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Kinetics of Accumulation of γδ Receptor-Bearing T Lymphocytes in Mice Infected with Live Mycobacteria

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The kinetics of accumulation of T cells bearing the γδ heterodimer form of the T-cell receptor in mice infected with live *Mycobacterium bovis* BCG or *M. tuberculosis* was studied. Substantial numbers of γδ T cells accumulated in mice given primary mycobacterial infections, although this accumulation was in parallel to, but not preferential to, that of αβ receptor-bearing T cells. In contrast, no accumulation of γδ cells was observed in memory immune mice upon rechallenge, thus suggesting that γδ cells play no role in the anamnestic response. The results of the study show, further, that large accumulations of γδ T cells can also be induced by inoculation with oil adjuvant vehicles containing heat-killed mycobacteria, although not by inoculation of the heat-killed bacteria alone.

The initial phase of acquired protective immunity to *Mycobacterium tuberculosis* infection in mice is primarily mediated by $\alpha\beta^+$ $CD4^+$ $CD8^-$ cyclophosphamide-sensitive T lymphocytes which act to halt bacterial proliferation within infected macrophages and give rise to the onset of bacterial elimination (8). If the animal is later rechallenged with *M. tuberculosis*, accelerated reemergence of resistance to this challenge is mediated by $\alpha\beta^+$ $CD4^+$ cyclophosphamide-resistant memory T cells (9). Recently, however, in addition to $\alpha\beta^+$ T cells, a second population of $CD3^+$ T cells which use the γδ heterodimer form of the T-cell receptor have been identified (1). Among other functions, these cells have been implicated in immunity to mycobacterial infections. For example, γδ cells can be detected in increased numbers within mycobacterium-induced lesions (6), where they may be in the process of recognizing mycobacterial antigens, such as heat shock proteins (7) and perhaps even nonproteinaceous materials (10).

A variety of mycobacterial antigens have been used to stimulate γδ cells, but little information is available concerning the kinetics of emergence of this cell population in experimental models in which live infection has been used. In view of this, we document here the kinetics of accumulation of αβ and γδ T-cell receptor-bearing lymphocytes in the draining lymph nodes of mice infected with live mycobacteria. This report shows that while a substantial accumulation of γδ cells was seen in the primary cellular response to the infection, no accumulation was observed in mice undergoing a memory immune response to rechallenge. These data are interpreted as suggesting that γδ cells, even if sensitized to a specific antigen during the course of the primary infection, do not appear to share a role with αβ cells in the expression of the anamnestic response. In addition, this report shows the absence of preferential accumulation of γδ cells, following either subcutaneous or intraperitoneal inoculation of mycobacteria. Finally, data are presented that demonstrate that a large accumulation of γδ cells can also be induced by inoculation with dead mycobacteria in an adjuvant oil vehicle but that, contrary to a recent report (4), this accumulation appears to be in response to the oil vehicle itself, not the dead mycobacteria within it.

These experiments were performed by using specific-pathogen-free female C57BL/6 mice obtained from Jackson Laboratory, Bar Harbor, Maine. All mice weighed 20 to 25 g and were 6 to 8 weeks old at the start of the experiment. They were maintained under barrier conditions for the duration of the experiment. *M. tuberculosis* (strain Erdman) and *M. bovis* BCG (strain Pasteur) were originally obtained from the Trudeau Mycobacterial Collection, Saranac Lake, N.Y. Subcutaneous challenges consisted of inoculation of 5×10^6 live bacteria suspended in 33 μl of sterile phosphate-buffered saline into both hind footpads. Memory immune mice were generated via lateral tail vein injection of 10^5 viable *M. tuberculosis* organisms suspended in 200 μl of sterile phosphate-buffered saline. At 30 days postinfection, mice were exposed to chemotherapy consisting of administration of isoniazid (200 mg/liter) in drinking water for an additional 30 days (9). These mice were allowed to rest for a minimum of 10 days before subsequent subcutaneous challenges. In some experiments, heat-killed mycobacteria were emulsified into incomplete Freund's adjuvant (Sigma, St. Louis, Mo.).

Accumulation of cell types following infection was assessed by flow cytometry. On the days indicated, groups of three mice were euthanized and popliteal lymph nodes were aseptically removed, passed through fine-mesh screens, washed twice, and suspended in Dulbecco's minimal essential tissue culture medium. Single cell suspensions were incubated for 30 min at 4°C with a biotinylated anti-αβ T-cell receptor (TCR) monoclonal antibody (H57-597.2; a kind gift of Ralph Kubo) or an anti-γδ TCR monoclonal antibody (403 A.10.2; Pharmingen, San Diego, Calif.). The cells were then washed three times and incubated with phycoerythrin-conjugated strepavidin (Tago Inc., Burlingame, Calif.) and fluorescein isothiocyanate-conjugated anti-CD3 (145-2C11; kindly provided by J. Bluestone) for 30 min at 4°C. Cells were washed three times and analyzed on an EPICS Profile II flow cytometer (Coulter Electronics, Hialeah, Fla.). The fluorescenceseeninnegativecontrolsincubatedwithphycoerythrin-conjugated streptavidin alone was not significantly different from that seen with cells alone. Numbers of cells per lymph node were calculated by multiplying the percent-

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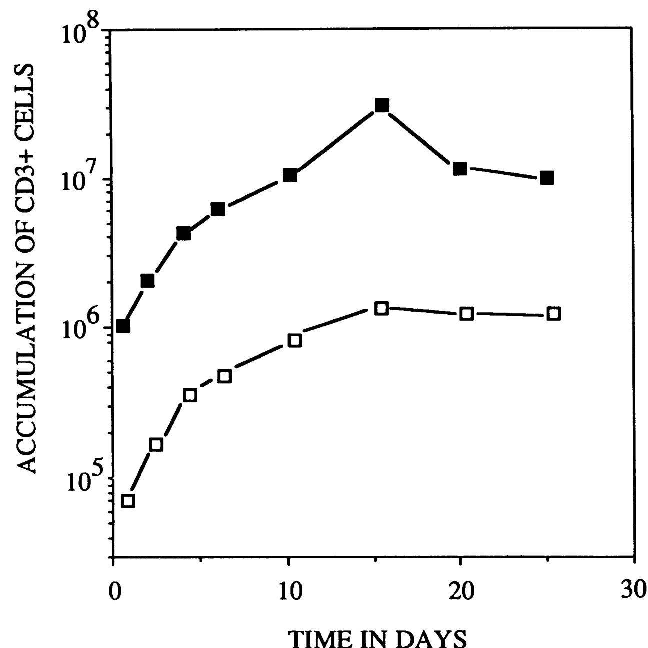

FIG. 1. Kinetics of accumulation of $CD3^+$ T cells bearing either αβ (■) or γδ (□) T-cell receptors in the draining lymph nodes of mice subcutaneously infected with *M. bovis* BCG. The data shown are mean numbers of lymphoid cells per lymph node ($n = 12$). Standard error bars have been omitted; they did not exceed 11% of the mean.

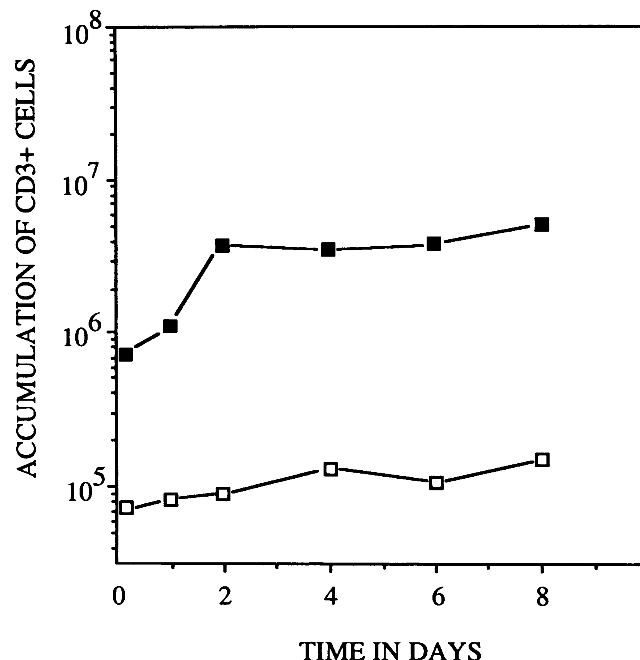

FIG. 2. Kinetics of accumulation of $CD3^+$ T cells bearing either αβ (■) or γδ (□) T-cell receptors in the draining lymph nodes of mice subcutaneously rechallenged with BCG following a primary infection with *M. tuberculosis* and a course of isoniazid chemotherapy. The data are expressed as in Fig. 1. The sharp increase in αβ numbers is a reproducible event seen in three separate experiments.

age of cells staining positive for two antigens by the total number of cells harvested per node.

Because the numbers of cells harvested from the draining lymph nodes were relatively low, six mice were harvested at each indicated time point, yielding 12 nodes. The cell suspensions prepared from these nodes were pooled, and the data presented represent the mean number of cells bearing a given marker per individual node. In a statistical analysis of the data, the variability of the flow cytometric counting technique (standard error) was calculated as 3% of the mean for αβ cells and 6% for γδ cells within samples ($n = 4$) obtained from individual mice and 11% for αβ cells and 10% for γδ cells when data for separate mice ($n = 4$) were compared.

Accumulation of $CD3^+$ T cells in the draining popliteal nodes of naive mice following inoculation with BCG is shown in Fig. 1. Both the $CD3^+$ $\alpha\beta^+$ and $CD3^+$ $\gamma\delta^+$ populations increased progressively, in essentially parallel fashion, with peak numbers observed on day 15 in both cases.

In contrast, very different patterns of accumulation were observed following rechallenge of mice rendered memory immune by *M. tuberculosis* infection followed by isoniazid chemotherapy (Fig. 2). In the case of $CD3^+$ $\alpha\beta^+$ cells, a rapid sixfold increase in T-cell numbers was observed in the first 2 days postchallenge, followed by a plateau in cell numbers. Within the population staining $CD3^+$ $\gamma\delta^+$, however, only a marginal rise in cell numbers was observed over the 8 days of the experiment.

In a second series of experiments, accumulation of $\gamma\delta^+$ cells following injection of dead bacteria in an adjuvant oil vehicle was compared with accumulation following injection of live bacteria, given an earlier report (4) that the former procedure results in a large accumulation of such cells. It was found (Fig. 3) that while the dead-bacterium–oil adjuvant combination gave a substantial accumulation of γδ cells, almost identical numbers were observed when the oil adjuvant vehicle was given alone. Live bacteria gave a more modest accumulation, while dead bacteria alone were ineffective compared with saline-inoculated controls.

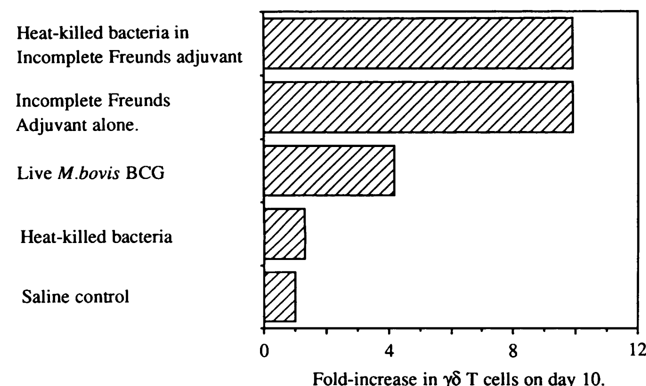

FIG. 3. Relative increase in $CD3^+$ γδ T-cell numbers in the draining lymph nodes of mice inoculated in the footpad 10 days earlier with the materials indicated to the left. The data shown were calculated from the mean number of cells per lymph node of each experimental group ($n = 12$). The saline control value was defined as 1.0 (no increase); these mice had a mean value of 7×10^4 cells per node on day 10. An emulsified form of incomplete Freund's adjuvant was injected into mice either alone or with 5×10^6 heat-killed BCG strain Pasteur organisms.

TABLE 1. Accumulation of $CD3^+$ T cells in the peritoneal cavity

Mice	Total no. of cells in washout (10^6)	% CD3 cells	% of total no. of cells expressing:	
			CD3 αβ cells	CD3 γδ cells
Naive	2.05	63.4	57.9	5.5
Infected	2.12	65.2	61.3	3.9

Finally, the data obtained following subcutaneous inoculation were compared with events following intraperitoneal inoculation. This was performed in view of the recent observation (3) of a rapid and preferential accumulation of $\gamma\delta^+$ cells in the peritoneal cavity following injection of mycobacteria, while in the present study no such trend was observed after subcutaneous inoculation. It was found (Table 1) that no significant infiltration of mononuclear cells occurred in the 7 days following inoculation with 10^5 *M. tuberculosis* organisms and the relative percentages of αβ and γδ cells did not change to any appreciable extent.

These data thus confirm that γδ cells accumulate within sites of mycobacterial infection. In the present subcutaneous-infection model, a steady accumulation of γδ cells was observed, essentially in parallel with the much larger αβ response. Thus, on the basis of this evidence alone, it can be hypothesized that the accumulating γδ T cells contribute to expression of the primary cellular response.

On the other hand, however, the data also clearly indicate that such cells do not appear to possess an anamnestic response following rechallenge of memory immune mice. A rapid increase in αβ cells was observed in such mice, but no increase at all in γδ cells was seen over the same time period. One explanation for this could be that αβ memory cells reemerge at a faster rate and quell the secondary infection before γδ memory cells are triggered; another could be that antigens recognized by γδ memory T cells are not presented very early during secondary infection. However, a more recent and perhaps more likely explanation is that γδ cells do not possess a memory component because they undergo apoptosis following their initial encounter with an antigen, as recently suggested by the findings of Janssen and colleagues (5).

In a second series of experiments, accumulation of $\gamma\delta^+$ cells following injection of dead bacteria in an adjuvant oil vehicle was compared with that following injection of live bacteria, given an earlier report (4) that the former procedure results in a large accumulation of such cells. It was found, however, that accumulation of γδ cells in the draining nodes was a consequence of the oil vehicle itself, not the dead mycobacteria (Fig. 3). The reasons for these discordant results are unclear but may include the mouse strain used (B10.A versus C57BL/6 in the present study), the stimulus (heat-killed strain H37Ra versus BCG), or the ingredients (such as emulsifiers) in the adjuvant stock used. Whatever the reasons, however, the results of the present study show that inocula of dead bacteria alone do not induce accumulation of γδ cells to lymphoid tissue draining the site of implantation. In this regard, this finding is consistent with a recent report on a study of human T-cell response (2) in which a live infection was needed to expand the γδ T-cell population.

The data obtained following subcutaneous inoculation were compared with those on events following intraperitoneal inoculation. This comparison was performed in view of the recent observation (3) of a rapid and preferential accumulation of $\gamma\delta^+$ cells in the peritoneal cavity following injection of BCG, while in the present study no such trend was observed after subcutaneous inoculation. Similarly, in the experiments reported here, in which an injection of virulent *M. tuberculosis* was used as the stimulus, no significant infiltration of mononuclear cells occurred in the 7 days following inoculation and the relative percentages of αβ and γδ cells did not change to any appreciable extent. While these findings are not consistent with those of Inoue et al. (3), we do not regard our results as surprising. It has been known for many years that an intraperitoneal injection of mycobacteria or other intracellular parasites is usually quickly ingested by resident macrophages, many of which pass within hours into the draining thoracic lymph node chain. Those that are not immediately ingested quickly pass into the bloodstream, from which they are then removed by phagocytic cells in the spleen and liver, thus giving rise 10 to 20 days later to a population of circulating sensitized T cells. We have no explanation for the observation of Inoue et al. (3) that T cells, 26% of which were $\gamma\delta^+$, accumulated in the peritoneal cavity 7 days after local inoculation.

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