatment [open diamonds] or without IFN-γ pretreatment [open circles]. Treatment with anti-APO-1 without CHX for 18 h [closed triangles]. [B] Anti-APO-1 + CHX treatment of monocytes infected with MAI [circles] or uninfected with MAI [squares]. Results [A,B] are means ± SD of triplicate cultures, expressed as percent specific release of ^{51}Cr from prelabelled 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.


A
B
C
% Specific ^{51}Cr Release
100
80
60
40
20
0
0.001
0.01
0.1
1
Anti-APO-1 (μg/ml)
Fas-R (fluorescence units)
3.6
3.2
2.8
2.4
2.0
1.6
0
2
4
6
8
Days in culture

3. Induction of DNA fragmentation in cultured monocytes by anti-APO-1 with CHX. DNA fragmentation is a key feature of apoptotic cell death (165,138). In the experiments reported here, phase contrast microscopy of treated cells revealed a decrease in cell density compared to control cultures [Figure 4-3B] and alterations in morphology induced by anti-APO-1 + CHX [Figure 4-3D]. Following treatment, many monocytes had detached and 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 anti-APO-1 + CHX was accompanied by fragmentation of chromatin, which was not observed in control cells [Figure 4-3A]. When free ends of DNA were assayed by fluorescent labeling, pyknotic nuclei were brightly fluorescent [Figure 4-3C], indicating that the chromatin had been fragmented. Discrete nucleosome-sized DNA fragments were also detected by agarose gel electrophoresis of treated cells [Figure 4-4]. Anti-APO-1 treatment in the absence of CHX did not induce fragmentation of target cell DNA.

4. The effect of anti-APO-1 + CHX treatment on the viability of intracellular MAI. MAI-infected monocytes were treated with anti-APO-1 + CHX to determine the effect of host cell death on bacillary viability. Survival of bacteria was assayed by quantitation of the number of CFU in the cultures after the various treatments. When MAI-infected monocytes were treated

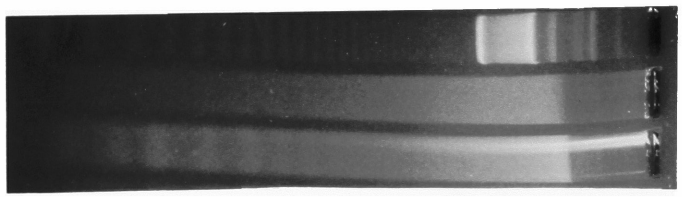
Figure 4-3. Fragmentation of chromatin following treatment of monocytes with anti-APO-1 + CHX. 10^5 monocytes on coverslips were cultured for 4 d, not treated [A and B], or treated with 0.1 μg/ml anti-APO-1 and 1 μg/ml CHX for 6 h [C and D]. Free ends of DNA were visualized by FITC-conjugated antibodies. [A and C are fluorescent micrographs, and B and D are the corresponding phase micrographs.] Magnification X250.


A
B
C
D

with anti-APO-1 + CHX for 18 h, no reduction in recovery of viable bacilli was observed [Table 4-1]. This suggests that Fas-mediated cytotoxicity is not involved in killing of intracellular mycobacteria. Similarly, when infected cells were sonicated before treatment with anti-APO-1 + CHX, there was no reduction in mycobacterial CFU. Even when infected monocytes were pretreated with IFN-γ, which led to enhanced expression of Fas receptor on these cells [Figure 4-2C] and resulted in increased killing of infected monocytes by anti-APO-1 + CHX treatment [Figure 4-2A], no significant effect on the viability of intracellular bacteria was observed [Table 4-2]. Treatment of MAI infected monocytes with IFN-γ for the 4 day culture period did not modulate bacterial growth or survival in this system. Thus, Fas-induced apoptosis did not appear to affect MAI survival in human monocytes cultured *in vitro*.

5. Morphology of MAI-infected monocytes. Electron microscopy of MAI-infected cells revealed that MAI were localized within perinuclear membrane bound vacuoles. Each mycobacterium was found singly in a tight vacuole, and the organisms appeared to be intact [Figures 4-5A and 4-5a]. On day 4 post infection, the MAI-infected monocytes were morphologically intact [Figure 4-5A]. When the infected monocytes were treated with anti-APO-1 + CHX, the treated cells exhibited morphological changes typical of cells undergoing apoptosis [Figure 4-5B]. Nuclear condensation and cytoplasmic contraction

Figure 4-4. Internucleosomal DNA fragmentation. DNA ladder visualized by agarose gel electrophoresis in monocytes treated for 6 h with anti-APO-1 + CHX.



123 bp Marker
Control
anti-APO-1/CHX, 6 hrs

Table 4-1. MAI viability after treatment of infected monocytes with anti-APO-1 + CHX

Treatment	Percent MAI Viability[@]	
	Intact monolayers	Sonicated cultures
Culture medium	100	109.6 ± 1.7
Anti-APO-1 + CHX	106.2 ± 3.5	113.3 ± 1.2

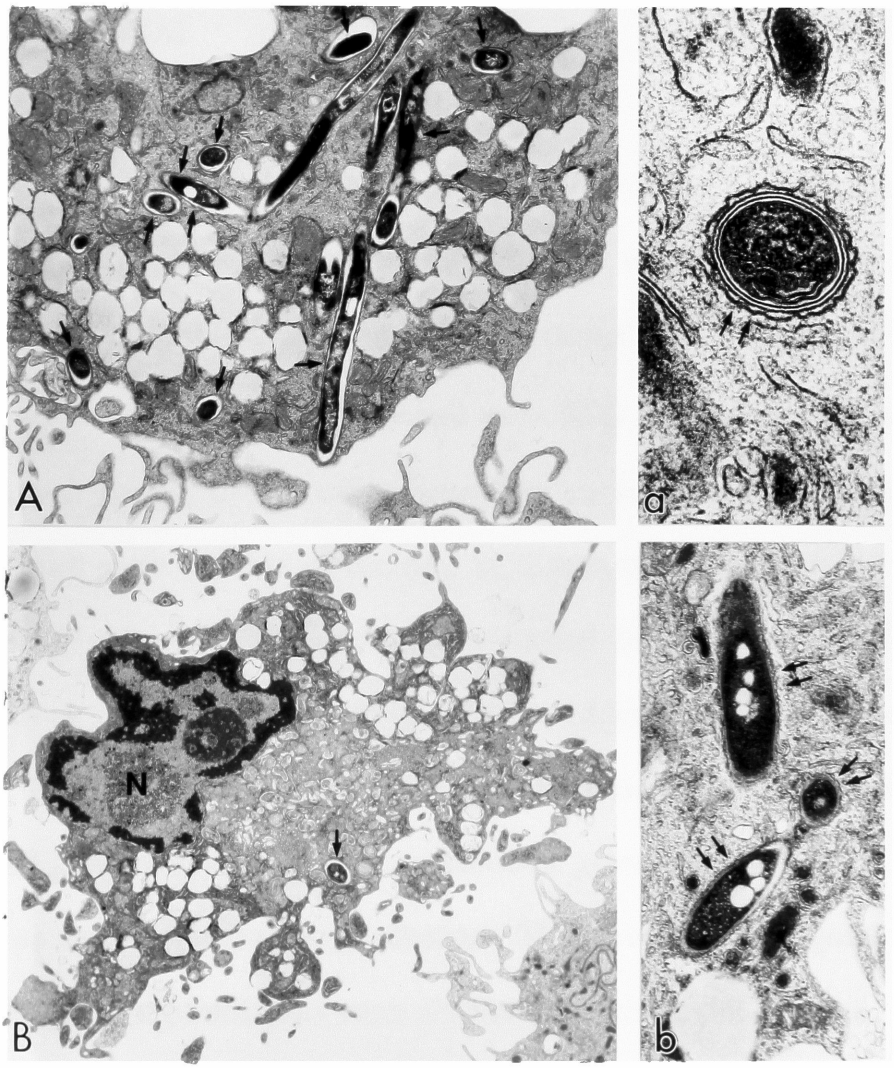
[@] Four-day-infected monocytes were treated and incubated for 18 h at 37°C, 5% CO_2 before cultures were harvested for CFU. Results are means ± SEM from 2-3 experiments, each done in triplicate.

Table 4-2. The effect of IFN-γ pretreatment on anti-APO-1 + CHX induced killing of MAI@

Treatment	Amount [μg/ml]	Colony forming units [10^{-4}]	
		Control	+ IFN-γ
Culture medium		21.1 ± 1.2	23.6 ± 1.0
Anti-APO-1	0.1	21.1 ± 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

@ Four-day-infected monocytes were treated as above and incubated for 18 h at 37°C, 5% CO_2. Parallel cultures were incubated with 100 U/ml IFN-γ from the time of monocyte infection. Results are means ± SEM of triplicate cultures.

Figure 4-5. Electron micrographs of MAI-infected monocytes. Freshly explanted monocytes were infected with one viable bacillus per cell and cultured for 4 d. [A] MAI-infected monocytes, untreated or [B] MAI-infected monocytes treated with anti-APO-1 + CHX for 6 h. [N] Cell nucleus. Magnification: [A] x 6,600; [a] x 45,000; [B] x 5,000; [b] x 13,500.


A
a
N
B
b

were apparent. Cell surface blebbing as well as budding of apoptotic bodies [cellular components packaged within membranes] were also evident. However, the intracellular MAI of the treated cells appeared to be intact [Figure 4-5b]. These findings confirmed our CFU data that Fas-induced apoptosis of infected monocytes did not affect the viability of the intracellular MAI.

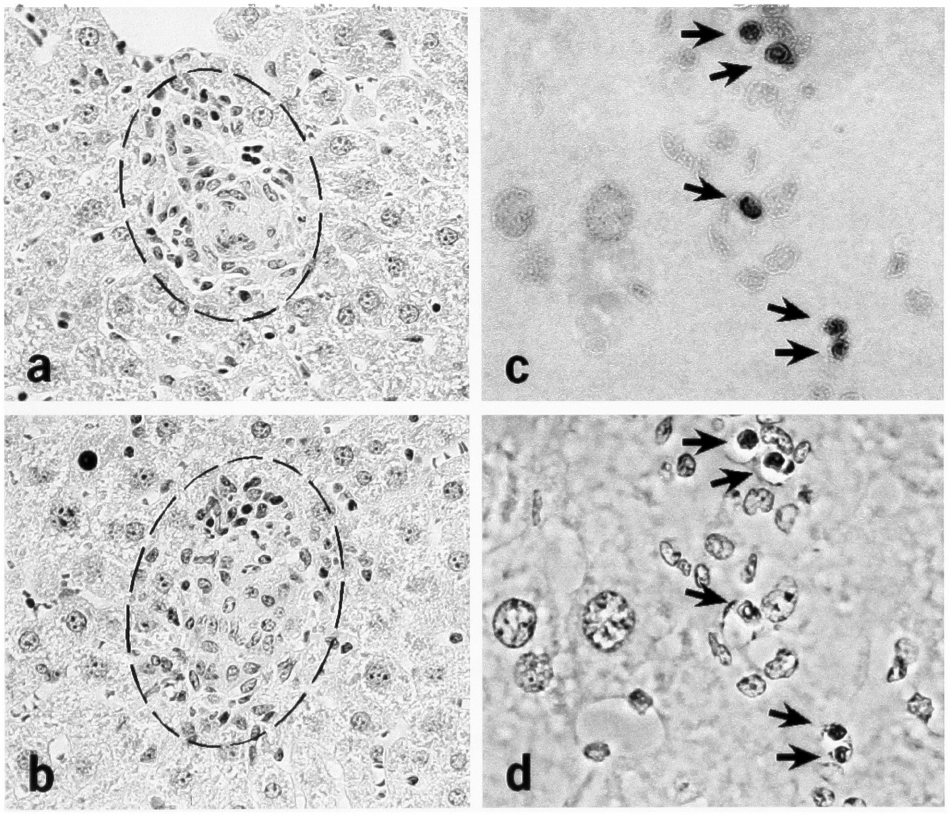
6. Mycobacterial infection of Fas-receptor defective mice. To investigate whether Fas-induced apoptosis contribute to the control of mycobacterial infection *in vivo*, Fas-receptor defective mice [CBA/lpr^{cg}] and control mice [CBA$^{+/+}$] were infected with BCG. CFU data obtained from infected lungs, livers and spleens indicated that Fas-receptor defective mice were capable of limiting BCG infection as effectively as the control [Table 4-3]. Granuloma formation in response to BCG infection was rarely observed in the lungs of both types of mice. However, both Fas-receptor defective [Figure 4-6a] and control mice [Figure 4-6b] infected with BCG demonstrated granuloma formation in the livers as early as 1 week post infection. The morphology [Figures 4-6a and 4-6b] of the granulomas were similar for both strains of mice. In addition, the number of granuloma in response to BCG infection was also examined. Both CBA/lpr^{cg} and control CBA$^{+/+}$ mice demonstrated no significant difference in the number of granuloma following BCG

Table 4-3. CFU per organ in Fas-receptor defective [CBA/lpr^{cg}] and control mice [CBA$^{+/+}$] infected with BCG.

Infected tissues		Weeks post infection [CFU in $\log_{10}$]*		
		1	3	5
Lung	CBA/lpr^{cg}	3.44 ± 0.30	4.64 ± 0.06	4.38 ± 0.07
	CBA$^{+/+}$	3.62 ± 0.97	4.66 ± 0.05	4.56 ± 0.21
Liver	CBA/lpr^{cg}	5.84 ± 0.07	5.59 ± 0.19	5.39 ± 0.59
	CBA$^{+/+}$	5.91 ± 0.07	5.59 ± 0.57	5.39 ± 0.80
Spleen	CBA/lpr^{cg}	4.96 ± 0.21	4.80 ± 0.73	4.10 ± 0.70
	CBA$^{+/+}$	5.13 ± 0.13	5.44 ± 0.82	4.57 ± 0.04

* Results are means ± SD from 2 separate experiments with 2-3 mice per group.

Figure 4-6. Histology of mycobacteria-infected tissues. Livers of Fas-receptor deficient [CBA/lpr^{cg}] [a] and control [CBA$^{+/+}$] mice [b] at 3 weeks post BCG infection. The granulomas are seen within the enclosed ellipses. Staining of apoptotic cells in the granulomas in the livers of Fas-receptor deficient [CBA/lpr^{cg}] mice infected with BCG [week 3] shown in bright field [c] and phase contrast [d]. The apoptotic cells [arrows] are stained with ApopTag Peroxidase as described in Materials and Methods, and seen here as condensed phagocytosed nuclei. Magnification: [a-b] x 100; [c-d] x 250.


a
c
b
d

infection [Figure 4-7]. Taken together, these findings suggest that Fas-mediated cytotoxicity is not involved in the early protective immune response to BCG challenge *in vivo*.

Moreover, in spite of significantly reduced Fas-receptor activity, apoptosis of leukocytes in the granulomas in livers of Fas-receptor defective mice was observed and the frequency of apoptotic cells was similar to that observed in the control mice [Figures 4-6c and 4-6d]. This finding suggests that even in the absence of an intact Fas-induced killing pathway, [an] alternative apoptotic pathway[s] contribute[s] to cell turnover in the granuloma.

7. Systemic cytokine activation in Fas-receptor defective mice. To investigate the immune response to mycobacterial infection in the two strains of mice, we investigated systemic leukocyte cytokine activation. RT-PCR analysis of cytokine mRNA levels in the spleen revealed that Fas-receptor defective mice had enhanced cytokine mRNA levels at baseline. IL-6 mRNA was increased by 15.8 fold in the spleens of Fas-receptor defective mice compared to the control [Table 4-4]. In addition, Fas-receptor defective mice had 3.0-3.6 times higher levels of IFN-γ mRNA, IL-12p35 mRNA and IL-10 mRNA in their spleens relative to the uninfected control littermates [Table 4-4]. Baseline IL-1β and IL-12p40 were similar in both strains of mice. Thus, it appeared that the

Figure 4-7. The numbers of granulomata in livers of CBA/lpr^{cg} mice [circles] and CBA$^{+/+}$ mice [squares] infected with BCG. Ten random fields of infected livers were examined for each infected animal, and the numbers of granulomata counted. Results are means $\pm$ SEM for 2-3 infected animals per each test group.

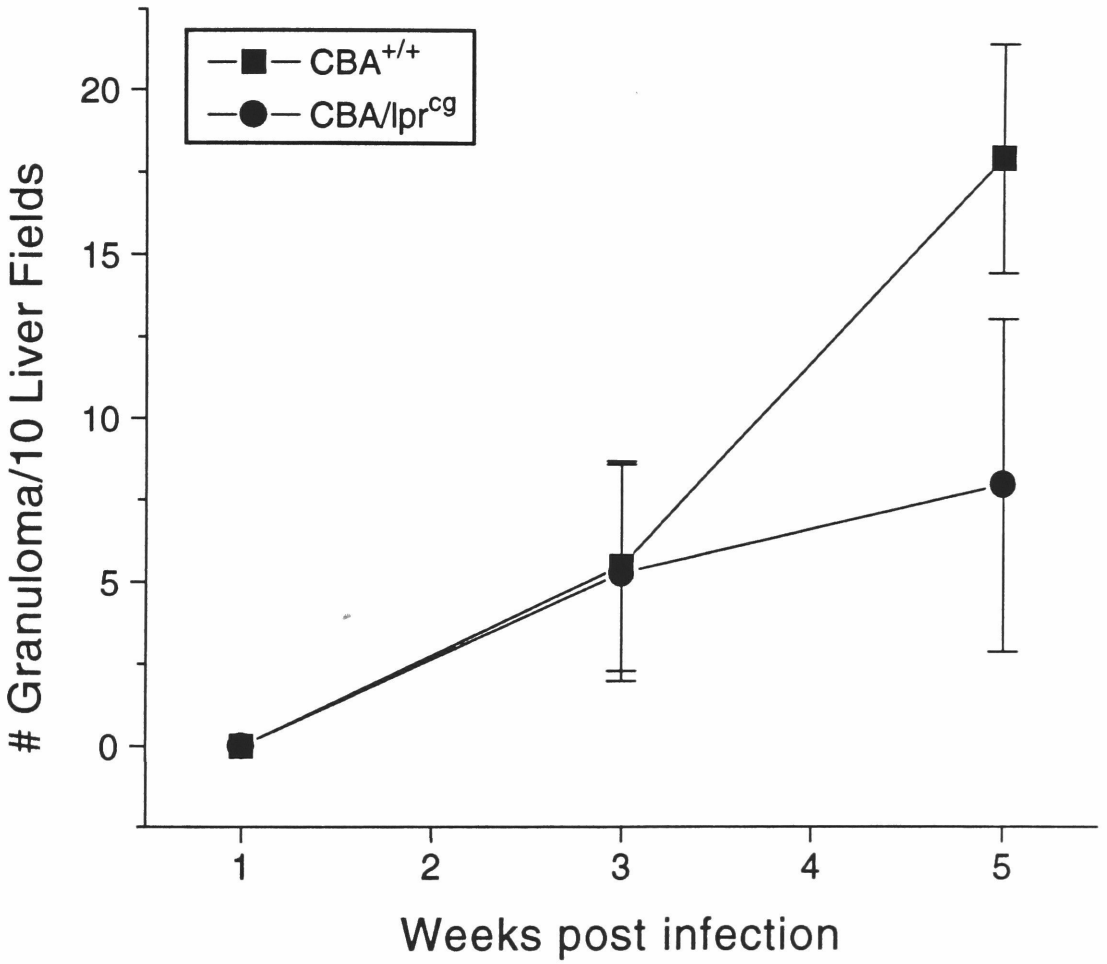

CBA+/+
CBA/lprcg
Granuloma/10 Liver Fields
0
5
10
15
20
1
2
3
4
5
Weeks post infection

Table 4-4. Baseline Cytokine Activation of Fas Receptor Defective Mice [CBA/lpr^{cg}]

Cytokine	mRNA in spleen@ [fold increase]
IL-1β	1.54 ± 0.37
IL-6	15.8 ± 12.3
IL-10	3.56 ± 0.45
IL-12p35	3.30 ± 0.71
IL-12p40	1.57 ± 0.89
IFN-γ	2.96 ± 0.31

@ Cytokine mRNAs were first normalized to amount of β-actin mRNA in each lane, and then expressed as fold increase over the amount of cytokine message expressed by the uninfected CBA$^{+/+}$ control mice at time 0. Results are expressed as means ± SEM for 4 mice per group.

loss of functional Fas receptor in CBA/lpr^{cg} mice was associated with a compensatory immunologic activation of leukocytes and the expression of increased levels of leukocyte cytokine mRNAs, even in the absence of experimental infection.

C. Discussion

MAI, 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 intraphagosome killing of MAI, we examined whether Fas-mediated apoptosis of the infected host cell affected the viability of the intracellular organisms. In the *in vitro* system described here, the isolate of MAI used was an obligate intracellular pathogen. MAI 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 MAI. However, the mean viable bacillary count on day 14 increased from that of day 7, indicating that the MAI infection persisted over time, rendering human monocytes somewhat more permissive to the growth of the intracellular organism [Figure 4-1]. Since MAI survive in monocytes and gradually replicate with a doubling time of 2 weeks in this system, any significant reduction in colony forming units [CFU] would indicate a bacteriocidal effect.

Our laboratory previously reported that ATP^{4-}-induced apoptosis of BCG-infected macrophages led to killing of the intracellular bacilli (57). This implied that apoptosis may perhaps be responsible for killing intracellular mycobacteria. In this study, Fas-mediated apoptosis of MAI-infected cells did not lead to killing of intracellular MAI, even after IFN-γ pretreatment which upregulated the expression of Fas receptors on human monocytes and enhanced killing of the cells. Similarly, the *in vivo* study revealed that Fas-receptor defective mice were as capable of handling BCG infection as control littermates. Taken together, these findings suggest that Fas-mediated apoptosis of infected monocytes does not affect the survival or death of intracellular mycobacteria. It is conceivable that ATP^{4-}treatment of infected cells initiates some bactericidal mechanism[s] against intracellular mycobacteria which are not directly related to the death of the host cell, and that apoptosis is simply an epiphenomenon. At present, how ATP^{4-} induces killing of intracellular bacilli remains unknown.

Previous studies suggested that in mycobacterial infection cell turnover within the granuloma may contribute to the control of mycobacterial infection (144). The results described here have now established that Fas-mediated apoptosis and perforin-induced necrosis had no effect on the survival of intracellular mycobacteria *in vitro*. In addition, the *in vivo* studies of $P^{-/-}$ mice and CBA/lpr^{cg} mice experimentally infected with mycobacteria confirm the *in vitro* observations and demonstrate that both

perforin- and Fas-mediated cytotoxicity are not necessary for the early control of mycobacterial infection *in vivo*. Taken together, these results suggest that cell turnover within granuloma of mycobacteria-infected tissues may not directly affect the viability of mycobacteria. The question of how human monocytes control the growth of and/or kill intracellular mycobacteria will be explored in subsequent chapters.

V. Reactive oxygen intermediates and survival of mycobacteria within mononuclear phagocytes

A. Introduction

Mycobacteria survive and/or replicate within phagosomes of human alveolar macrophages and other mononuclear phagocytes. It is these cells that are ultimately responsible for host resistance to mycobacterial infection (166), although how human monocytes/macrophages kill intracellular mycobacteria is still not known. The antibacterial activity of mononuclear phagocytes has long been associated with the ability of the macrophages to generate an oxidative burst. The production of reactive oxygen intermediates [ROI] by phagocytes has been correlated with growth restriction and/or killing of a variety of intracellular pathogens including *Candida albicans*, *Listeria monocytogenes*, *Toxoplasma gondii* and *Leishmania* (104,105,106,107,108, 109,103).

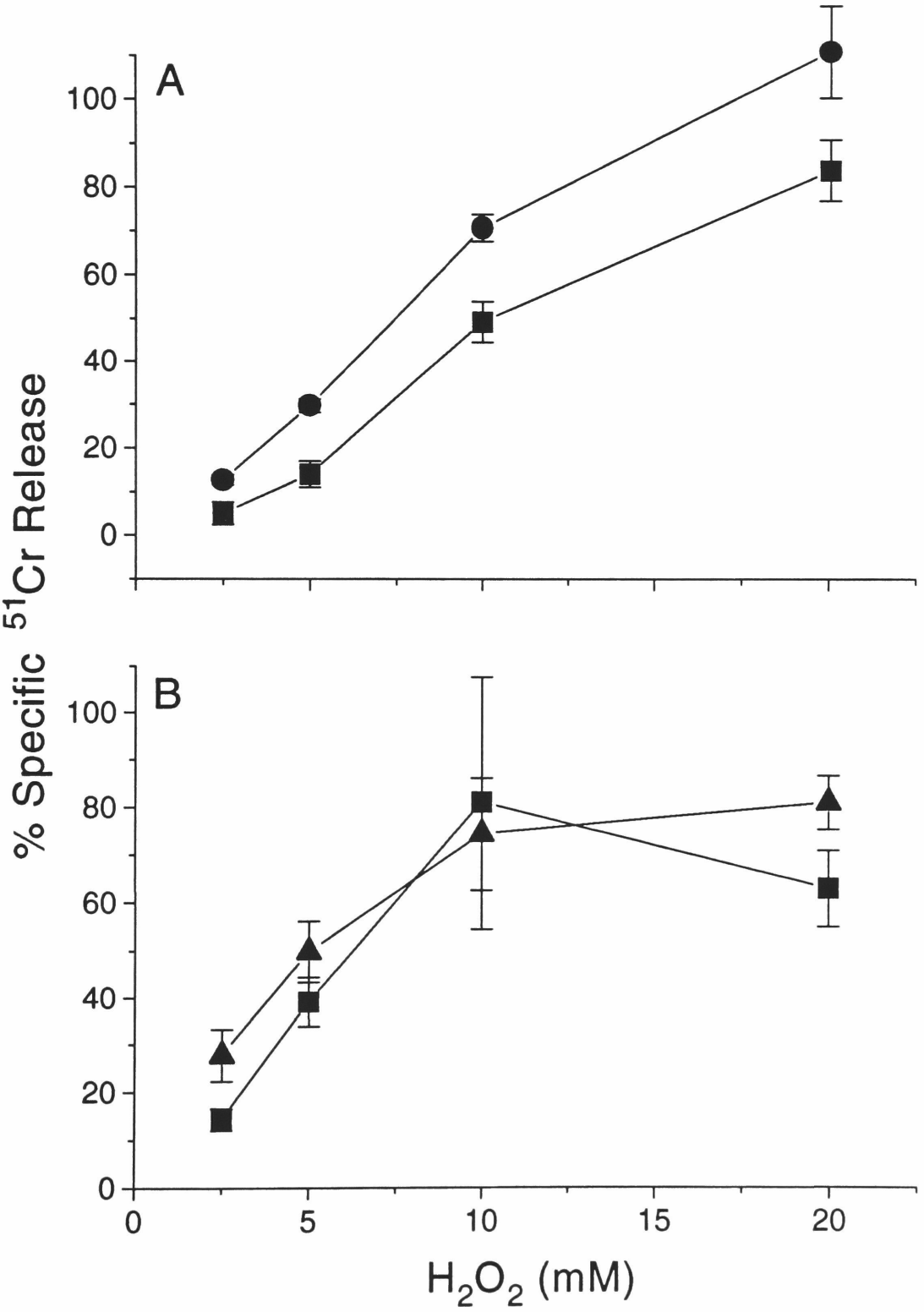
Previous studies have suggested that oxygen and reactive oxygen intermediates contribute to the control of mycobacteria. For instance, ROI generated by peroxidase or catalase-H_2O_2-halide have been shown to directly kill *M. leprae*, *M. tuberculosis*, and MAI (111,112,113). Human monocytes activated by IFN-γ (114) or by mycobacterial cell wall products (115) have been shown to be more oxidatively active and better capable of killing *M. leprae*. In addition, release of ROI have been documented during phagocytosis of

mycobacteria by monocytes (99). To evaluate whether ROI play a role in the control of mycobacterial survival within infected cells, newly isolated human monocytes were infected with a patient isolate of MAI. On day 4 post infection, the infected monocytes were treated with various concentrations of H_2O_2 [a reactive oxygen molecule]. The viability of both the host cells and the intracellular MAI were assessed by ^{51}Cr release assay and CFU assay respectively. In addition, we examined another MAI strain [ATCC 25291] known to be virulent in mice, and compared the growth rates and the viability of the two MAI strains following H_2O_2 treatment of infected monocytes.

B. Results

1. The effect of H_2O_2 treatment on 4 d cultured monocytes. To determine whether oxidative stress induced death of human monocytes in culture, 4 d 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 MAI-infected cells were performed. Host cell death occurred in a dose-dependent manner, and the dose-response curve for H_2O_2-induced cell death was reproducible with monocytes from different donors [Figure 5-1A]. The results suggest that intracellular MAI rendered cultured monocytes more sensitive to death induced by H_2O_2 [p values for 5 mM and 10 mM H_2O_2 are 0.001 and 0.002

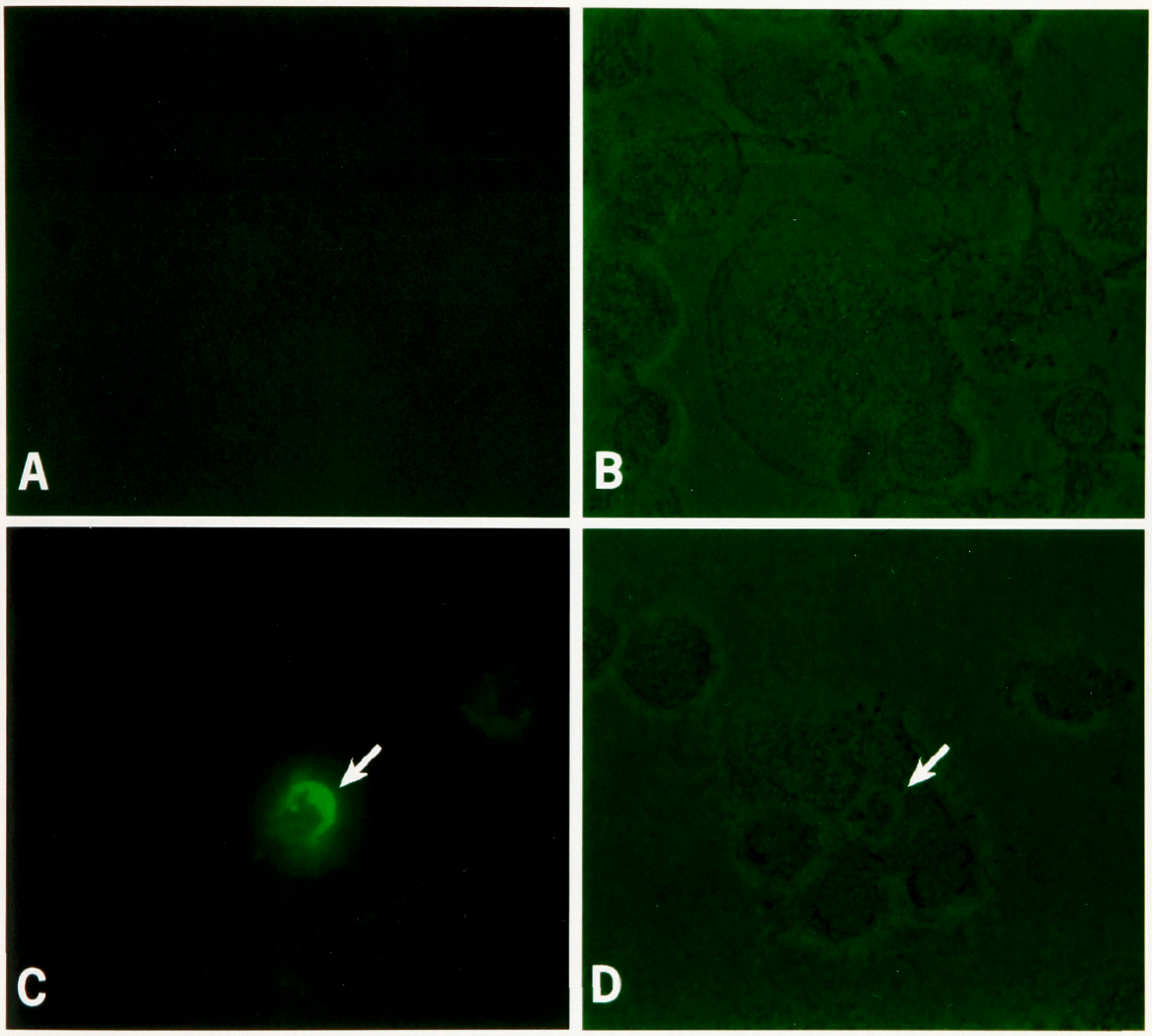
Figure 5-1. ^{51}Cr release from 4 d monocytes treated with H_2O_2 for 6 h. [A] 6 x 10^4 uninfected monocytes [squares] and MAI-infected monocytes [circles]. [B] 6 x 10^4 uninfected monocytes were pretreated with 100 U/ml IFN-γ in R-20 [triangles] or with R-20 alone [squares] before H_2O_2 treatment. Results are means $\pm$ SD of triplicate cultures.


A
B
% Specific ^{51}Cr Release
H_2O_2 (mM)
0
20
40
60
80
100
0
5
10
15
20

respectively] [Figure 5-1A]. Because IFN-γ has been shown to be important in expression of protective immunity to mycobacterial infection in IFN-γ-gene-disrupted mice (167), the effect of IFN-γ pretreatment 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 [Figure 5-1B].

2. Induction of DNA fragmentation in cultured monocytes by H_2O_2. One of the hallmark of apoptosis is DNA fragmentation (165,138). Similar to the observations of Fas-induced apoptosis [chapter 4], phase contrast microscopy of H_2O_2-treated cells revealed a decrease in cell density and alterations in morphology [Figure 5-2D] compared to control cultures [Figure 5-2B]. When treated with low dose H_2O_2, many of the monocytes which remained adherent were rounded and small, and pyknotic nuclei were apparent as dense shrunken bodies of condensed chromatin. When free ends of DNA were assayed by fluorescent labeling, pyknotic nuclei induced by H_2O_2 treatment were brightly fluorescent [Figure 5-2C], suggesting that the chromatin had been fragmented. No DNA fragmentation was observed in control cells [Figure 5-2A]. Furthermore, discrete nucleosome-sized DNA

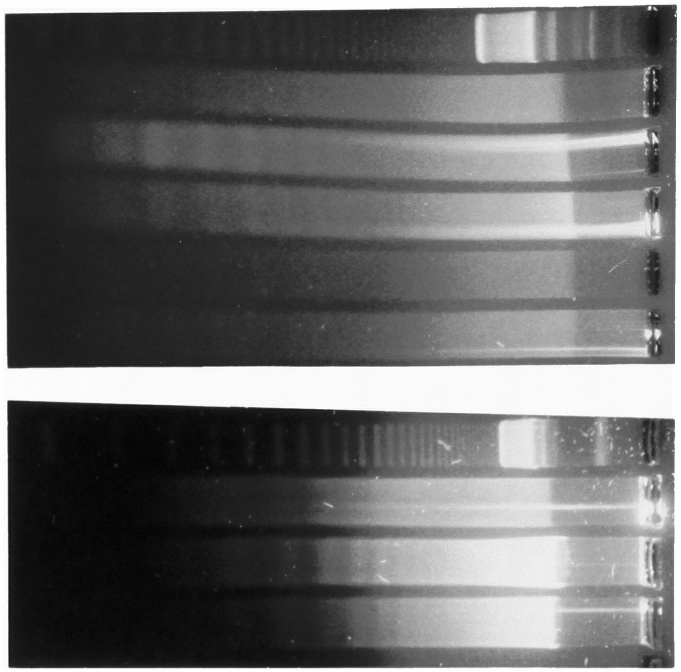
Figure 5-2. Fragmentation of chromatin following treatment of monocytes with H_2O_2. 10^5 monocytes on coverslips were cultured for 4 d, not treated [A and B], or treated with 2 mM H_2O_2 for 6 h [C and D]. Free ends of DNA were visualized by FITC-conjugated antibodies. [A and C are fluorescent micrographs, and B and D are the corresponding phase micrographs.] Magnification X250.


A
B
C
D

fragments were detected by agarose gel electrophoresis of H_2O_2-treated cells. H_2O_2 treatment at 2, 5, and 10 mM resulted in internucleosomal DNA fragmentation [Figure 5-3]. However, when monocytes were treated with 20 mM H_2O_2 which has previously been reported to induce necrosis, no DNA fragmentation was observed [Figure 5-3].

3. The effect of H_2O_2 treatment on the viability of intracellular MAI. The effect of oxidatively induced host cell death on the viability of intracellular MAI was assayed by comparing CFU counts in parallel sonicated and intact cultures treated with H_2O_2. Sonication destroys infected monocytes and releases the bacilli without affecting bacillary viability. Therefore, any differences in CFU observed between the two parallel cultures following H_2O_2 treatment must be due to specific process induced in dying cells during H_2O_2-mediated host cell death. When MAI-infected monocytes were treated with varying doses of H_2O_2 for 6 h, killing of intracellular mycobacteria [a reduction in CFU] was observed [Figure 5-4]. Bacillary killing was observed at doses between 2 and 20 mM H_2O_2. The optimal dose of 10 mM H_2O_2 resulted in the killing of approximately 60% of intracellular bacteria. If infected monocytes were lysed by sonication before the beginning of the assay, the presence of H_2O_2 up to 10 mM had no significant effect on the viability of the bacilli [Figure 5-4]. However, treatment of extracellular bacilli [released by

Figure 5-3. Internucleosomal DNA fragmentation. DNA ladder visualized by agarose gel electrophoresis in 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.



123 bp Marker
Control
anti-APO-1/CHX, 6 hrs
2mM H_2O_2, 6 hrs
20mM H_2O_2, 2 hrs
20mM H_2O_2, 6 hrs

123 bp Marker
Control
5mM H_2O_2, 6 hrs
10mM H_2O_2, 6 hrs

sonication] with 20 mM H_2O_2 resulted in the killing of about 20% of the organisms [Figure 5-4]. These results indicated that H_2O_2-induced host cell death was coupled with the induction of a microbicidal activity in dying, but not dead, infected monocytes.

4. Role of nitric oxide production in killing of intracellular MAI. Nitric oxide [NO] production has been demonstrated to be an important part of the antimicrobial activities of activated murine macrophages against many pathogens including mycobacteria (168,169). We therefore investigated whether NO played a role in the control of MAI viability in human monocyte cultures *in vitro*. 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] and aminoguanidine. Four day MAI-infected monocytes were treated with either L-NMMA or aminoguanidine for 16 h before treatment with 10 mM H_2O_2 and maintained in the culture throughout the experiment [Table 5-1]. Our results revealed that treatment of infected monocytes with either L-NMMA or aminoguanidine alone at 1 mM had no effects on the viability of MAI. In addition, pretreatment of the infected cells with either L-NMMA or aminoguanidine did not affect H_2O_2-induced killing of intracellular MAI. In fact, MAI killing seemed to be increased in the presence of both inhibitors.

Figure 5-4. Effect of H_2O_2-induced monocyte apoptosis on killing of MAI. 3 x 10^5 monocytes were infected immediately after isolation and cultured for 4 d. H_2O_2 diluted in culture medium was added to intact monolayers [intracellular MAI] [open bars], or to monolayers that had first been sonicated [extracellular MAI] [hatched bars]. Cultures were harvested for CFU assay after 6 h. Results are means ± SEM of 3-7 experiments in triplicate, expressed as percent killing of MAI.

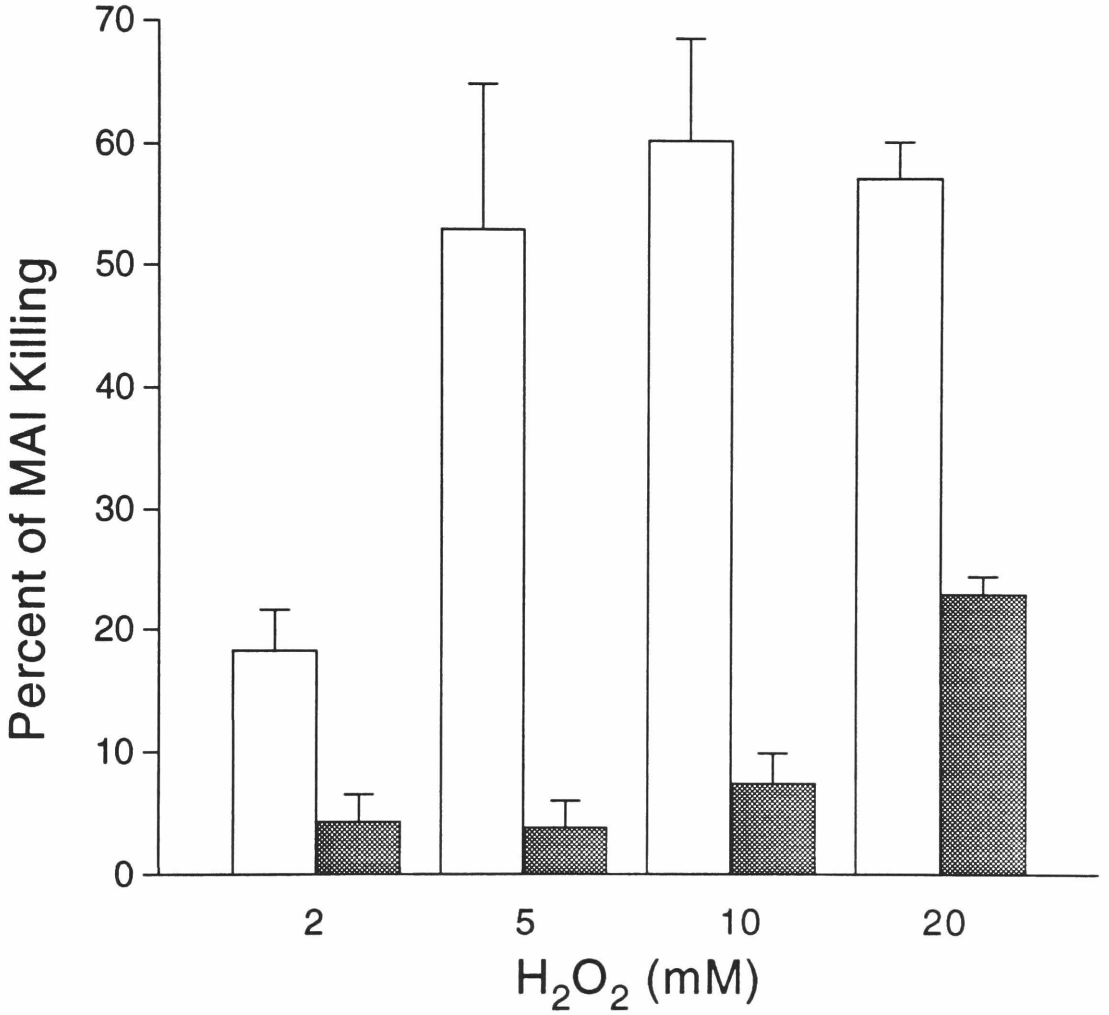

70
60
50
40
30
20
10
0
Percent of MAI Killing
2
5
10
20
H_2O_2 (mM)

Table 5-1. Bacillus killing after treatment of infected monocytes with H_2O_2, L-NMMA, and aminoguanidine

Treatment	Concentration	Colony forming units@ [10^{-4}]	
		Intact monolayers	Sonicated cultures
None [control]		17.7 ± 0.2	21.1 ± 2.1
L-NMMA	1 mM	19.4 ± 1.0	19.8 ± 1.2
Aminoguanidine	1 mM	15.7 ± 0.9	15.6 ± 0.7
H_2O_2	10 mM	10.4 ± 0.3	18.7 ± 1.6
H_2O_2 + L-NMMA	10 mM + 500 μM	6.6 ± 0.5	17.6 ± 1.1
H_2O_2 + L-NMMA	10 mM + 1 mM	8.5 ± 0.5	17.5 ± 0.9
H_2O_2 + Aminoguanidine	10 mM + 1 mM	8.4 ± 0.7	16.1 ± 0.9

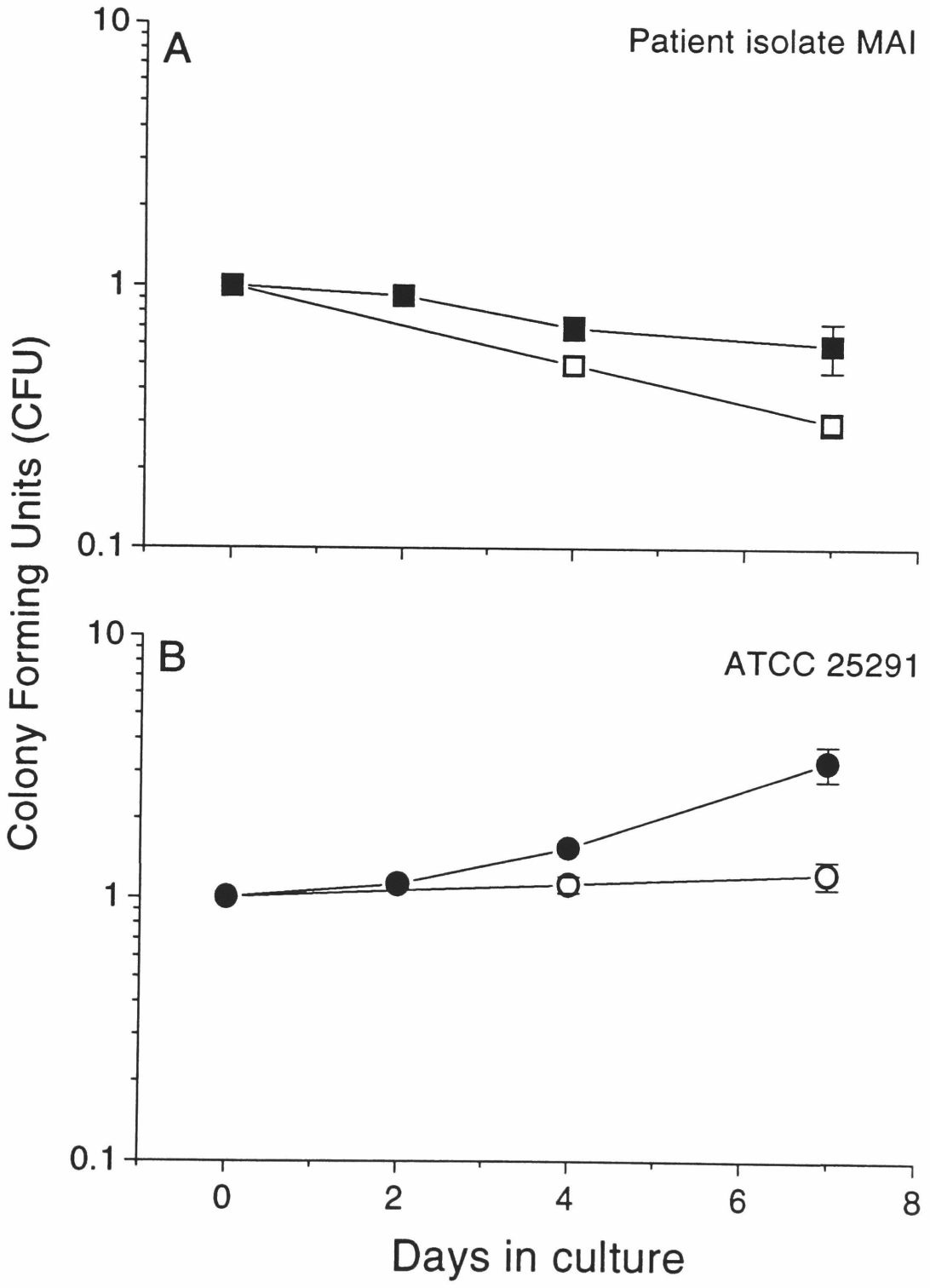
@ Four-day-infected monocytes were treated with either L-NMMA or aminoguanidine 16 h before the addition of H_2O_2, and then incubated for 6 h at 37°C, 5% CO_2. Results are means ± SEM of 1-2 experiments, each done in triplicate cultures.

Whether this is physiologically relevant requires further investigation. These findings suggest that H_2O_2-mediated killing of intracellular MAI in cultured human monocytes did not depend on NO production.

5. Growth of different MAI strains in freshly isolated human monocytes. Freshly explanted monocytes were infected at a multiplicity of infection of one viable MAI per cell with either the patient isolate or the mouse virulent strain [ATCC 25291] [mMAI] immediately after isolation and cultured. At the time points indicated, CFU assays were performed to assess the number of viable bacilli. Unlike the patient isolate of MAI which failed to grow in human monocytes during the first 7 d in culture [Figure 5-5A], the mMAI grew immediately in freshly explanted human monocytes with the doubling time of 96 h [Figure 5-5B]. In the absence of monocytes, mMAI remained viable without replicating in the culture medium while the patient isolate MAI lost viability with time in culture [Figures 5-5A and 5-5B]. Thus, within cultured human monocytes mMAI replicated more readily than the patient isolate MAI.

6. Susceptibility of different MAI strains to killing by H_2O_2. To examine whether mMAI was as sensitive to killing by H_2O_2 as the patient isolate MAI, freshly explanted monocytes were infected with either the patient isolate MAI or mMAI immediately after isolation and cultured for 4 days. On day 4 post

Figure 5-5. MAI growth in cultured monocytes. [A] patient isolate MAI and [B] mouse virulent MAI [ATCC 25291]. 3×10^5 viable MAI were added to wells containing 0.5 ml culture medium alone [open symbols] or 0.5 ml culture medium containing 3×10^5 freshly isolated adherent monocytes [closed symbols]. At the time point indicated, colony forming units [CFU] were assayed. For comparison, CFU data for each MAI strain were normalized as the fold change in number of CFU relative to CFU in cultures on the day of infection. Results are expressed as means $\pm$ SD of 2 experiments, each done in triplicate.

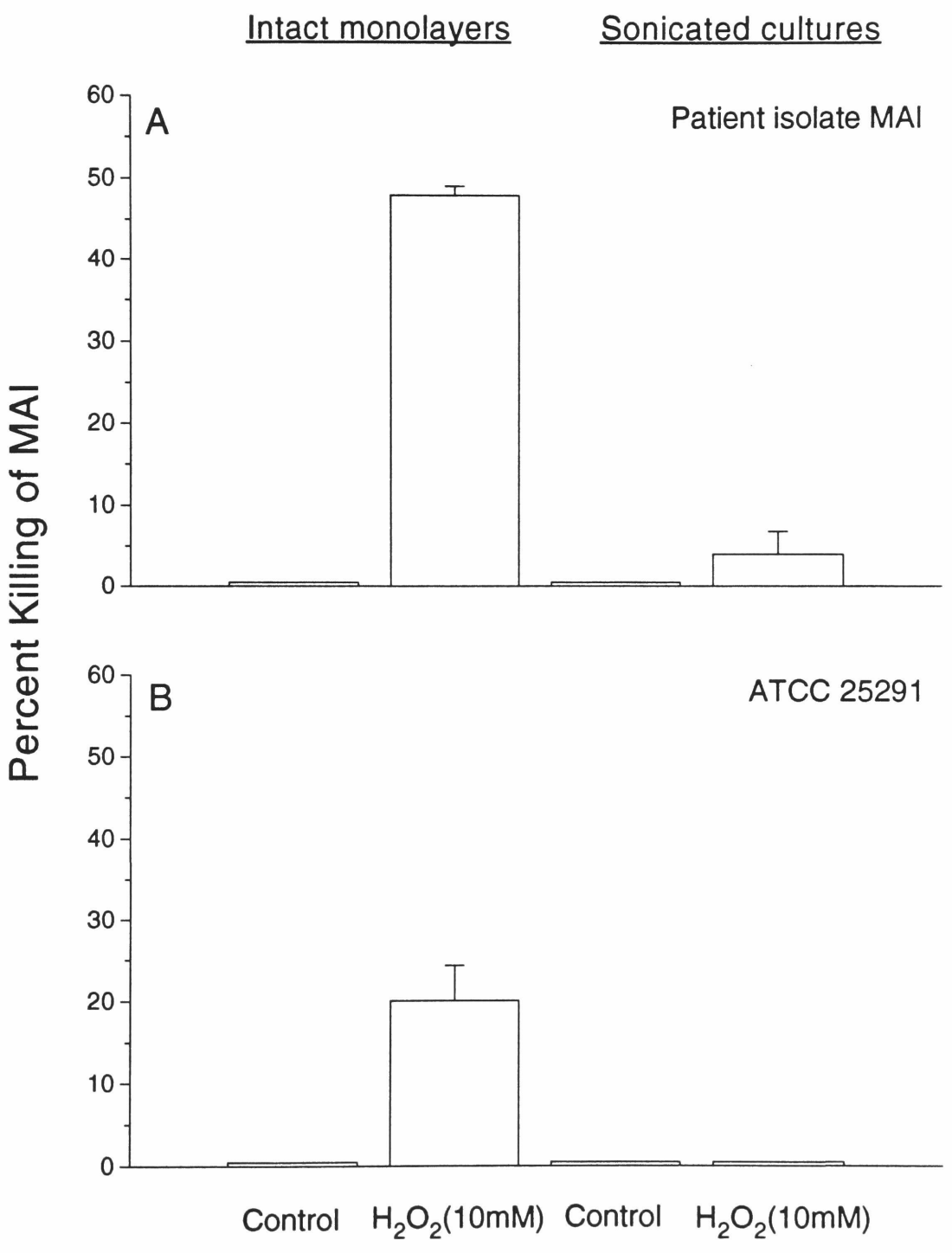

A
Patient isolate MAI
10
1
0.1
B
ATCC 25291
10
1
0.1
Colony Forming Units (CFU)
0
2
4
6
8
Days in culture

infection, the infected monocytes were treated with 10 mM H_2O_2 for 6 h. The mean percent killing of patient isolate MAI and mMAI were 48% and 20% respectively [Figures 5-6A and 5-6B].

We also investigated the susceptibility of the two MAI strains to the toxic effects of H_2O_2 when infected monocytes were disrupted by sonication. With increasing concentration of H_2O_2 added, the two strains of MAI became progressively less viable [Figure 5-7A]. Under these conditions, no significant difference in the susceptibility of the two MAI strains was observed.

To determine whether the difference observed in intracellular killing [Figures 5-6A and 5-6B] depends on the endogenous resistance of the two MAI strains to neutralize the toxic effects of H_2O_2, frozen aliquots of the two MAI strains were thawed, resuspended in R-20 and cultured for 1 h before treatment with varying dilutions of H_2O_2 for 6 h. Dose-dependent killing of both MAI strains were observed between 5 and 80 mM H_2O_2 [Figure 5-7B]. mMAI appeared to be more sensitive than patient isolate MAI to H_2O_2 treatment between the concentrations of 10 and 40 mM [Figure 5-7B]. These results indicated that within cultured human monocytes the patient isolate MAI was more sensitive to H_2O_2-induced killing than mMAI. The different susceptibility to H_2O_2-induced killing was independent of the organisms' ability to neutralize H_2O_2 both in cell-free culture medium and in cell lysates.

Figure 5-6. Effects of H_2O_2-induced monocyte death on MAI killing. [A] patient isolate MAI and [B] mouse virulent MAI [ATCC 25291]. 3 x 10^5 monocytes were infected with one viable MAI per cell after isolation and cultured in R-20. On day 4, H_2O_2 diluted in culture medium was added to intact monolayers [intracellular mycobacteria], or to monolayers that had first been sonicated [extracellular mycobacteria] at the final concentration of 10 mM. Cultures were harvested for CFU assay after 6 h. Results are means $\pm$ SD of 2-3 experiments in triplicate, expressed as percent killing of MAI.

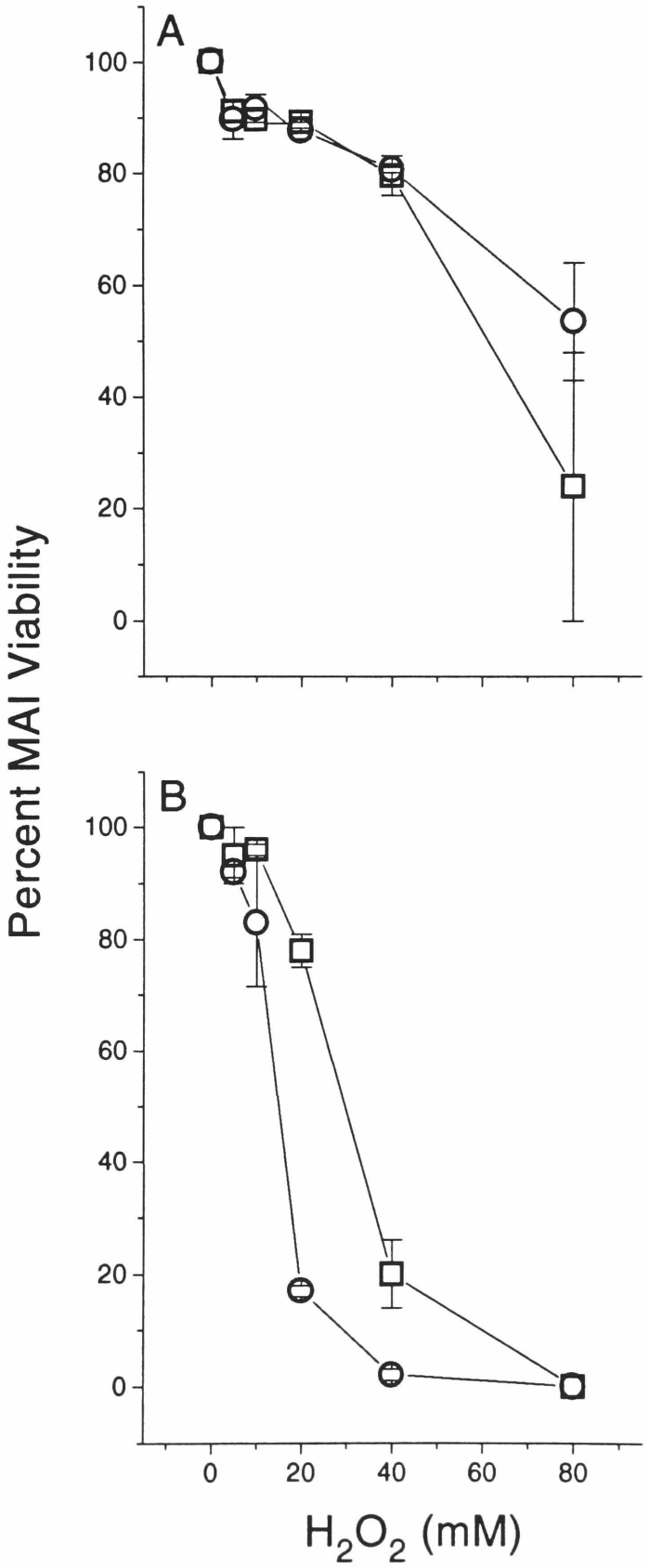

Intact monolayers
Sonicated cultures
A
Patient isolate MAI
B
ATCC 25291
Percent Killing of MAI
60
50
40
30
20
10
0
Control
H2O2(10mM)
Control
H2O2(10mM)

Moreover, there is an inverse relationship between the ability of MAI to replicate intracellularly and the susceptibility to H_2O_2-induced killing.

C. Discussion

Macrophages employ oxidative bacteriocidal mechanisms to combat many intracellular pathogens such as *Candida albicans* and *Listeria monocytogenes* (105,104,170). In this study, H_2O_2-induced death of MAI-infected monocytes/macrophages was associated with killing of intracellular MAI. This may be a physiological mechanism of host defense against MAI, since release of H_2O_2 and other reactive oxygen metabolites have been documented following phagocytosis of different mycobacteria including MAI (99,100,101). A previous study reported that different MAI strains have differing resistance 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 replicate intracellularly (171). This is consistent with the study reported here in that the patient isolate of MAI which failed to replicate in cultured human monocytes was more susceptible to H_2O_2-induced killing than mMAI which began replicating immediately after infection. Based on these observations, the control of MAI may depend to some extent on the balance between the ability of infected monocytes to restrict the growth of the intracellular mycobacteria and the

Figure 5-7. Effects of H_2O_2 treatment on extracellular MAI. [A] 3 x 10^5 newly explanted human monocytes were infected with either one viable patient isolate MAI [squares] or with one viable mouse virulent MAI [ATCC 25291] [circles] per cell and cultured for 4 days. On day 4 post infection, infected monolayers were sonicated and then treated with varying dilutions of H_2O_2. [B] 3 x 10^5 bacilli were added to R-20 culture medium and incubated for 1 h before treatment with varying dilutions of H_2O_2. All cultures were harvested for CFU assay after 6 h treatment. Results are expressed as % viability and are the means $\pm$ SD of 2-4 experiments in triplicate.


A
B
100
80
60
40
20
0
0
20
40
60
80
Percent MAI Viability
H_2O_2 (mM)

ability of the bacteria to withstand oxidative stress-induced killing.

This study has demonstrated that H_2O_2 induced apoptosis of the host cells. Reactive oxygen intermediates [ROI] can damage many cellular macromolecules, or initiate a chain reaction whereby the free radical is shuttled from one molecule to the next causing extensive damage to cellular structures (172). It is conceivable that either ROI or free radicals generated during this chain of events kill the monocytes. It is worth noting that H_2O_2 has been shown to induce apoptosis in cell types other than monocytes (173). Dosages used in these reports ranged from 10-100 μM H_2O_2. In this study, the dose range of H_2O_2 efficacious in monocyte killing is much higher, and this may reflect the relative resistance of peripheral blood monocytes to oxidative damage.

Nitric oxide [NO] production has been demonstrated to be an important component of the antimicrobial capacity of activated murine macrophages (168,169). The existence of similar bacteriocidal pathways in human monocytes/macrophages has been difficult to demonstrate. A number of studies failed to report NO synthesis in human macrophages stimulated with either bacterial products or lymphokines (120,121,122,123). This may be explained by the inability of human mononuclear phagocytes to synthesize tetrahydrobiopterin, an essential cofactor of NO synthase (124,125,126). In addition, induction of the NO synthase has not been clearly observed in human peripheral blood monocytes or peritoneal macrophages (127). Only one study

individuals and from AIDS patients were comparable in their abilities to phagocytose and restrict the growth of MAI (176). However, monocytes obtained from AIDS patients have been reported to have a reduced capacity to release H_2O_2 (177). Whether or not killing of MAI *via* H_2O_2-induced apoptosis is compromised in HIV-1 infected individuals is not known. Altered macrophage function may be important but the relationship between this and the primary $CD4^+$ T cell deficit remains undefined. Further investigations on the susceptibility to oxidative stress of MAI in infected monocytes obtained from AIDS patients may elucidate the role of H_2O_2-induced killing of these organisms.

VI. Growth and resistance to H_2O_2 killing of different mycobacterial species in human monocytes *in vitro*

A. Introduction

Mycobacteria comprise a large family of organisms with a wide spectrum of virulence as defined by their ability to establish infection and cause active disease in humans. Among these agents, *Mycobacterium tuberculosis* are the most virulent in humans with an estimated one billion infected individuals worldwide (1). Approximately 10% of infected immunocompetent individuals develop active disease over their lifetime. In comparison, *M. avium-M. intracellulare* [MAI], a common contaminant of soil and water, are usually nonvirulent [do not establish infection or cause disease] in humans, but have recently emerged as a life-threatening opportunistic infection in immunocompromised AIDS patients (3,4).

In 1953, Pierce *et al.* showed that strains of mycobacteria which caused disease in humans were also capable of unrestricted growth in organs of infected mice (87). Recently, North *et al.* demonstrated in mice that virulent strains of mycobacteria had faster doubling times *in vivo* than their less virulent related species (93).

In an attempt 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 with *M. tuberculosis* [H37Ra] and a

patient isolate of *M. avium-M. intracellulare* [MAI]. Previously, we had demonstrated that low doses [5-10 mM] H_2O_2 induced death of MAI-infected monocytes and reduced the viability of the intracellular MAI (96). In this chapter, we compare the growth rates of H37Ra and the patient isolate of MAI within human monocytes maintained in culture for up to 14 days. We also compare the susceptibility of these mycobacterial species to H_2O_2-induced killing as well as their profile of cytokine induction during infection of human monocytes *in vitro*.

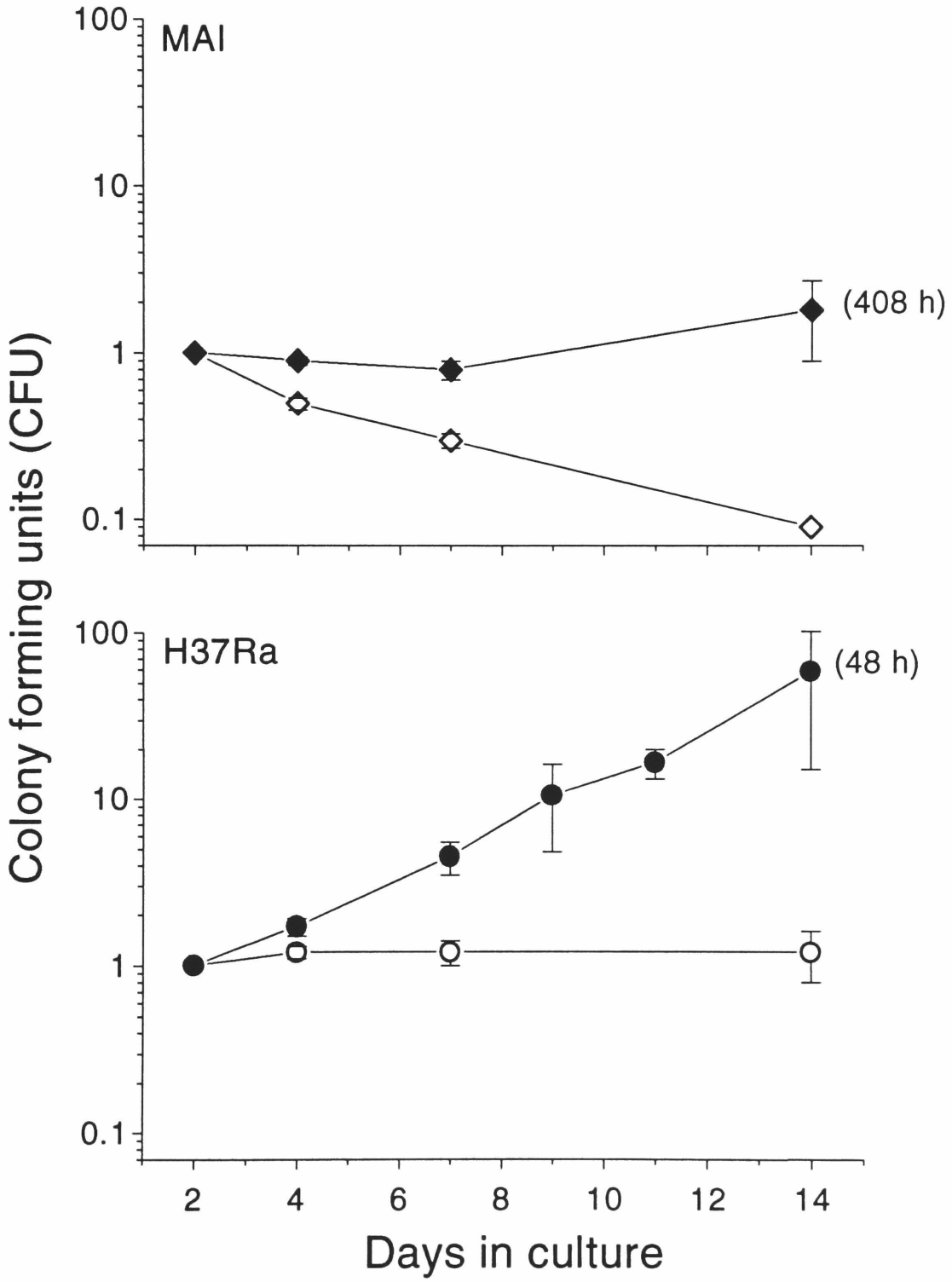
B. Results

1. Mycobacterial growth in freshly isolated human monocytes. Monolayers of adherent human monocytes were infected with either MAI or H37Ra at a multiplicity of infection [MOI] of one viable bacillus per cell. Infection at the MOI of 1 was chosen because at this multiplicity of infection monolayers of infected monocytes remained fully intact for at least the first 7 d of culture. Our previous studies have shown that when various mycobacteria were added to adherent monocytes at this MOI, the bacilli were phagocytosed by the cells, survived, and/or replicated within a unique phagocytic vacuole (57,79). As a control, parallel cultures containing R-20 medium alone without monocytes were inoculated with the same number of organisms as 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 MAI neither replicated nor remained viable in the culture medium [Figure 6-1]. In the presence of human monocytes, only a slight change in the number of viable MAI was observed during the first 7 d in culture, while H37Ra appeared to replicate in cultured monocytes without any apparent lag time [Figure 6-1]. After the first week of culture, H37Ra-infected cultures began to show microscopic signs of disruption of the monolayers and detachment of some infected cells. This change was not observed in MAI-infected cultures during the 14 d assay. The calculated doubling times of H37Ra and MAI during 14 d of culture in human monocytes were 48 h and 408 h respectively. Thus, within cultured human monocytes the intracellular growth of MAI was restricted compared to the growth of H37Ra.

2. ***The effects of exogenously added H_2O_2 on the survival of intracellular and extracellular mycobacteria.*** To explore the H_2O_2-induced killing of intracellular mycobacteria, the susceptibility of the two mycobacterial species to exogenous H_2O_2 were examined. Monocytes were infected with either MAI or H37Ra immediately after introduction into culture and then incubated for 4 d. When 4 d mycobacteria-infected monocytes were treated for 6 h with varying doses of H_2O_2 [2-20 mM], a reduction in the number of

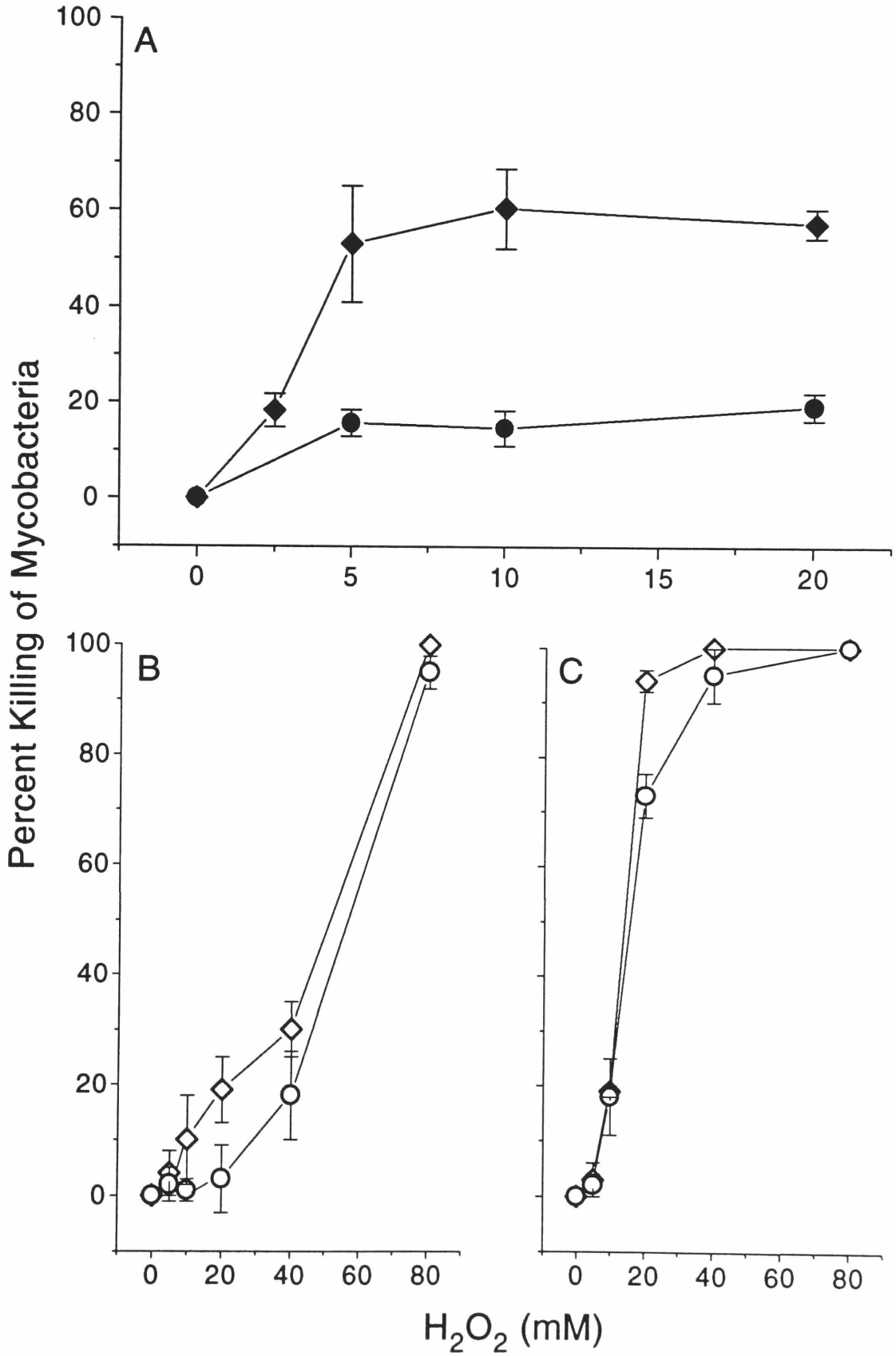
Figure 6-1. Mycobacterial growth in cultured monocytes. 3×10^5 viable mycobacteria were added to wells containing 0.5 ml culture medium alone [open symbols] or 0.5 ml culture medium containing 3×10^5 freshly isolated adherent monocytes [closed symbols]. At the time points indicated, CFU were assayed. 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 MAI and $49 \pm 1 \times 10^4$ for H37Ra. Results are expressed as means $\pm$ SEM of 2 experiments, each done in triplicate. Numbers in parentheses denote the mean generation time for each strain of mycobacteria.


MAI
100
10
1
0.1
(408 h)
H37Ra
100
10
1
0.1
(48 h)
Colony forming units (CFU)
2
4
6
8
10
12
14
Days in culture

CFU, indicating killing of intracellular bacilli was observed [Figure 6-2]. For MAI, killing was maximal when infected cells were treated with 10 mM H_2O_2. For H37Ra, the extent of intracellular killing was low when infected cells were treated with H_2O_2 concentrations between 5 and 20 mM. The percent of killing of bacilli within infected cells at 10 mM H_2O_2, as evaluated by the CFU assay, were 60% for MAI and 15% for H37Ra [Figure 6-2A].

The ability of the mycobacteria to withstand the toxic effects of H_2O_2 when the infected monocytes were disrupted by sonication, which rendered the bacilli extracellular in culture medium containing monocyte fragments, was investigated. Under these conditions, the mycobacteria became more resistant to H_2O_2-induced killing. The addition of H_2O_2 at concentrations up to 20 mM to the cell sonicates had no effect on the viability of extracellular H37Ra [Figures 6-2B]. At 10 and 20 mM H_2O_2, sonicated cultures of MAI-infected cells showed a slight decrease in viability of the bacilli [10-20%]. Our results indicated that intracellular bacilli were more susceptible than extracellular bacilli to the presence of H_2O_2 in the culture medium. This suggested that H_2O_2 induces microbicidal activity in living, but not dead, human monocytes. As the concentration of exogenously added H_2O_2 was increased above 20 mM, the two mycobacterial species became less viable [Figure 6-2B] and were completely killed at 80 mM H_2O_2.

Figure 6-2. Killing of mycobacteria by H_2O_2 treatment. MAI [diamonds]; and H37Ra [circles]. [A] 3 x 10^5 newly explanted human monocytes were infected with one viable bacillus per cell and cultured for 4 d. H_2O_2 diluted in culture medium was added to intact monolayers [intracellular mycobacteria]. Cultures were harvested for CFU assay after 6 h. [B] 3 x 10^5 newly explanted human monocytes were infected with one viable bacillus per cell and cultured for 4 days. On day 4 post infection, H_2O_2 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 $\pm$ SEM of 3-7 experiments each performed in triplicate. [C] 3 x 10^5 bacilli were added to R-20 culture medium for 1 h before treatment with varying dilutions of H_2O_2. All cultures were harvested for CFU assay after 6 h treatment. Results are expressed as percent killing of mycobacteria and are the means $\pm$ SD of 2 experiments, each done in triplicate.


A
B
C
Percent Killing of Mycobacteria
H_2O_2 (mM)
100
80
60
40
20
0
0
5
10
15
20
0
20
40
60
80

To determine the effect of H_2O_2 on the viability of mycobacteria in the culture medium in the absence of any monocyte components, frozen aliquots of MAI and H37Ra were thawed, resuspended in R-20 and cultured for 1 h before treatment with varying dilutions of H_2O_2 for 6 h. CFU assays were performed at the end of 6 h, and the percent killing at different concentrations of H_2O_2 was determined for the two species of mycobacteria [Figure 6-2C]. Dose-dependent killing of mycobacteria in R-20 medium was observed between 5 and 40 mM H_2O_2. Extracellular MAI appeared to be slightly more sensitive to H_2O_2 treatment than H37Ra. The results show that extracellular mycobacteria in monocyte lysates were less sensitive to H_2O_2 treatment than extracellular mycobacteria in cell-free culture media.

3. Morphology of intracellular mycobacteria. Electron microscopy of mycobacteria-infected monocytes revealed that at all times the bacilli were localized within perinuclear membrane bound vacuoles [Figure 6-3]. 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 [Figure 6-3a and 6-3c]. Some bacilli appeared to be in the process of dividing, coincident with division of the vacuoles [Figure 6-3C]. Direct counting of electron micrographs of infected monocytes cultured for 4 days before fixation and processing revealed that

Figure 6-3. Electron micrographs of mycobacteria-infected monocytes. Freshly explanted monocytes were infected with one viable bacillus per cell and cultured for 4 d. [A] MAI-infected monocytes, untreated or [B] MAI-infected monocytes treated with 2 mM H_2O_2 for 6 h. [C] H37Ra-infected monocytes, untreated or [D] H37Ra-infected monocytes treated with 5 mM H_2O_2. [N] Cell nucleus. Magnification: [A] x7,600; [a] x44,800; [B] x11,500; [b] x23,000; [C] x13,000; [c] x32,500; [D] x3,000; [d] x36,800.

A
N
a
B
N
b
C
N
c
D
N
N
d

H37Ra-infected and MAI-infected monocytes contained an average of 10.3 and 2.5 bacilli per infected cell respectively [Table 6-1]. This is consistent with the observation in the CFU assay that the growth of MAI inside human monocytes is more restricted than that of H37Ra.

Following treatment of infected cells with low dose [2-10 mM] H_2O_2, changes in the phagocytic vacuoles containing MAI were observed. MAI-containing vacuoles which were originally tightly apposed to discrete bacilli [Figures 6-3A and 6-3a] became swollen or irregular in appearance [Figures 6-3B and 6-3b]. Most impressively, some of the bacilli in these altered vacuoles appeared to be degraded [Figures 6-3B and 6-3b]. These morphologic changes were not seen in the majority of vacuoles containing H37Ra. H_2O_2-treated intracellular H37Ra remained predominantly intact [Figures 6-3D and 6-3d]. Electron microscopic examination of monocytes infected for 4 d with MAI or H37Ra and treated for 6 h with 2.5 - 5 mM H_2O_2 revealed that the percent of morphologically degraded bacilli present within phagocytic vacuoles was 34% and 9% respectively [Table 6-1]. This finding confirms our CFU results and indicates that MAI is more susceptible to H_2O_2-induced intracellular killing than H37Ra.

4. Cytokines released by monocytes in response to mycobacterial infection.

Many studies have shown that cytokines activate monocytes better able to

Table 6-1. Mycobacterial infection of human monocytes *in vitro*.

Mycobacteria	Total infected cells*	#Bacilli/infected cells	% Degraded@
MAI	34	2.5	34% [29/84]
H37Ra	35	10.3	9% [33/362]

* Electron micrographs of 4 day infected monocytes were scored for the presence of intracellular mycobacteria and for the percent of organisms which appeared intact. A total of 34-35 infected cells were scored for each experiment.

The mean number of bacilli per infected cell was calculated by dividing the total number of intracellular bacteria by the total number of infected monocytes.

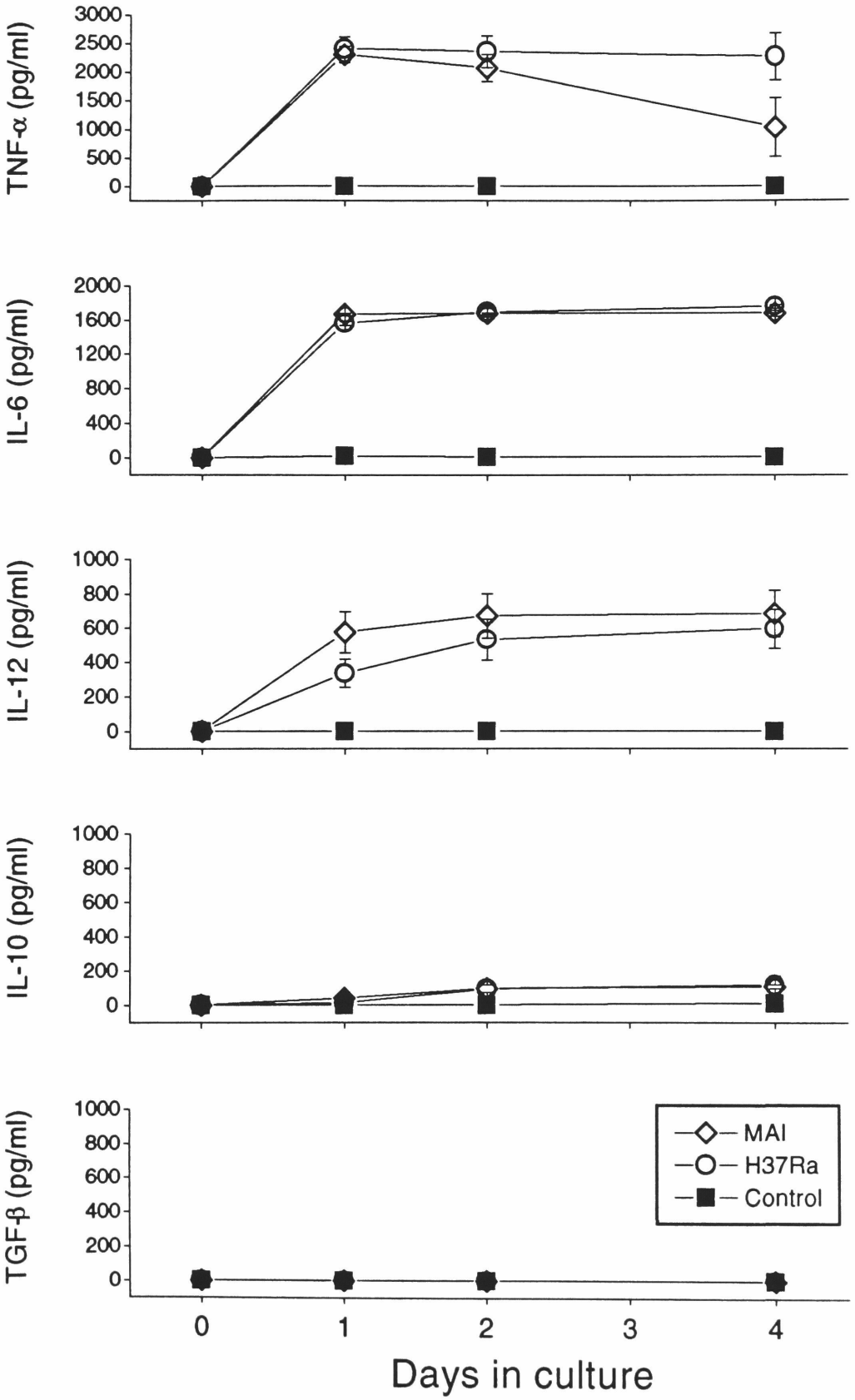
@ The percent of degraded mycobacteria was determined by dividing the total number of morphologically damaged organisms by the total number of organisms [shown in brackets], multiplied by 100.

restrict the growth of or even kill intracellular mycobacteria (178,169,119,97,65). To examine the role of monocyte cytokines in this system, the amount of various cytokines released into the culture supernatants of freshly explanted monocytes infected with either MAI or H37Ra were analyzed by ELISA. Following infection with either of the 2 mycobacterial species, the same cytokine profile was induced [Figure 6-4]. In response to infection, human monocytes produced relatively high levels of the pro-inflammatory cytokines TNF-α, IL-6, and IL-12 and small amounts of IL-10. TGF-β was not detected in any of the mycobacteria-infected cultures. Since infection with MAI and H37Ra appeared to induce human monocytes to produce similar amounts of TNF-α, IL-6, IL-12, and IL-10, the findings suggest that the efficient intracellular growth rate of H37Ra and the very slow growth rate of MAI may be independent of the profile of cytokines produced by infection of the phagocytes.

C. Discussion

Oxidative burst has long been implicated as an important part of the antimicrobial activities of mononuclear phagocytes. The T-cell cytokine IFN-γ, and the monocyte cytokine TNF-α, have been shown to prime human and murine macrophages to secrete increased amounts of ROI during phagocytosis of bacteria and better restrict the growth and survival of

Figure 6-4. Cytokine release by freshly explanted monocytes infected with different mycobacteria. Monocytes were infected with one viable organism per cell, and culture supernatants were analyzed by ELISA for cytokine production at different time points [open symbols]. To control for spontaneous release of cytokines, monocytes were cultured in the absence of experimental infection, and culture supernatants were analyzed by ELISA [closed symbols]. The results are means $\pm$ SEM from two independent experiments, each done in triplicate.


TNF-α (pg/ml)
3000
2500
2000
1500
1000
500
0
IL-6 (pg/ml)
2000
1600
1200
800
400
0
IL-12 (pg/ml)
1000
800
600
400
200
0
IL-10 (pg/ml)
1000
800
600
400
200
0
TGF-β (pg/ml)
1000
800
600
400
200
0
MAI
H37Ra
Control
0
1
2
3
4
Days in culture

intracellular pathogens (102,103). In experiments with human monocytes, treatment of the cells with TNF-α alone or in combination with IL-2 have been reported to reduce the survival of intracellular mycobacteria (95). On the other hand, treatment of human monocytes with TGF-β has been reported to render the phagocytes more permissive to *M. tuberculosis* growth (179). In the experiments reported here, the composition of the cytokine milieu of the mycobacteria-infected monocytes was not directly related to the intracellular growth rates of the two species of mycobacteria in freshly explanted monocytes.

The ability of an intracellular organism to withstand oxidative stress may be an important determinant of its pathogenicity *in vivo*. 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 (180,181). Macrophages have been shown to release H_2O_2 and superoxide anion during phagocytosis of intracellular pathogens, including mycobacteria such as MAI and BCG (99,100,101). The production of ROI by phagocytes has been correlated with growth restriction and/or killing of a variety of intracellular pathogens including *Candida albicans*, *Listeria monocytogenes*, *Toxoplasma gondii*, and *Leishmania* (104,105,106,107,108,109,103).

In the present study, the survival of two different species of mycobacteria within human monocytes *in vitro* was examined. The

susceptibility of the intracellular mycobacteria to H_2O_2-induced killing varies, and appears to relate to their relative ability to establish chronic infection in man. The clinical isolate of MAI, a mycobacterium that is usually nonvirulent and does not establish infection or cause disease in immunocompetent individuals, is more susceptible to H_2O_2-induced killing, while H37Ra, a strain of *M. tuberculosis*, is less susceptible [Figures 6-1 and 6-2].

The relative ability of an intracellular pathogen to establish infection and persist may be due to the interaction of several factors. It has been shown that the relative virulence of mycobacterial strains in infected mice [as defined by their ability to kill the animal], correlates with the relative growth rates *in vivo* [as determined by colony counts] of the mycobacteria (182). Using 2 different mycobacteria, the present study demonstrates a relationship between the infectivity in humans and growth rates *in vitro* and between the growth rates *in vitro* and the susceptibility of the intracellular mycobacteria to killing by exogenously added H_2O_2. In these experiments, MAI [the less pathogenic in man] hardly grows at all, and is more susceptible to killing by H_2O_2 *in vitro* [60% killing]. In contrast, H37Ra [an attenuated strain of pathogenic mycobacteria], which grows with a generation time of 48 h, is also significantly less susceptible to H_2O_2 *in vitro* [15% killing]. A relationship between growth rate and susceptibility to H_2O_2 treatment has also been reported with *Listeria*

monocytogenes (183). Log phase *L. monocytogenes* has been shown to be less susceptible to killing by H_2O_2 than lag phase bacteria. In that system, the resistance to H_2O_2 killing was associated with increased levels of catalase activity in log phase cells. Thus, the induction of catalase molecules during log phase growth may be an important defense mechanism for survival of *L. monocytogenes*.

The ability of microorganisms to detoxify ROI is an adaptation to survival either extracellularly or within a host cell. *OxyR*, a bacterial gene which regulates an inducible defense response against ROI (184,185,186), is both a sensor of reactive oxygen species and a transcriptional activator. *OxyR* controls the expression of nine hydrogen peroxide-inducible proteins, among which are catalase, an alkyl hydroperoxide reductase and glutathione reductase. Unlike other bacteria, pathogenic mycobacteria do not possess a functional *OxyR* gene. Only the saprophytic mycobacteria, such as *M. smegmatis*, have a protective oxidative response analogous to the *OxyR* response of gram-negative bacteria (187). However, the absence of this inducible response does not render pathogenic mycobacteria incapable of withstanding the toxic effects of H_2O_2. In fact, pathogenic mycobacteria [*M. tuberculosis*] have been shown to be much more resistant to H_2O_2 and ROI than *M. smegmatis* (187) and more resistant than *E. coli* and *S. typhimurium* (188). Thus, pathogenic mycobacteria appear to possess other responses to

defend against reactive oxygen species. Recently, Sherman *et al.* reported that pretreatment of BCG with a sub-bactericidal concentration of H_2O_2 resulted in increased expression of the *KatG* catalase-peroxidase gene (189) causing a 35-fold increase in resistance to organic hydroperoxides. In our preliminary studies, BCG replicated in monocytes with a generation time of 96 h and were found to be relatively resistant to low dose H_2O_2-induced killing [a maximum of 37% killing at 10 mM]. In addition, the ability of mycobacteria to upregulate the expression of the *AhpC*, an alkyl hydroperoxidase gene, may provide the pathogen with another defense against the deleterious effects of organic hydroperoxides (189). Also, a thioredoxin [*Trx*] of *M. tuberculosis* has recently been identified and characterized as a further detoxifier of ROI (190). Finally, cyclopropanation of mycobacterial cell wall mycolic acids has been proposed as an important adaptation of mycobacteria to oxidative stress; the *cma1* gene, a homologue of the cyclopropane fatty acid synthetase of *E. coli*, is present in *M. tuberculosis*, but absent in *M. smegmatis* (191,192), which may account for the relative H_2O_2 resistance of the pathogenic organism. When the *cma1* gene of *M. tuberculosis* is transfected into and expressed in *M. smegmatis*, it confers a 10-fold increase in resistance to H_2O_2 treatment (192). At present, it is not yet known whether MAI and H37Ra express the *cma1* gene.

Taken together, the results reported here demonstrated that different species of mycobacteria [H37Ra and MAI] replicated at different rates within

freshly explanted monocytes. The growth rates appear to be an intrinsic property of the mycobacteria, and is inversely related to the resistance of the organisms to H_2O_2-induced killing. In response to infection with either H37Ra or the patient isolate of MAI, freshly explanted monocytes produced the same cytokine profile. Therefore, the production of cytokines [TNF-α, IL-6, IL-12, IL-10, and TGF-β] does not appear to determine the differential growth rates of different species of mycobacteria. In the following chapter, the effect of host cell maturation in culture on the control of *M. tuberculosis* infection will be investigated.

VII. The effect of host cell differentiation in culture on the control of *M. tuberculosis* infection

A. Introduction

During *M. tuberculosis* infection of humans, the organism is phagocytosed by pulmonary mononuclear phagocytes, in which it survives and replicates within the phagosome. In the majority of infected individuals, initial infection leads to the development of a cell-mediated immune response which results in the containment of the infection. However, in about 10% of infected individuals active disease ensues. The outcome of the infection depends on the dynamic interaction between the host's cellular immune response and the organisms' ability to withstand the response and replicate within the infected mononuclear phagocytes. Once established, infection by *M. tuberculosis* is usually not eradicated even by a competent human immune response.

The previous chapter demonstrated that MAI and H37Ra replicated with different doubling times within freshly explanted human monocytes. These infected cells produced similar cytokine profiles in response to infection by the two mycobacterial species. This suggests that the intracellular growth rate of these 2 strains of mycobacteria may be determined by an inherent property of the organisms themselves. In this study, human monocytes were allowed to mature in culture for 8 days prior to infection

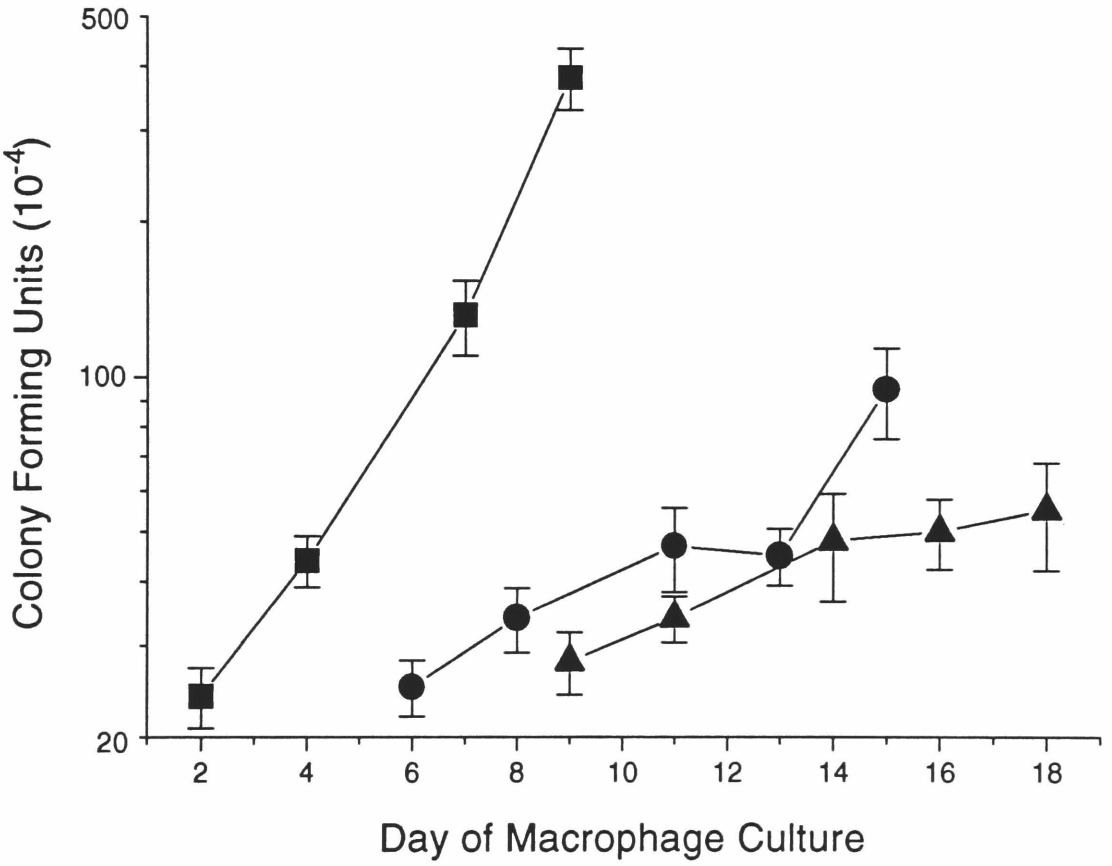
with H37Ra. The intracellular growth rate, susceptibility to H_2O_2-induced killing of H37Ra, and cytokine induction in infected freshly isolated monocytes and in infected 8 d old monocytes were compared. The effect of altering monocyte cytokines on the growth rates of H37Ra was also examined.

B. Results

1. The effect of monocyte culture on growth of intracellular H37Ra. For this study, monocytes were infected with one viable H37Ra per cell immediately after isolation, or monocytes from the same donors were cultured for 5 d or 8 d before mycobacterial infection. At the time points indicated, CFU assays were performed to determine the numbers of viable bacilli in the cultures. The results indicate that H37Ra began replicating immediately in all cultured monocytes [Figure 7-1]. The bacterial generation time [determined by the CFU assay] in monocytes infected on day 0 in culture was 44 h; in monocytes infected on day 5 in culture, the generation time was 103 h, and in monocytes infected on day 8, 189 h. Thus, bacillary growth was more restricted, the longer the monocytes were allowed to age in culture.

2. H_2O_2-induced killing of intracellular H37Ra in freshly explanted and 8 d old monocytes. Next, the sensitivity to H_2O_2-induced killing of intracellular H37Ra growing in either freshly isolated monocytes or 8 d old cultured

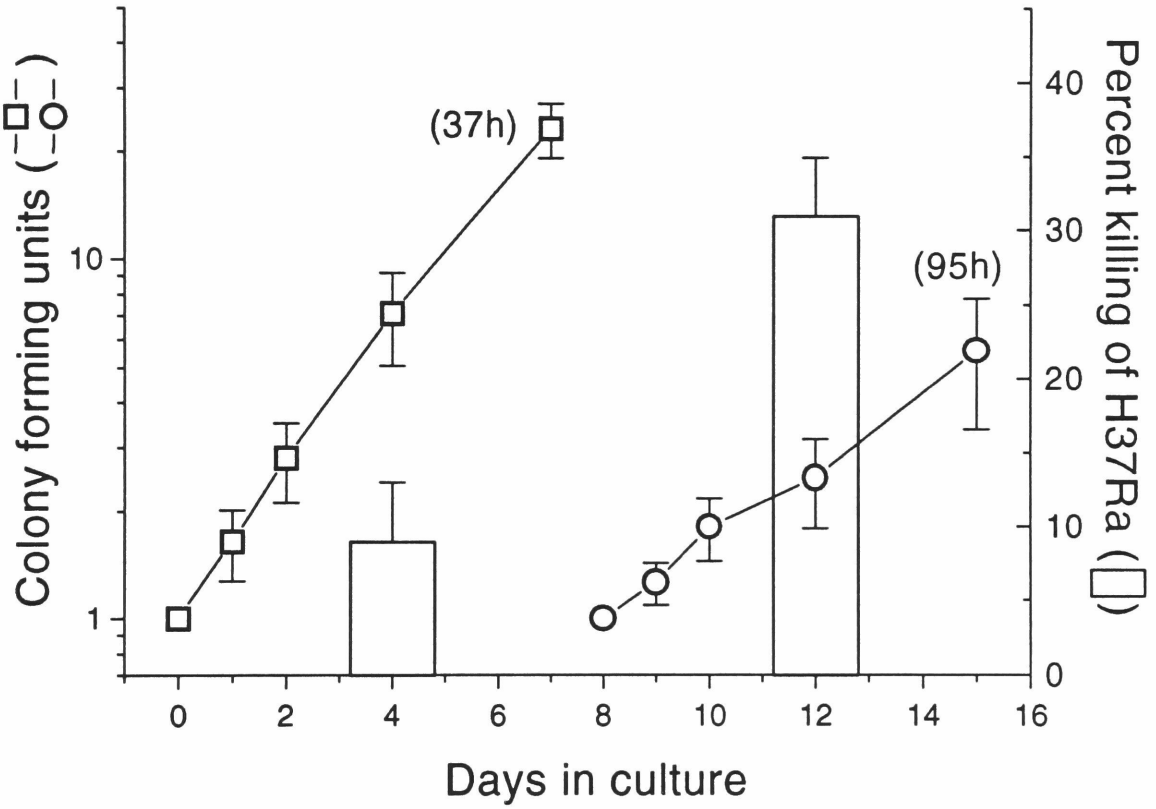
Figure 7-1. Effect of duration in culture of monocytes on growth of *M. tuberculosis* H37Ra. Monocytes on day 0 [square], day 5 [circle], or day 8 [triangle] after isolation and introduction into culture were infected with 3 x 10^5 CFU of H37Ra. To allow infection of identical cell populations at each time point, monolayers were not washed before or after infection [adherence varies with duration in culture]. At the time points indicated, colony counts were performed. Results are mean $\pm$ SEM of triplicates for each point.


500
100
20
Colony Forming Units (10^{-4})
2
4
6
8
10
12
14
16
18
Day of Macrophage Culture

monocytes derived from the same donors were compared. Three separate donors were used in this study. Freshly explanted and 8 d old monocytes derived from the same donors were infected with H37Ra, cultured for 4 d, and then treated with 10 mM H_2O_2 for 6 h. The mean percent H_2O_2-induced killing of H37Ra, evaluated by the CFU assay, was 9% for freshly explanted infected monocytes and 31% for 8 d old infected monocytes [Figure 7-2]. The calculated doubling time of H37Ra in freshly explanted monocytes and 8 d old monocytes were 37 h and 95 h respectively [Figure 7-2]. Our results suggest that as monocytes differentiate in culture, the intracellular growth of H37Ra becomes more restricted. The inhibition of H37Ra growth within 8 d old monocytes renders the bacilli more susceptible to H_2O_2-induced killing. Therefore, there appears to be an inverse relationship between monocyte permissiveness for intracellular growth of H37Ra and susceptibility of H37Ra to killing by H_2O_2.

3. Cytokines released by freshly explanted and 8 d old monocytes in response to H37Ra infection. To study the effect of monocyte differentiation *in vitro* on cytokine production during mycobacterial infection, the amounts of TNF-α, IL-6, IL-12, IL-10, and TGF-β proteins in the culture supernatants of both freshly explanted and 8 d old monocytes infected with H37Ra were analyzed by ELISA. In response to the infection, freshly explanted monocytes produced

Figure 7-2. H37Ra growth in cultured monocytes. 3×10^5 viable mycobacteria were added to wells containing 3×10^5 freshly isolated adherent monocytes [squares] or wells containing 3×10^5 monocytes that had been cultured for 8 d [circles]. At the time point indicated, CFU were assayed. CFU data from each experiment were normalized to the number of CFU at the start of the experiment and expressed as the fold change in number of CFU over time. The baseline CFU were 48 ± 7 and $44 \pm 23 \times 10^4$ for freshly explanted and 8 d old infected monocytes respectively. Results are means $\pm$ SEM of 3 experiments, each done in triplicate. BARS: H_2O_2-induced killing of intracellular H37Ra. On day 4 post-infection, freshly isolated monocytes and 8 d old monocytes were treated with 10 mM H_2O_2 diluted in culture medium. After 6 h, cultures were harvested for CFU assay. Results are expressed as percent killing of mycobacteria and are means $\pm$ SEM of 4 experiments, each done in triplicate.


Colony forming units (□–□, ○–○)
Percent killing of H37Ra (□)
(37h)
(95h)
10
1
40
30
20
10
0
0
2
4
6
8
10
12
14
16
Days in culture

significantly more TNF-α, IL-6, and IL-12 than 8 d old monocytes, while 8 d old monocytes released substantially more IL-10 [Figures 7-3A and 7-3B]. No TGF-β was found in any of the infected cultures. The spontaneous production of cytokines by human monocytes in the absence of experimental infection was also evaluated. A small amount of IL-10 was found in the supernatants of monocytes after 4 d in culture [Figure 7-3C]. The other monocyte cytokines were not detected in the culture supernatants in the absence of mycobacterial infection. These findings suggest that as monocytes age in culture, they produce a different cytokine profile in response to H37Ra infection.

4. The effect of monocyte conditioned medium on the intracellular growth of H37Ra. Throughout the experiment, the culture medium was not changed; therefore, it was possible that H37Ra grew better in freshly isolated monocytes because the medium was fresh. To investigate the effects of conditioned culture medium on bacillary growth rate, freshly explanted monocytes were infected with H37Ra, and then cultured in conditioned culture medium from 8 d old monocytes. CFU data revealed that in monocyte-conditioned medium, the growth of H37Ra in freshly explanted monocytes became more restricted [Table 7-1]. These findings suggest that monocyte-conditioned medium was capable of partially inhibiting the intracellular growth rate of H37Ra. However, the growth inhibition of H37Ra failed to fully mimic the

Figure 7-3. Cytokine release by freshly explanted monocytes and 8 d old monocytes following H37Ra infection. Monocytes were infected at the multiplicity of infection of 1 bacillus per cell, and culture supernatants were analyzed by ELISA for cytokine production [open symbols]. To control for spontaneous release of cytokines, monocytes were cultured in the absence of experimental infection, and culture supernatants were analyzed by ELISA [closed symbols]. The results are means $\pm$ SEM from 2-7 independent experiments, each done in triplicate.

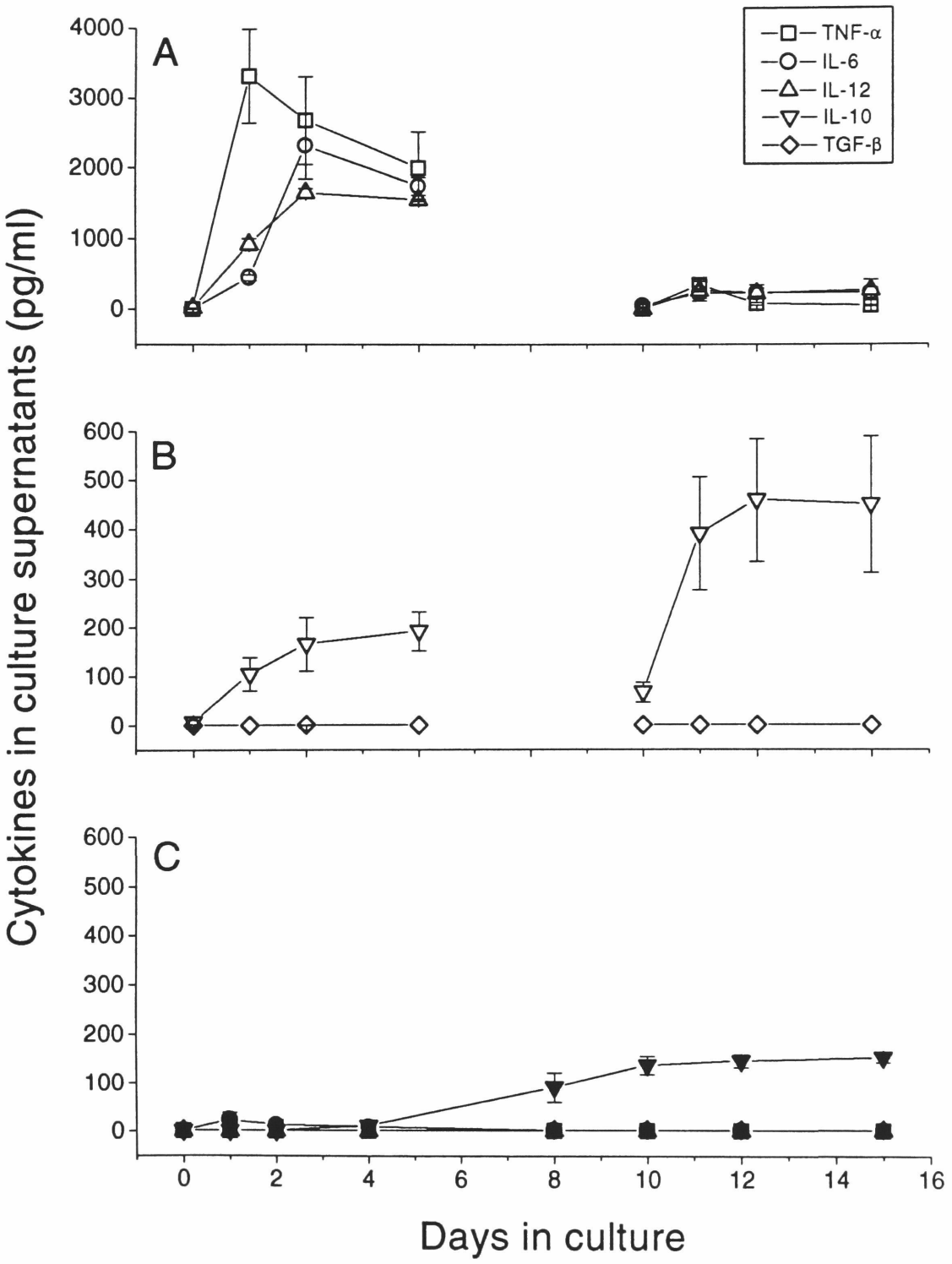

A
B
C
TNF-α
IL-6
IL-12
IL-10
TGF-β
Cytokines in culture supernatants (pg/ml)
Days in culture
4000
3000
2000
1000
0
600
500
400
300
200
100
0
0
2
4
6
8
10
12
14
16

Table 7-1. H37Ra growth rate in freshly explanted monocytes

Treatment conditions@	Colony forming units [CFU]#			
	Day 0	Day 4	Day 7	Doubling time [h]†
Control	1*	6.4 ± 0.8	20.9 ± 2.4	40 ± 2[a]
+ 20-40 μg/ml anti-TNF-α[$]	1	9.0 ± 2.0	26.0 ± 3.0	36 ± 1[b]
+ 2-10 ng/ml rhIL-10	1	7.0 ± 0.5	24.0 ± 2.0	37 ± 1[b]
+ 40 μg/ml anti-TNF-α, 10 ng/ml rhIL-10	1	3.0 ± 0.2	7.2 ± 0.3	58 ± 2[c]
+ 100 μg/ml anti-IL-6[¥]	1	4.1 ± 0.1	20.9 ± 2.1	38 ± 1 [c]
+ 40 μg/ml anti-TNF-α, 20 μg/ml anti-IL-6, 20 μg/ml anti-IL-12, 10 ng/ml rhIL-10	1	5.3 ± 0.2	7.6 ± 0.7	55 ± 2[c]
+ 8 d conditioned media	1	3.0 ± 0.3	11.0 ± 2.0	53 ± 6[b]

@ Neutralizing polyclonal Abs against human TNF-α, IL-6, and IL-12 or rhIL-10 were added daily to the infected monocyte cultures at the indicated concentration.

CFU numbers were calculated as fold increase with respect to the initial infecting inoculum on Day 0 normalized to 1.

* Monocytes were infected with MOI of 1:1. CFU on Day 0 = 40 ± 6 x 10^4.

$ 40 μg/ml anti-TNF-α Ab inhibited 100% of TNF-α in the culture supernatants.

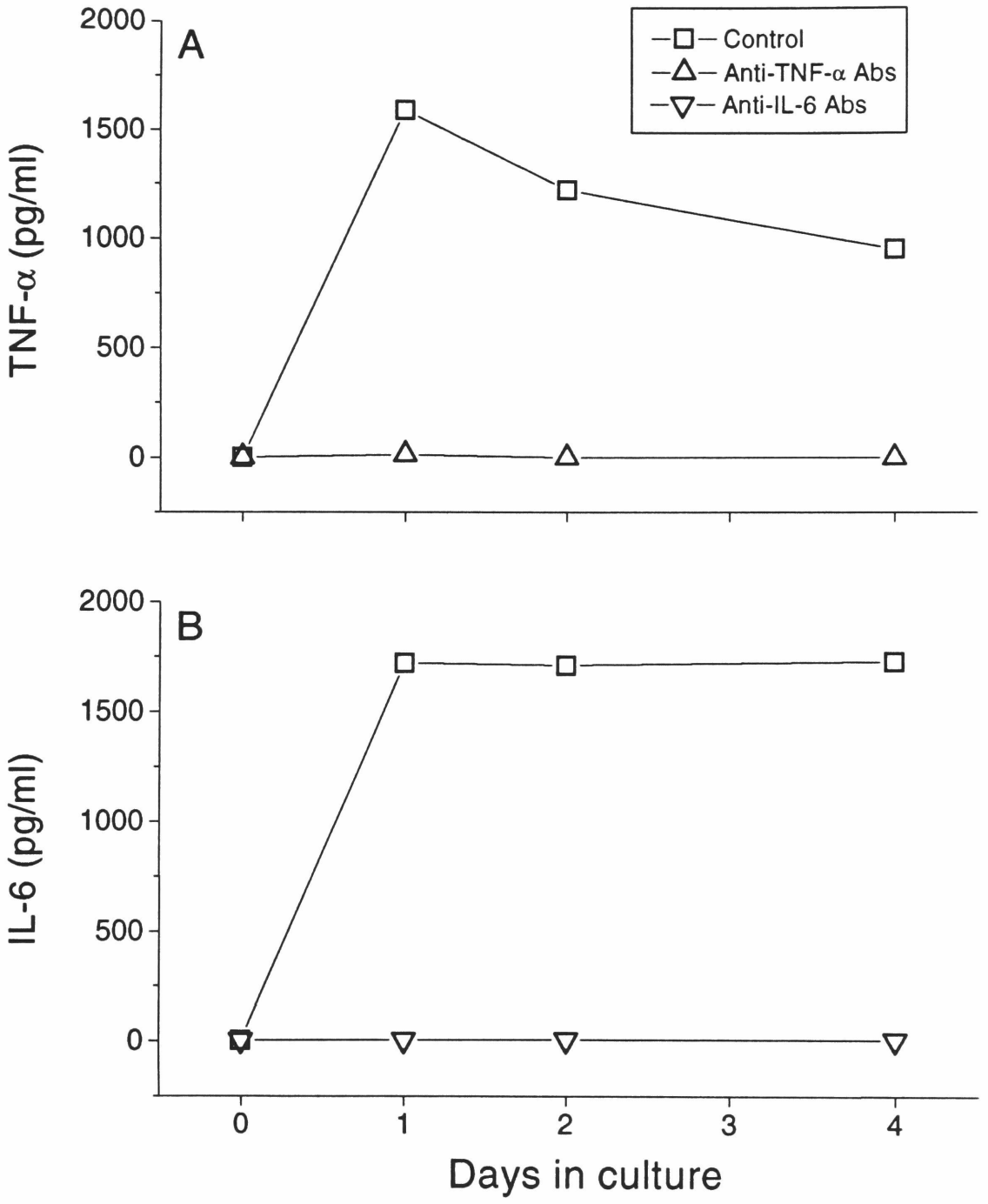
¥ 100 μg/ml anti-IL-6 Ab was added on day 0 and 30 μg/ml was added daily thereafter. This concentration of Ab inhibited 100% of IL-6 in the culture supernatants.

† Results are expressed as mean ± SEM of triplicate cultures derived from: [a] 8 experiments; [b] 2 experiments; [c] 1 experiment.

growth restriction observed in H37Ra-infected 8 d old monocytes.

5. The effect of the cytokines on growth of intracellular H37Ra. We examined whether experimentally altering the cytokine milieu of infected freshly explanted cells to mimic the cytokine environment of infected 8 d old cells would have an effect on the intracellular growth rate of H37Ra. Freshly isolated monocytes were immediately infected with H37Ra and simultaneously treated with neutralizing antibodies to TNF-α, IL-6, IL-12, and recombinant human IL-10 [rhIL-10]. Anti-TNF-α antibody [40 μg/ml] and anti-IL-6 antibody [100 μg/ml] inhibited 100% of TNF-α and IL-6 in the culture supernatants respectively [Figure 7-4]. In these cultures, bacillary growth was monitored over time using the CFU assay. When infected freshly explanted monocytes were treated daily with either anti-TNF-α antibody, or with anti-IL-6 antibody, or with rhIL-10 for 4 d, the growth rate of intracellular H37Ra was not affected [Table 7-1]. However, simultaneous treatment of infected cultures with both anti-TNF-α antibody and rhIL-10 resulted in a partial decrease in the growth rate of H37Ra [Table 7-1]. The intracellular growth rate of H37Ra in freshly explanted monocytes was not further affected when infected cells were treated with a combination of anti-TNF-α, anti-IL-6, anti-IL-12 antibodies and rhIL-10. In none of the treatment conditions, were we able to fully reduce the growth rate of H37Ra in freshly explanted monocytes

Figure 7-4. Neutralization of monocyte cytokines. 3 x 10^5 freshly isolated adherent monocytes were infected at the multiplicity of infection of 1 bacillus per cell. [A] 40 μg/ml anti-TNF-α antibody was added daily to the infected monocyte cultures and was shown to inhibit 100% of TNF-α in the culture supernatants by ELISA. [B] 100 μg/ml anti-IL-6 was added on day 0 and 30 μg/ml was added daily thereafter. This concentration of antibody inhibited 100% of IL-6 in the culture supernatants as determined by ELISA. The results are mean ± SEM of triplicates for each point.


A
B
Control
Anti-TNF-α Abs
Anti-IL-6 Abs
TNF-α (pg/ml)
IL-6 (pg/ml)
2000
1500
1000
500
0
0
1
2
3
4
Days in culture

to the extent observed in 8 d old monocytes. Therefore, the changes in the cytokine environment did not fully account for the inhibitory activity of 8 d old monocytes, suggesting that other cellular factors may contribute to the regulation of intracellular bacillary growth. Investigations are currently underway to examine how monocyte differentiation in culture affects the intracellular growth of H37Ra.

C. Discussion

In an attempt to understand how human monocytes contribute to the control of mycobacterial infection, growth of *M. tuberculosis* H37Ra was assayed in monocytes at different stages of *in vitro* maturation. The present study demonstrates that as monocytes age in culture, the cells appear to acquire the ability to restrict the growth of intracellular H37Ra. This growth inhibition of H37Ra observed in 8 d old monocytes also rendered the bacilli more susceptible to H_2O_2-induced killing. These findings confirm the earlier observation that growth rate of mycobacteria is inversely related to the organisms' susceptibility to H_2O_2-induced killing [chapters 5 and 6]. Furthermore, in response to *M. tuberculosis* H37Ra infection 8 d old monocytes produced small amounts of pro-inflammatory cytokines [TNF-α, IL-6, and IL-12] and released substantially more IL-10 compared to freshly explanted monocytes. This suggested that cytokines may be involved in the control of mycobacterial infection in human monocytes.

The importance of cytokines in the successful containment of mycobacterial infection has been clearly demonstrated in *in vivo* studies. For instance, neutralization of TNF-α in mice experimentally infected with mycobacteria interfered with granuloma formation and exacerbated the infection (59). Mycobacterial infection of IFN-γ gene-disrupted mice resulted in a progressive course of infection and early death of the infected animals (34). In the murine *in vitro* model, the host cell's killing of mycobacteria has been associated with cytokine-induced activation of the phagocytes. Chan *et al.* demonstrated that treatment of murine macrophages either with IFN-γ and LPS or with IFN-γ and TNF-α activated the anti-mycobacterial activity of murine macrophages, resulting in the inhibition and killing of *M. tuberculosis* (52). This inhibition/killing of *M. tuberculosis* was found to be dependent on reactive nitrogen intermediates produced by activated murine macrophages (52). In contrast to the murine model, how cytokine-induced activation of human mononuclear phagocytes contributes to the control of mycobacterial survival is as yet unknown.

In the *in vitro* model described here, human monocytes released a small amount of IL-10 into the culture supernatant in the absence of experimental infection. Previously, IL-10 has been shown to inhibit monocyte cytokine production (70). Therefore, endogenous production and accumulation of IL-10 by 8 d old monocytes may help dampen the excessive

inflammatory response [TNF-α, IL-6, and IL-12 production] due to H37Ra infection which may contribute to the growth restriction of H37Ra in 8 d old monocytes. However, neutralization of TNF-α together with the addition of IL-10 to the fresh monocytes infected with H37Ra, failed to fully mimic the growth restriction of H37Ra observed in infected 8 d old monocytes. This indicates that although these cytokines may be of some importance, other cytokines or cellular factors may also contribute to the growth restriction.

Our laboratory has shown that the addition of IFN-γ to BCG-infected or MAI-infected human monocytes *in vitro* [chapter 4] did not affect the viability of the these intracellular mycobacteria (57,96). Treatment of human monocytes with TNF-α alone or in combination with IL-2 have been reported to reduce the survival of intracellular mycobacteria (95). Other published studies suggested that *M. tuberculosis* may be killed in human macrophages activated by IFN-γ and vitamin D_3 (97,58). On the other hand, treatment of human monocytes with TGF-β has been reported to render the phagocytes more permissive to *M. tuberculosis growth* (179). Therefore, it appears that the contribution of cytokines whether of T cell or monocyte origin to the regulation of intracellular mycobacterial growth is complex. Additional studies examining the effects of cytokines on mycobacterial growth in monocytes may help elucidate how activated human mononuclear phagocytes kill intracellular mycobacteria.

VIII. The mechanism of H_2O_2-induced killing of intracellular mycobacteria

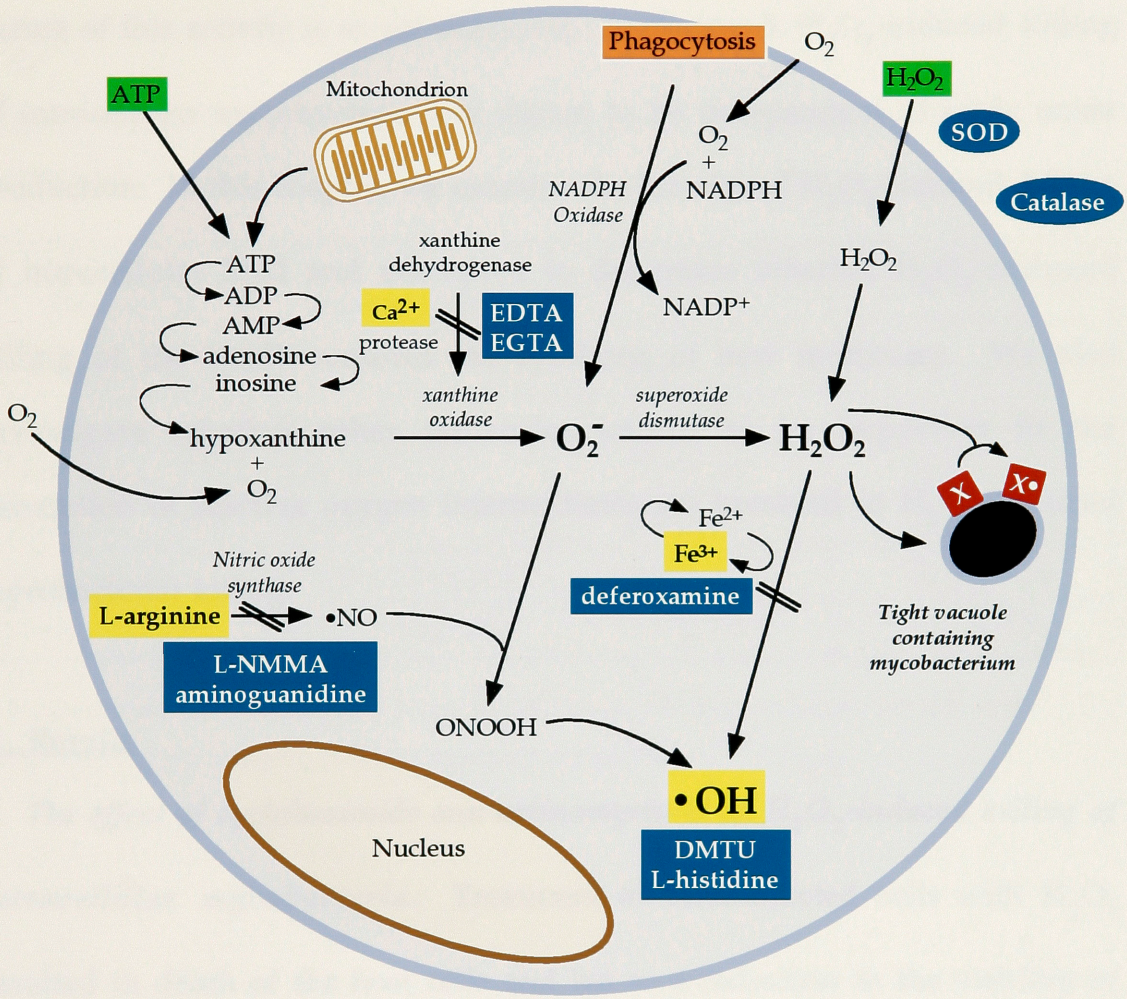
A. Introduction

During phagocytosis of intracellular pathogens including mycobacteria, human monocytes/macrophages have been shown to produce superoxide anion, H_2O_2, and hydroxyl radicals (193,103). Figure 8-1 shows the major pathways involved in the generation of ROI and RNI as well as the inhibitors that block their production. The respiratory burst depends on the activation of an enzyme complex associated with the plasma membrane. B.M. Babior showed that electrons provided by NADPH was used to reduce oxygen to superoxide anion [Figure 8-1] (194), and it is this process that accounts for most of the intracellular superoxide anion produced. Intracellular superoxide dismutase converts superoxide anion into H_2O_2 which can undergo iron-catalyzed Haber-Weiss reaction to form hydroxy radicals [Figure 8-1]. Once generated, H_2O_2 can diffuse freely through cell membranes and exert its toxicity at distant locations while hydroxyl radicals react with the molecules in their immediate surroundings. The production of these ROI has been correlated with growth restriction and/or killing of a variety of intracellular pathogens including *Candida albicans*, *Listeria monocytogenes*, *Toxoplasma gondii*, and *Leishmania* (104,105,106,107,108,109,103).

In the *in vitro* infection model described here, killing of intracellular

Figure 8-1. The major pathways involved in the generation of ROI and RNI as well as the inhibitors used in this 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 agents [H_2O_2 and ATP] are shown in green. In the proposed mechanism of oxidative killing of mycobacteria within human monocytes, the potential host cell molecule responsible for mycobacterial killing is shown in red.

Proposed Mechanism of Oxidative Killing of Mycobacteria in Human Monocytes



Adapted from D.G. Remick and L. Villarete (1996) *J. Leuko. Biol.* 59, 471-475.

mycobacteria was observed following treatment of the infected monocytes with H_2O_2. The killing of mycobacteria was more pronounced when the infected cells were intact. This suggests that human monocytes have a mycobactericidal activity that is induced by H_2O_2 or oxidative stress. The nature of this activity is as yet unknown. In chapter 5, H_2O_2-induced killing of intracellular mycobacteria was shown to be independent of nitric oxide production. In this chapter, we examine the kinetics of H_2O_2-induced killing of intracellular MAI and attempted to determine whether H_2O_2-induced killing of the bacilli requires the synthesis of new molecules. We also investigate whether other molecules known to be important in the generation of reactive oxygen intermediates are involved in H_2O_2-induced mycobacterial killing.

B. Results

1. The effect of cycloheximide and actinomycin-D on H_2O_2-induced killing of intracellular mycobacteria. Treatment of MAI-infected cells with H_2O_2 resulted in death of the host cells and led to a reduction in the viability of intracellular bacilli. It is conceivable that in addition to its direct cytotoxic effects on both the host cells and the intracellular mycobacteria, H_2O_2 may also induce a secondary effector molecule involving gene transcription and/or protein synthesis which contribute to the killing of intracellular MAI.

Table 8-1. Bacillus killing after treatment of infected monocytes with H_2O_2, actinomycin-D and cycloheximide

Treatment	Concentration	Colony forming units@ [10^{-4}]	
		Intact monolayers	Sonicated cultures
None [control]		18.0 ± 0.9	24.1 ± 1.0
CHX	20 µg/ml	20.1 ± 1.0	24.0 ± 1.3
Act-D	5 µg/ml	17.2 ± 0.9	21.2 ± 1.1
H_2O_2	10 mM	10.8 ± 0.7	21.0 ± 1.0
H_2O_2 + CHX	10 mM + 20 µg/ml	8.9 ± 0.7	18.4 ± 0.9
H_2O_2 + Act-D	10 mM + 5 µg/ml	6.7 ± 0.6	17.1 ± 0.9

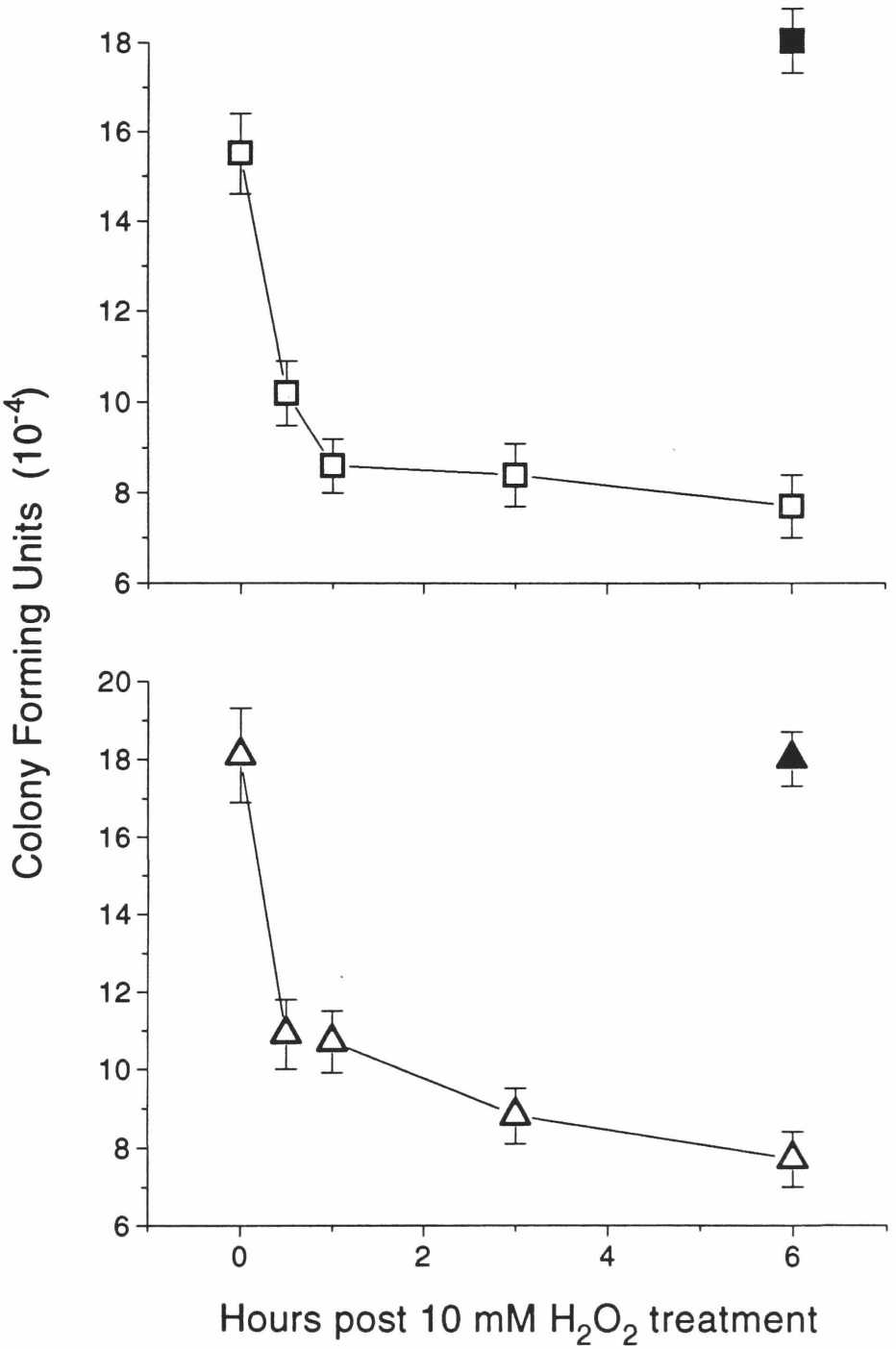
@ Four-day-infected monocytes were treated as above and incubated for 6 h at 37°C, 5% CO_2. Results are means ± SEM of triplicate cultures.

To investigate whether H_2O_2-induced killing of intracellular MAI requires transcription of new genes, MAI-infected monocytes were treated with either 5 μg/ml actinomycin-D, or 10 mM H_2O_2, or a combination of both for 6 h. The results revealed that actinomycin-D alone had little effect on bacterial viability and did not affect the H_2O_2-induced killing of intracellular MAI [Table 8-1].

To determine whether new protein synthesis was required for H_2O_2-induced killing, 20 μg/ml CHX was added together with 10 mM H_2O_2 to cultures of infected monocytes for 6 h. The extent of bacillus killing observed under these conditions was identical to that of cultures treated with H_2O_2 alone [Table 8-1]. These results indicated that neither transcription nor new protein synthesis was required for H_2O_2 -induced killing of intracellular MAI.

2. ***The kinetics of H_2O_2-induced killing of intracellular mycobacteria.*** To determine whether H_2O_2-induced killing of intracellular mycobacteria required the presence of H_2O_2 in the culture medium for the entire 6 h treatment, 4 d MAI-infected monocytes were treated with 10 mM H_2O_2. At the time points indicated, exogenous H_2O_2 in the treated cultures was neutralized by either washing of the cultures and adding fresh culture medium [Figure 8-2A] or by the addition of 100 μg/ml catalase to the culture

Figure 8-2. Kinetics of H_2O_2-induced killing of intracellular MAI. 3 x 10^5 monocytes were infected immediately after isolation and cultured for 4 d. On day 4 post infection, H_2O_2 diluted in culture medium was added to intact monolayers at the final concentration of 10 mM. At the time points indicated, the exogenously added H_2O_2 was either [A] washed away or [B] neutralized by the addition of 100 µg/ml catalase into the cultures, and further incubated. At the end of the 6 h H_2O_2 treatment, treated cultures [open symbols] and parallel untreated control cultures [closed symbols] were harvested for CFU assay. Results are means ± SEM of triplicate cultures.


Colony Forming Units (10^{-4})
18
16
14
12
10
8
6
20
18
16
14
12
10
8
6
0
2
4
6
Hours post 10 mM H_2O_2 treatment

medium [Figure 8-2B]. Cultures were then incubated for the remaining duration of the 6 h H_2O_2 treatment. The results demonstrated that the addition of catalase or removal of H_2O_2 from the culture supernatant by washing at time 0 completely protected the intracellular bacilli from H_2O_2-induced killing. However, neutralization of H_2O_2 after 30 minutes of H_2O_2 treatment did not block the killing of intracellular MAI [Figures 8-2A and 8-2B]. These experiments suggest that addition of exogenous H_2O_2 triggers a killing mechanism that is irreversible within 30 minutes and is independent of the continued extracellular presence of H_2O_2.

3. The effect of scavengers of ROI on H_2O_2-induced killing of intracellular mycobacteria. H_2O_2-induced killing of intracellular mycobacteria was completely inhibited by the addition of 100 μg/ml catalase at time 0 [Figure 8-2]. To investigate whether pretreatment of MAI-infected monocytes with other scavengers of ROI would affect the H_2O_2-induced killing of intracellular bacilli, MAI-infected monocytes were treated with various scavengers of ROI 1 h prior to the addition of 10 mM H_2O_2 and maintained in the culture throughout the experiment [Table 8-2]. The molecular target[s] of superoxide dismutase is superoxide anion, of L-histidine are singlet oxygen and hydroxyl radicals, and of dimethylthiourea are H_2O_2, hydroxyl radicals and

Table 8-2. Bacillus killing after treatment of infected monocytes with H_2O_2, and anti-oxidants

Treatment	Concentration	Colony forming units@ [10^{-4}]	
		Intact monolayers	Sonicated cultures
None [control]		20.0 ± 1.1	21.9 ± 1.4
Catalase	5-10 μg/ml	17.1 ± 0.7	20.3 ± 0.2
SOD	5-10 μg/ml	18.6 ± 0.1	20.2 ± 0.5
DMTU	5 mM	20.7 ± 0.9	23.6 ± 1.4
L-histidine	10 mg/ml	24.1 ± 1.3	25.3 ± 1.1
H_2O_2	10 mM	9.4 ± 0.7	18.1 ± 1.6
H_2O_2 + Catalase	10 mM + 5-10 μg/ml	15.2 ± 0.9	17.7 ± 1.4
H_2O_2 + SOD	10 mM + 5-10 μg/ml	8.4 ± 0.8	15.9 ± 0.3
H_2O_2 + DMTU	10 mM + 5 mM	6.5 ± 0.4	23.6 ± 1.4
H_2O_2 + L-histidine	10 mM + 10 mg/ml	11.5 ± 0.6	18.7 ± 1.2

@ Four-day-infected monocytes were treated with each anti-oxidant 1 h before the addition of H_2O_2, and then incubated for 6 h at 37°C, 5% CO_2. Results are means ± SEM of 1-4 experiments, each done in triplicate cultures.

hypochlorous acid. These anti-oxidants failed to inhibit the killing of intracellular MAI. Since treatment of cells with L-histidine and dimethylthiourea have been shown to result in the accumulation of these molecules in the cytoplasm of the treated cells (195,196,197), the lack of inhibitory effect of these scavengers in this system suggests that H_2O_2-induced killing of intracellular mycobacteria did not involve either singlet oxygen, hydroxyl radicals, or hypochlorous acid.

4. The effect of deferoxamine mesylate on H_2O_2-induced killing of intracellular mycobacteria. The role of iron in H_2O_2-induced killing of intracellular mycobacteria was examined. Iron ions have been shown to catalyze the Haber-Weiss reaction to generate hydroxyl radicals (198). When cells were treated with deferoxamine mesylate, a powerful chelator of Fe^{3+}, the intracellular concentration of Fe^{3+} decreased, and interfered with this iron-dependent free radical reaction (199,196). To evaluate the contribution of iron ions to the killing of intracellular mycobacteria by H_2O_2, 4 d MAI-infected monocyte cultures were treated with 100 μM deferoxamine mesylate for 2 h before treatment with 10 mM H_2O_2 for 6 h. The same percentage of intracellular MAI were killed in cultures treated with deferoxamine mesylate as in untreated cultures following 10 mM H_2O_2 treatment [Table 8-3]. Thus, Fe^{3+} does not appear to be involved in the H_2O_2-induced killing of

Table 8-3. Bacillus killing after treatment of infected monocytes with H_2O_2 and deferoxamine mesylate

Treatment	Concentration	Colony forming units@ [10^{-4}]	
		Intact monolayers	Sonicated cultures
None [control]		22.7 ± 3.8	23.8 ± 3.9
DFX	100 μM	21.4 ± 3.4	23.2 ± 3.1
H_2O_2	10 mM	12.1 ± 2.7	21.7 ± 4.2
H_2O_2 + DFX	10 mM + 100 μM	10.9 ± 2.8	20.9 ± 3.2

@ Four-day-infected monocytes were treated with deferoxamine mesylate [DFX] 1 h before the addition of H_2O_2, and then incubated for 6 h at 37°C, 5% CO_2. Results are means ± SEM of 2 experiments, each done in triplicate cultures.

intracellular MAI.

5. The effect of EDTA and EGTA on H_2O_2-induced killing of intracellular mycobacteria. The respiratory burst of activated human monocytes has been shown to be dependent on both Ca^{2+} and Mg^{2+} (200,201). To investigate whether chelation of the divalent cations by EDTA or EGTA would affect the intracellular killing of MAI by H_2O_2 treatment, 4 d MAI-infected monocyte cultures were treated with 1-10 mM EDTA or 10 mM EGTA 1 h prior to the addition of 10 mM H_2O_2. The addition of either EDTA and EGTA resulted in detachment of these infected cells, but the percent killing of intracellular MAI was unaffected [Table 8-4]. This suggests that both Ca^{2+} and Mg^{2+} are not involved in H_2O_2-induced killing of intracellular MAI. These results indicated that scavengers of ROI [apart from catalase], and chelators of Fe^{3+}, Ca^{2+}, and Mg^{2+} did not affect H_2O_2-induced killing of intracellular MAI.

C. Discussion

Reactive oxygen intermediates [ROI] including H_2O_2 can directly damage many cellular macromolecules or initiate a chain reaction whereby the free radical is shuttled from one molecule to the next causing extensive damage to cellular structures (172). Killing of intracellular mycobacteria following H_2O_2 treatment was independent of transcription of new genes or

Table 8-4. Bacillus killing after treatment of infected monocytes with H_2O_2, EDTA and EGTA

Treatment	Concentration	Colony forming units@ [10^{-4}]	
		Intact monolayers	Sonicated cultures
None [control]		18.7 ± 0.9	21.2 ± 2.2
EDTA	10 mM	19.7 ± 1.4	22.8 ± 1.3
EGTA	10 mM	16.3 ± 1.0	15.7 ± 0.9
H_2O_2	10 mM	10.0 ± 0.1	19.4 ± 2.1
H_2O_2 + EDTA	10 mM + 1 mM	9.4 ± 0.7	22.9 ± 1.1
H_2O_2 + EDTA	10 mM + 10 mM	9.9 ± 0.9	16.6 ± 0.7
H_2O_2 + EGTA	10 mM + 10 mM	5.2 ± 0.4	15.2 ± 0.8

@ Four-day-infected monocytes were treated with either EDTA or EGTA 1 h before the addition of H_2O_2, and then incubated for 6 h at 37°C, 5% CO_2. Results are means ± SEM of 1-2 experiments, each done in triplicate cultures.

new protein synthesis, and was unaffected by treatment of the infected cells by inhibitors of ROI. This suggests that the exogenously added H_2O_2 either directly destroy the intracellular mycobacteria or induce a secondary effector molecule already present within the infected phagocytes to mediate the killing of intracellular mycobacteria. In the experiments reported here, the presence of H_2O_2 during the first 30 minutes of the treatment period was both necessary and sufficient to induce killing of intracellular mycobacteria. Since H_2O_2 diffuses freely through cell membranes, it is conceivable that treatment of the infected phagocytes with H_2O_2 may lead to a high concentration of H_2O_2 accumulated within the phagosomes containing mycobacteria [Figure 8-1] which may be responsible for the killing of mycobacteria. However, since H_2O_2 diffuses freely, it may not concentrate in intracellular compartments. Thus, the concentration of H_2O_2 in the mycobacterial vacuole is unlikely to exceed the overall 10 mM concentration which cannot explain the level of killing seen in the intact cell cultures.

On the other hand, H_2O_2 treatment of the infected phagocytes may induce a secondary effector in the cell, which is not inhibited by catalase, either because it is not accessible to the enzyme 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, detoxification of the exogenously added H_2O_2 by the effector molecule results in the generation of

a reactive effector molecule such as a lipid peroxide. Indeed, organic hydroperoxides have been shown to be toxic to mycobacteria (189). Lipid peroxides produced by reaction with ROI, are resistant to anti-oxidants (202). In the present study, anti-oxidants such as superoxide dismutase, 1,3-dimethyl-2-thiourea, and L-histidine did not affect the H_2O_2-induced killing of intracellular MAI [Table 8-2].

One previous study reported a close correlation between degrees of the anti-mycobacterial activity of various cell fractions and the amount of nonesterified fatty acids (203). In that study, lysosome extracts of normal and activated liver cells from guinea pigs did not inhibit growth of *M. bovis*, BCG while lysosomal membranes and cell extracts exhibited some growth-inhibiting activity. The strongest growth-inhibiting activity was, however, in the cell membrane fractions (203). Since mycobacteria derive their initial vacuoles from the plasma membranes of the mononuclear phagocytes, H_2O_2-induced lipid peroxides may accumulate within the vacuolar membrane, reducing the viability of the mycobacteria in proportion to their sensitivity to the toxic effects of the lipid peroxides [Figure 8-1]. Taken together, these findings suggest that perhaps some reactive lipid peroxides on the vacuoles containing mycobacteria may be responsible for the killing of mycobacteria following H_2O_2 treatment.

Furthermore, the possible accumulation of lipid peroxides within the cell might explain the observation that sonication of the infected monocytes

and subsequent dilution of the cellular components in a larger volume of culture medium reduced the susceptibility of mycobacteria to H_2O_2-induced killing. Even if cellular lipids detoxify the experimentally added hydrogen peroxide thus becoming reactive themselves, their relative concentration in the extracellular medium may be insufficient to kill the extracellular organisms. In cell-free culture medium, H_2O_2 is not inactivated by cellular components and remains able to interact directly with the organisms, resulting in more efficient killing of mycobacteria. Future studies are underway in our laboratory to determine the mechanism[s] by which infected monocytes utilize oxidative intermediates to restrict the growth of intracellular mycobacteria and how the mycobacteria respond to oxidative stress.

IX. General discussion

A. The role of the host monocytes and lymphocytes in the control of intracellular mycobacteria

Scientists have long been interested in determining how mycobacteria survive or are killed by the host mononuclear phagocytes. In the 1950's, Lurie and Suter demonstrated that macrophages obtained from immune animals exhibited tuberculostatic activities whereas the cells from naive animals failed to control the growth of the *M. tuberculosis* bacilli (166,204). Subsequently, Mackaness showed that the activation of murine macrophages' anti-mycobacterial activity was dependent on lymphocytes (205). A few years later, the lymphoid cytokine IFN-γ was identified as the molecule responsible for macrophage activation (103). *In vitro*, treatment of murine macrophages with IFN-γ endows the phagocytes with the ability to kill *M. tuberculosis* bacilli (56,206,52). The degree of killing of *M. tuberculosis in vitro* correlates closely with the ability of the murine macrophages to produce reactive nitrogen intermediates [RNI] following IFN-γ treatment (52). These data indicate that the control of mycobacterial infection in murine macrophages is dependent on lymphokine activation of the host cells.

In contrast to the murine system, human monocytes *in vitro* have not been shown to effectively kill *M. tuberculosis* bacilli. We have shown that

when mycobacteria including *M. tuberculosis* H37Ra, and *M. tuberculosis* H37Rv are phagocytosed by human monocytes *in vitro*, the organisms remain intracellular, survive and replicate intracellularly (57,79,96). These observations indicate that human monocytes are not capable of eliminating these mycobacteria. Moreover, how T cells contribute to the elimination of mycobacteria *in vivo* is unclear. Indirect evidence suggests that $CD4^+$ T cells are involved in the anti-tuberculosis response in humans. For instance, the responsiveness of $CD4^+$ T cells to mycobacterial antigens appears to correlate inversely with disease progression among *M. tuberculosis*-infected individuals. In addition, HIV-infected patients are extremely susceptible to both primary and reactivated tuberculosis (35). Therefore, it is believed that $CD4^+$ T cells are primarily responsible for the production of Th1 type cytokines including IFN-γ which activate the mononuclear phagocytes to kill *M. tuberculosis* bacilli. To date, the demonstrated effects of IFN-γ treatment *in vitro* on *M. tuberculosis*-infected human mononuclear phagocytes vary widely, ranging from enhanced *M. tuberculosis* replication to inhibition of bacillary growth (55,97).

In his studies, Koch observed that many of the tubercle bacilli found in the "necrotized" lesions of infected guinea pigs were dead (207); bacillary death was attributed to the lack of oxygen and nutrients as a result of death of the host cells. Since that time, host cell death [necrosis and apoptosis] has

been considered important in the control of mycobacterial infection. I have shown here that perforin-induced necrotic death of the infected host *in vitro* had no effect on the viability of the intracellular mycobacteria. In addition, the absence of the perforin gene did not render mice more susceptible to either virulent or avirulent mycobacterial infection [chapter 3], confirming that perforin-induced necrosis does not play a role in the control of mycobacterial infection *in vivo*.

Apoptosis is another pathway leading to cell death and has been observed in both *M. tuberculosis*-infected and *M. leprae*-infected tissues (143,144). Apoptosis of epithelioid cells within a granuloma of *M. leprae*-infected tissue has been proposed to be critical for the healing of tuberculoid leprosy lesions (144). When the infected human monocytes undergo apoptosis following treatment with either ATP^{4-} [previous study from our laboratory] or low dose H_2O_2 [chapter 5] killing of the intracellular bacilli was observed. These findings suggest that apoptosis of the infected host cells may be involved in the control of mycobacterial survival. However, in the studies reported here, Fas-mediated apoptosis of MAI-infected human monocytes *in vitro* failed to induce killing of intracellular bacilli. The *in vivo* observations that Fas receptor defective mice responded to mycobacterial infection in the same way as control littermates confirmed the *in vitro* findings that Fas-mediated host cell death did not contribute significantly to the control of mycobacterial infection [chapter 4]. Thus, apoptosis of the infected host cells

per se does not have a role in the control of mycobacteria. Rather, apoptotic death of the infected host cells appears to be an epiphenomenon which occurs when ATP^{4-} or low dose H_2O_2 induces killing of intracellular mycobacteria. At present, it is not known how ATP^{4-} or H_2O_2 mediates the killing of intracellular mycobacteria. Since ATP^{4-} is involved in the generation of ROI [Figure 8-1], it is possible that treatment of the infected phagocytes with either ATP^{4-} or H_2O_2 produces a toxic ROI which mediates the killing of intracellular mycobacteria. This concept will be discussed below [Section E].

B. The role of cytotoxic lymphocytes in the protective response of mycobacterial infection *in vivo*

In studies *in vivo*, I examined the contribution of cell-mediated cytotoxicity to the protective response to mycobacterial infection. Previously, the contribution of cytotoxic lymphocytes [$CD8^+$ T cells, NK cells and $CD4^-CD8^-TCR\gamma\delta^+$ T cells] to the anti-tuberculosis response was not clearly understood. For instance, $CD8^+$ T cells had been implicated in resistance to *M. tuberculosis* infection because mice with a disruption in the β2-microglobulin [β2m] gene failed to develop functional $CD8^+$ T cells and succumbed to virulent *M. tuberculosis* infection much more rapidly than control mice (39). The contribution of $CD8^+$ T cells to the protective response was thought to

involve their cytotoxic activity. In addition, *in vivo* depletion of NK cells has been shown to enhance the multiplication of MAI in mice (208). However, it is not known whether the lytic activity of these cytotoxic cells confers any protection *in vivo* against mycobacterial infection.

In the studies reported here, cell-mediated cytotoxicity was not involved in the protective response in mice experimentally infected with mycobacteria. The loss of perforin gene or the absence of intact Fas pathway did not render mice more susceptible to infection with either virulent or avirulent mycobacteria [chapters 3 and 4]. Since cytotoxic cells including $CD4^+$ T cells, $CD8^+$ T cells, $CD4^-CD8^-\gamma\delta^+$ T cells, and NK cells destroy their target cells by either perforin-mediated or Fas-dependent cytotoxicity, these findings indicate that the contribution of "cytotoxic" cells to the anti-tuberculosis response appears to be independent of their cytotoxic activity. Previous studies reported that killer lymphocytes including $CD8^+$ T cells and $CD4^-CD8^-\gamma\delta^+$ T cells are capable of producing IFN-γ (209,40). Also, IL-12-stimulated NK cells was shown to produce soluble factors which can activate the anti-mycobacterial activity of human macrophages (210). Taken together, these findings suggest that the contribution of cytotoxic cells in murine tuberculosis infection may be mediated by the production of cytokines or by as yet unidentified molecules.

C. The role of human monocytes in mycobacterial infection

In vivo, a granuloma is characterized by the accumulation of differentiated myeloid cells including blood monocytes, tissue macrophages, epithelioid cells and multinucleated giant cells, surrounded by a mantle of lymphocytes [Figure 1-1]. Although it is not known whether each of these myeloid cells differs in the ability to control the growth of *M. tuberculosis* bacilli, it is believed that newly recruited blood monocytes are more capable of controlling the growth of *M. tuberculosis* than tissue macrophages. A constant influx of blood-derived monocytes is thought to maintain the integrity of the granuloma by phagocytosing the bacilli released from dead infected mononuclear phagocytes.

In the *in vitro* infection model described here, the ability of mycobacteria to replicate intracellularly appears to depend on the interplay between the infected host cells and the intracellular bacteria. In the present experiments, *M. tuberculosis* H37Ra began replicating immediately following infection of human monocytes *in vitro*. However, differentiation of human monocytes in culture prior to mycobacterial infection appeared to endow the phagocytes with the ability to restrict the intracellular growth of H37Ra [chapter 7]. In contrast to the hypotheses described above, the present findings indicate that aged monocytes are better able to control the replication of intracellular H37Ra than freshly explanted monocytes. The results suggest that the contribution of freshly recruited blood-derived monocytes to the

control of mycobacteria within a granuloma may not be limited solely to monocyte phagocytosis of bacilli released from dead infected host cells.

Freshly explanted human monocytes are known to have a more marked respiratory burst than differentiated macrophages (194). Thus, within the granuloma freshly recruited monocytes may also control the mycobacterial infection by producing H_2O_2 which mediates the killing of intracellular mycobacteria. In addition, human monocytes have been reported to contain a myeloperoxidase-like enzyme which disappears as they differentiate into macrophages (194). Previous studies have shown that ROI generated by myeloperoxidase/catalase-H_2O_2-halide were able to directly kill *M. leprae*, *M. tuberculosis*, and MAI (111,112,113). The myeloperoxidase activity of human monocytes may, therefore, be involved in the killing of mycobacteria within the granuloma.

D. The biological relevance of H_2O_2-induced killing of intracellular mycobacteria *in vitro*

In the *in vitro* infection model described here, H_2O_2 treatment of monocytes infected with a patient isolate of MAI resulted in an efficient killing [60%] of the intracellular bacilli while killing of intracellular *M. tuberculosis* H37Ra was less efficient [10%-30%] [chapter 6]. However, within a granuloma this low level of killing of H37Ra may be sufficient to control

H37Ra replication. For instance, within a 24 h period or an equivalent of 4 cycles of H_2O_2-induced killing of intracellular mycobacteria *in vitro*, a 30% bactericidal activity can theoretically reduce the number of viable H37Ra by 75%. Since H37Ra replicated in human monocytes with a doubling time of 36-96 h depending on the age of the monocytes in culture, this level of H_2O_2-induced killing may be sufficient to maintain the balance in favor of reducing the bacillary load, the ultimate determinant of the course of the infection. Whether the *in vitro* observations could account for the control of mycobacterial infection *in vivo* is a matter of speculation because such dynamics cannot be readily investigated *in vitro*. However, a hypothesis of H_2O_2-mediated control of mycobacterial infection is plausible since release of H_2O_2 has been documented during phagocytosis of mycobacteria by human monocytes. In a granuloma, cell turnover is a very dynamic process with a constant influx of blood-derived monocytes into the granuloma, replacing dead mononuclear phagocytes. During phagocytosis of the bacilli released by dead phagocytes, the newly recruited monocytes release ROI including H_2O_2. Since H_2O_2 diffuses freely through cell membranes, it is conceivable that the local accumulation of H_2O_2 within the granuloma can achieve a sufficiently high concentration capable of mediating the killing of intracellular mycobacteria. When the surviving mycobacteria begin to replicate, newly recruited monocytes locally release H_2O_2, reinitiating the cycle.

E. Potential oxidative molecules responsible for killing of intracellular mycobacteria in human monocytes

Identification of host cell molecules that mediate mycobactericidal effects of macrophages had been the focus of many investigations. In 1981, Walker and Lowrie discovered that H_2O_2 produced by lymphokine-activated murine macrophages were able to kill *M. microti* (211). Since then, many studies have reported the ROI generated *in vitro* are capable of killing mycobacteria including *M. tuberculosis*, MAI, and *M. leprae* (111,112,113). However, the mechanism responsible for the killing of intracellular mycobacteria in human mononuclear phagocytes has not been established.

This thesis demonstrated the killing of intracellular mycobacteria following H_2O_2 treatment of the infected human monocytes *in vitro* [chapters 5, 6, and 7]. In this model system, the killing of intracellular mycobacteria was independent of transcription of new genes, new protein synthesis, nitric oxide production, Ca^{2+}, Mg^{2+}, and Fe^{3+} . In addition, treatment of the infected cells with scavengers of ROI, besides catalase, failed to inhibit the killing of intracellular mycobacteria [Figure 8-1; chapter 8]. These observations suggest that H_2O_2 may either kill the intracellular mycobacteria directly or induce a secondary effector molecule, which is not sensitive to anti-oxidants, to mediate mycobacterial killing.

We favor the hypothesis that H_2O_2 induces an effector molecule which

is constitutively expressed within the infected host cell. Following treatment with exogenous H_2O_2, the putative molecule becomes activated and exerts its mycobactericidal activity. In the previous chapter, we hypothesized that this putative host cell molecule is probably an organic peroxide such as lipid peroxide. In fact, one previous study reported that the plasma membrane of liver cells from guinea pigs contains the strongest anti-mycobacterial activity when compared to lysosomal extracts, lysosomal membranes and cell extracts. In addition, there was a close correlation between the levels of anti-mycobacterial activity of various cell fractions and the amount of nonesterified fatty acids (203). Since mycobacteria derive their initial vacuoles from the plasma membranes of the mononuclear phagocytes, it is conceivable that H_2O_2-induced lipid peroxides may accumulate within the vacuolar membrane and exert their mycobactericidal activity locally [Figure 8-1].

Moreover, there was a close relationship between the growth rate of intracellular mycobacteria and their susceptibility to H_2O_2-induced killing [chapters 5, 6, and 7]. In the experiments described here, the ability to replicate intracellularly within the phagosomes appeared to be associated with resistance of the intracellular organisms to killing by H_2O_2 treatment. If lipid peroxides are indeed responsible for the killing of intraphagosomal mycobacteria, replication of the organisms within the phagosomes will, over time, dilute the concentration of toxic lipid peroxides per phagosome. This

may explain the finding that H37Ra, which replicated immediately following infection of freshly explanted monocytes and were observed in multiple vacuoles in the infected cells [Figure 6-3], were less susceptible to H_2O_2-induced killing than MAI which did not replicate appreciably in human monocytes [chapter 6]. In addition, 8 d old monocytes were better at controlling the replication of intracellular H37Ra. Moreover, the intracellular organisms are more susceptible to H_2O_2-induced killing [chapter 7], possibly because the limited replication may prevent the dilution of the putative toxic molecule. Therefore, it appears that the survival of intracellular mycobacteria is, in part, determined by the ability to replicate within the host cells.

At present, it is not clear what cytotoxic mechanisms determine the survival of intracellular mycobacteria in human cells and the nature of the cytotoxic molecules involved. Investigations are currently underway in our laboratory to characterize the putative host cell molecules which becomes reactive following H_2O_2 treatment of the infected host cells.

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