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The Course of *Mycobacterium tuberculosis* Infection in the Lungs of Mice Lacking Expression of Either Perforin- or Granzyme-Mediated Cytolytic Mechanisms

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CD8 T cells have been shown to be protective against *Mycobacterium tuberculosis* infections in the mouse. These cells have been shown to be cytolytic toward *M. tuberculosis*-infected cells and have also been shown to release the protective cytokine gamma interferon in response to mycobacterial antigen. It has therefore been unclear how these cells mediate their protective response. To dissect this problem, we compared the courses of *M. tuberculosis* infections in control, perforin gene-knockout, and granzyme gene-knockout mice exposed by the realistic pulmonary route. The inability to express either of these molecules limits the expression of the major lytic pathway but does not appear to influence the course of the infection or result in any discernible histologic differences. These data seem to rule against a lytic role for CD8 T cells in the lungs and hence tend to suggest instead that another type of mechanism, such as cytokine secretion by these cells, is their primary mode of action.

While the generation of specifically sensitized CD4 gamma interferon (IFN-γ)-secreting T cells is critical to the expression of acquired cellular immunity to infection with *Mycobacterium tuberculosis* in the mouse (5, 13, 31), there is increasing evidence that major histocompatibility complex class I-restricted CD8 T cells also play an important role, especially in the lungs. For instance, CD8 T cells from immune mice were shown to prolong the survival of irradiated syngeneic recipients exposed to acute infection by aerosol (32); more recently, mice in which the β2-microglobulin gene had been disrupted by homologous recombination were shown to suffer severe and fatal pathology in lungs following intravenous infection (14).

It is unknown what role CD8 T cells specifically play in the lungs, and why for that matter they seem less important in protecting other organs. Like CD4 T cells, they can recognize the secreted and export proteins of the bacillus and respond by secreting IFN (31). In addition, they can specifically lyse *M. tuberculosis*-infected macrophages (38), and we have therefore hypothesized that they may play a cytolytic function in the lungs, by lysing local epithelial or endothelial lung tissue cells into which the bacillus may have eroded, a fact that can be signaled to the immune response only by the use of class I major histocompatibility complex-encoded molecules (33, 34).

The primary cytolytic mechanism of lymphocytes involves the release of perforin (35)- and granzyme (18)-containing granules (11) upon contact with the target cell. Perforin is a complement-like molecule that polymerizes in the target cell membrane creating pores that allow the rapid influx of water and salts, causing the cell to swell and burst (28, 35). Granzymes are proteases which enter the cell through the perforin pore and augment the cell damage already induced (18); in addition, they are thought to be important in the induction of DNA fragmentation (30, 37).

In this study, we compared the courses of *M. tuberculosis* infections in control, perforin gene-knockout (PO), and granzyme B gene-knockout (GO) mice exposed by the realistic pulmonary route. The inability to express either of these molecules did not appear to influence the course of the infection or result in any discernible histologic differences. These data seem to rule against a lytic role for CD8 T cells in the lungs and hence tend to suggest instead that another type of mechanism, such as cytokine secretion by these cells, is their primary mode of action.

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MATERIALS AND METHODS

Mice. Male and female PO and PO control (P2) mice (40) were a kind gift of William R. Clark (University of California, Los Angeles). Male and female GO with their littermate controls (G2) (17) were purchased from the Jackson Laboratories, Bar Harbor, Maine. Both sets of knockout mice were generated by targeted gene disruption of embryonic stem cells (line AB-1 for PO and line D3 for GO) which were then introduced into C57BL/6 blastocysts. Heterozygous mice (+/−) were then bred to produce both homozygous knockouts (PO and GO) and wild types (P2 and G2).

Experimental infections. A virulent laboratory strain of *M. tuberculosis* (Erdman) was grown from a low-passage-number seed lot in Proskauer-Beck liquid media to mid-log phase, aliquoted, and frozen at −70°C. Mice were infected with approximately 100 bacteria, using a Middlebrook Airborne Infection apparatus (Middlebrook, Terre Haute, Ind.) as described previously (7). The numbers of viable bacteria in target organs were determined at various time points by plating serial dilutions of whole organ homogenates on nutrient Middlebrook 7H11 agar and counting bacterial colonies after 20 days of incubation at 37°C. The data are expressed as the $\log_{10}$ value of the mean number of bacteria recovered per organ ($n = 4$ animals).

Isolation of mRNA and detection of cytokine-specific message by reverse transcriptase PCR. Infected and control tissues were excised, placed in Ultraspec (Cinna/Biotecx, Friendswood, Tex.), and homogenized, and RNA was extracted as described previously (6). One microgram of total RNA was reverse transcribed, diluted, and subjected to PCR expansion of cytokine-specific cDNA. The amount of cytokine-related product was determined by the exposure of blotted cDNA PCR product to fluorescein-tagged cytokine sequence-specific probe. The fluorescein was detected with an enhanced chemiluminescence kit (Amersham, Arlington Heights, Ill.), which results in a light signal which can be detected on film. The number of cycles which generate a log-linear relationship between the signal on film and the dilution of the sample was determined empirically (6, 43), and data are expressed as the fold increase in signal in experimental points relative to the control value from the appropriate uninfected tissue. The significance of the fold increase is determined by an unpaired Student's *t* test comparing the means of the signals from control and infected tissue ($n = 4$ samples).

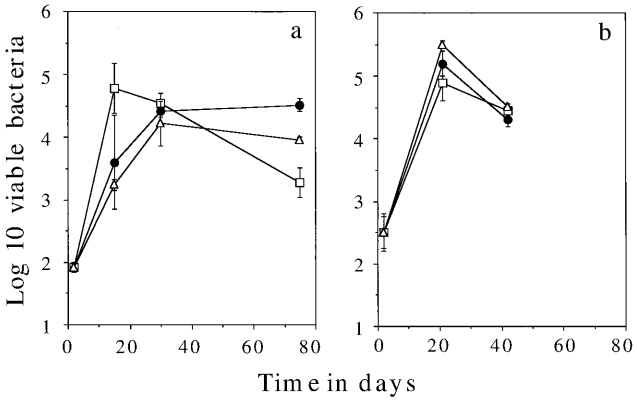

FIG. 1. Course of low-dose aerosol infection in PO and GO mice. PO (open squares) and GO (open triangles) mice were infected with either ~80 (a) or ~300 (b) bacteria (*M. tuberculosis* [Erdman]) in an aerosol cloud, and the number of viable bacteria present in the lung was enumerated throughout infection. Data for wild-type control mice (G2 and P2) were combined and are represented by closed circles. The values represent the means of four (eight in controls) animals ± standard errors.

Histological analysis. The lower right lobe of each mouse was inflated with 10% formal saline and blocked with the lobes from the other mice within the experimental group. Blocks were sectioned to allow the maximum area of each lobe to be seen, and sections were stained with hematoxylin and eosin. Slides were examined blind and analyzed for differences in the size of granuloma formation and the characteristics of cells within the granulomas.

Flow cytometric analysis. Thoracic lymph nodes were harvested from infected mice at various times and were made into single-cell suspensions. These were then stained and analyzed as described previously (15). Briefly, cells were stained with either fluoresceinated anti-CD4 (clone RM-4-5) or fluoresceinated anti-CD8 (clone 53-6.7) and counterstained with both biotinylated anti-CD45 (clone 16A) and phycoerythrin-conjugated anti-CD44 (clone IM7). The biotin was detected by using streptavidin Red-670 (Gibco-BRL, Grand Island, N.Y.). In addition cells were stained with phycoerythrin-conjugated anti-CD3 (clone 145-2C11) and counterstained with fluoresceinated anti-CD95 (clone Jo2). All antibodies were obtained from PharMingen (San Diego, Calif.). Cells were analyzed on a Coulter EPICS II flow machine.

RESULTS

Course of aerogenic *M. tuberculosis* infection in control and gene-disrupted mice. Several lines of evidence suggest a protective role for CD8 lymphocytes in the immune response to *M. tuberculosis* infection in the lung; however, the nature of this role has been unclear. To dissect the cytolytic activity of these T cells from their ability to produce protective cytokines, we infected mice whose lymphocytes are deficient in cytolytic activity. As the protective effect of CD8 cells is most pronounced in the lung, we used a low-dose aerosol infection route which has been previously well characterized (7). Levels of growth of bacteria in the lungs of both the control mice (P2 and G2 combined) and the cytolytically deficient mice (PO and GO) were similar in two different experiments (Fig. 1). The growth curves were similar to those already reported for C57BL/6 mice, and control of bacterial growth around day 20 correlates well with the upregulation of message for IFN-γ in the lungs of all three types of mice (Fig. 2). All three types of mice were able to maintain control of the infection for 75 days.

Histologic patterns in infected mice. Of special interest in this study was the nature of the granuloma formation. It was possible that absence of cytolytic activity could reduce tissue damage and therefore the severity of disease. We examined the lungs of mice throughout the course of infection and found very few differences in the nature of the granulomas. Very little inflammation was seen until day 30, when moderately sized accumulations of epitheloid macrophages and activated lymphoid aggregations were noted. These granulomas could be seen until the last time point at day 75 but did not increase in size in any of the mouse strains. One notable difference was observed in the reduced occurrence of the eosinophilic cells with fragmented nuclei, which represent apoptotic events in the granulomas from GO mice.

Lymphocyte activation in the infected mice. We were concerned that the removal of the perforin or granzyme gene may disrupt T-cell availability or function. Therefore, we determined the numbers of CD4- and CD8-positive CD3 cells and found that there were equivalent numbers in all animals tested and that the numbers of CD4 and CD8 cells increased as thoracic lymph nodes increased in size up to day 30 (data not shown). To dismiss the possibility that there was a defect in T-cell activation, we determined the numbers of both CD4 and CD8 cells which modulated their expression of the T-cell activation markers CD44 and CD45. CD4 cells which become activated downregulate expression of the CD45RB molecule and upregulate CD44 and are termed $CD44^{hi}$ $CD45^{lo}$ (15), while CD8 cells which become activated upregulate both CD44 and CD45 to become $CD44^{hi}$ $CD45^{hi}$ (8). Table 1 shows that the number of these activated cells per thoracic lymph node increases to day 30 in all three types of mouse.

In addition to T-cell activation, we were interested in the level of CD95 (Fas) antigen on the lymphocytes, as this reflects their ability to mediate non-perforin-mediated lysis of target cells. When we gated on CD3 cells from the thoracic lymph nodes of infected mice, we saw no upregulation of the Fas antigen (data not shown).

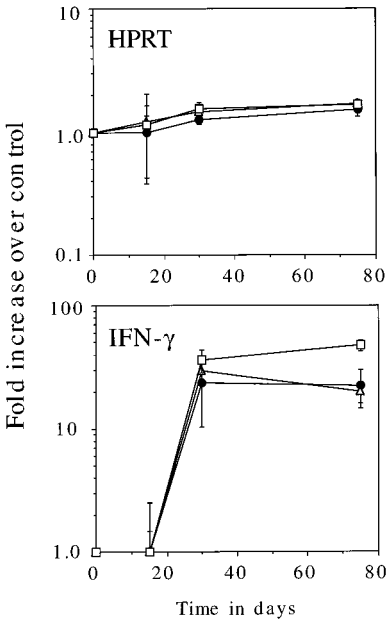

FIG. 2. Lung tissue was removed from mice infected as described for Fig. 1. Samples from four mice were analyzed for the presence of IFN-γ mRNA and hypoxanthine phosphoribosyltransferase (HPRT) mRNA by message-specific reverse transcriptase PCR. The signal from each of the four experimental mice was compared to the mean of the signal from the control mice, and the points on the graph represent the means and standard deviations of the fold increase in pixel signal of the experimental animals relative to uninfected control animals. The values for the gene-deficient mice were not significantly different from the values for the control mice. The data were obtained from experiment 1 (Fig. 1b) and are similar to the data from experiment 2.

TABLE 1. Mice which lack cytolytic mechanisms can generate activated T cells in response to infection[a]

Mouse group	Log_{10} no. of cells of the designated phenotype/organ on the indicated day					
	Activated CD4 T cells[b]			Activated CD8 T cells[c]		
	15	30	75	15	30	75
P2, G2	5.68	6.06	6.06	5.65	5.88	6.16
PO	5.81	5.96	5.76	5.74	5.93	6.00
GO	5.00	5.80	5.21	5.46	5.97	5.23

[a] Thoracic lymph nodes were pooled from four infected mice and analyzed by flow cytometry for the expression of surface markers. The statistical rationale is described in reference 15.
[b] $CD44^{hi}$ $CD45^{lo}$.
[c] $CD44^{hi}$ $CD45^{hi}$.

DISCUSSION

This study shows that disabling the primary mechanism of CD8 cytolytic T-cell activity by gene disruption does not influence the course of an *M. tuberculosis* infection in the lungs. This finding suggests that cytokine secretion may be the major mechanism of protective immunity expressed by CD8 T cells.

Although the primary function of CD8 T cells is usually described as their cytolytic activity, their ability to secrete cytokines plays an important role in modulating several diseases, including human immunodeficiency virus infection (4), listerial infection (41), *Rhodococcus aurantiacus* infection (1), toxoplasmosis (10), and chlamydial pneumonia (19, 29). The most important cytokine that these cells could produce in terms of a mycobacterial infection is probably IFN-γ, and indeed CD8 cells from *M. tuberculosis* can make this cytokine in an antigen-specific way (31). This ability to produce IFN-γ probably depends on the interleukin-12 (8) induced by *M. tuberculosis* infection of macrophages (6).

In addition to producing IFN-γ, CD8 cells have been shown to produce several other molecules which may influence tuberculosis infections. In particular, CD8 cells play a more noticeable role in the lungs than in other organs in both tuberculosis (32) and chlamydial infections (29), and this may be related to their ability to produce chemokines specific for CD4 T cells (2, 3, 9, 24). They may thereby aid in the accumulation of protective CD4 T cells in the lung. Their location at the periphery of the granuloma (27) and the diffuse granulomas seen in the lungs of β2-microglobulin-deficient mice (25) further support a possible role for CD8 cells as important mediators of granuloma formation in the lung.

There is, of course, a second major mechanism for the killing of target cells by lymphocytes which involves the tumor necrosis factor-related molecule Fas (CD95). The relative importance of the two distinct pathways in mediating protection from pathogens is still under debate; however, the greatly reduced cytolytic ability of perforin-deficient mice suggests that perforin pore formation is the major mechanism of killing used by CD8 T cells (22, 44). CD4 cytolytic T cells are more often associated with the CD95 pathway (16, 20, 26), and this mechanism is considered more important in control of activated immune cells (21, 39) than clearance of pathogens. The cytolytic mechanism mediated by CD95 involves the increased expression of both this molecule and its ligand on target cells and activated cytolytic cells; ligation of the molecules, after cell-cell contact, results in the induction of an apoptotic pathway (12, 22, 23). Our observation of a lack of induction of CD95 on the thoracic lymph node CD3 cells suggests that this mechanism is not important in the early stages of bacterial control. In addition, we have recently infected mice deficient in the CD95/CD95L pathway (*gld* mice) and have seen no increased bacterial growth in the lungs of mice exposed to aerogenic infection (34a). Our previous observations of CD4 cytolytic T cells from *M. tuberculosis*-infected mice (32) and the occurrence of apoptotic bodies in tuberculous granulomas suggest that the CD95 pathway may act not to control bacterial growth but conversely to control inflammation in tuberculosis.

Indeed, we were originally curious as to whether the cytolysis induced by perforin in the absence of granzymes would affect the type of inflammation and necrosis seen in the lungs. Granzymes are thought to be essential for the induction of apoptosis in the perforated cells (30, 36), and we did see a reduction in the occurrence of apoptotic bodies in the granulomas of the GO mice. The isolate used in these studies, however, induced limited inflammation in the lungs, and any differences in the nature of the inflammation between the PO and GO mice was difficult to see. Further studies using the much more inflammatory *M. tuberculosis* isolates which we have previously described (36) and mice deficient in the CD95 pathway may highlight a role for programmed cell death over necrosis in the control of tissue damage.

It may be that CD8 cells perform several minimal functions, the absence of all of which can lead to rapid death if a large enough challenge is given (14). If, however, only one component of CD8 function, i.e., cell lysis, is lost and a moderate challenge (such as described here) is faced, then the effect is insufficient to be detected. The role of CD8 cells remains somewhat enigmatic, and although our data strongly suggest that their true function in mediating protective immunity to *M. tuberculosis* is as producers of cytokines, their cytolytic abilities may function as a minor player in controlling inflammation.

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ADDENDUM IN PROOF

A recent publication in this journal reported very similar results with regard to the perforin knockout animal (P. Laochumroonvorapong, J. Wang, C.-C. Liu, W. Ye, A. L. Moreira, K. B. Elkon, V. H. Freedman, and G. Kaplan, Infect. Immun. **65:**127–132, 1997).

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