Memory T Cell-Mediated Resistance to Mycobacterium tuberculosis Infection in Innately Susceptible and Resistant Mice

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Inbred mouse strains have been designated as naturally susceptible (NS) or naturally resistant (NR) on the basis of the growth patterns of Mycobacterium bovis BCG seen in the spleens of minimally infected mice (5). The list of mouse strains which fit these two categories has been expanded to include infections with various mycobacterial species (1). Analysis of the innate immune response to BCG infection in the NR mice suggested that the poor response was largely due to limited growth in the spleen by the immunizing agent (14). On the other hand, Mycobacterium tuberculosis showed similar growth patterns in both NS and NR mice (18), and the resulting T cell-mediated resistance was cross-protective against infections caused by other mycobacterial species, regardless of the genetic background of the mouse strain (15, 16). In addition, such mycobacterial infections have been shown to nonspecifically activate macrophages which express enhanced tumoricidal, leishmanicidal, and bactericidal activities against a number of unrelated organisms (11, 19).

Development of a persistent memory immune response must be the goal of any rational immunization procedure. The presence of such cells in tuberculous mice can be demonstrated by means of prolonged antibiotic therapy, which eliminates the stimulating infection from the tissues without affecting the ability of the splenic T cells to protect the host against reinfection (15). Most of these experiments have been carried out in B6D2 (NR) mice, and relatively little is known about the ability of NS mice to generate a memory T cell-mediated immune response against a virulent Mycobacterium tuberculosis infection. Thus, the purpose of the present study was to compare the development of memory T cell-mediated immunity in NS versus that in NS mice after a sublethal Mycobacterium tuberculosis infection. The phenotype (L3T4* or Lyt2*) and the cyclophosphamide (CY) sensitivity of the T cells harvested from both mouse strains and the effect of depletion of L3T4* or Lyt2* T cells on the memory immune response were compared in susceptible versus resistant mouse strains and were shown to vary substantially.

MATERIALS AND METHODS

Mice. Six- to 8-week-old male C57BL/6 (NS) and (C57BL/6 × DBA/2)F1 hybrid (NR) B6D2 mice were obtained from the Trudeau Institute Animal Breeding Facility, Saranac Lake, N.Y. These mouse strains vary genetically at several loci, including those for innate resistance and H-2 haplotype. The mice were raised under barrier conditions and given sterilized food and acidified water ad libitum (12).

Organisms. Mycobacterium tuberculosis Erdman was obtained from the Trudeau Mycobacterial Collection, Saranac Lake, N.Y. The organisms were grown and stored at −70°C as previously described (2). The viability of the suspension was determined by thawing randomly selected ampoules and dispersing the bacteria by brief exposure to sonic oscillation before diluting and plating on Middlebrook 7H11 agar (Difco Laboratories, Detroit, Mich.). Bacterial colonies were counted after incubating the plates in sealed plastic bags at 37°C for 21 days.

Animal inoculation. A frozen ampoule of M. tuberculosis was thawed, sonicated briefly, and diluted appropriately in saline. Approximately 10⁵ CFU in 0.2 ml of saline was injected intravenously via a lateral tail vein (2). In the adoptive transfer studies, recipient mice were inoculated 1 day after receiving an infusion of splenic T cells. Growth of Mycobacterium tuberculosis in the spleens and lungs of infected mice was determined at weekly intervals by homogenizing the organs from five randomly selected mice in sterile saline and plating the 10-fold dilutions onto 7H11 agar. The limit of detection was 50 CFU for the spleen and 100 CFU for the lung.

Antibiotic therapy and memory T cells. The antibiotics were administered in the drinking water, as a 0.2% isoniazid (Sigma, St. Louis, Mo.)-0.1% rifampin (Merrill Dow Pharmaceuticals, Cincinnati, Ohio) solution. The mice drank an average of 6 ml of water per day, thus ingesting approximately 15 to 18 mg of drug daily (3). Antibiotic treatment was initiated 2 weeks after infection, and viable Mycobacterium tuberculosis could no longer be recovered from the spleens after 2 weeks of treatment or from the lungs after 6 weeks. Cortisone treatment confirmed the elimination of viable organisms from the tissues (15). Memory T cells were harvested from
donor animals 1 month after completion of the antibiotic treatment.

Adoptive transfer of acquired resistance by using splenic T cells. Single-cell suspensions were prepared from the spleens of donor mice by pressing the tissue through a stainless steel screen into RPMI 1640 medium (GIBCO, Grand Island, N.Y.) containing 10% heat-inactivated fetal calf serum (GIBCO), 1 mM sodium pyruvate, 2 mM L-glutamine, 10 mM HEPES (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid), and $5 \times 10^{-5}$ M 2-mercaptoethanol but not containing antibiotics (12). The cell suspensions were enriched for T cells by a double adherence protocol. To remove the macrophages, 20-ml aliquots of the cell suspension (10$^6$ cells per ml) were incubated for 45 min at 37°C in 10% CO$_2$-enriched air on tissue culture dishes (150 by 25 mm; Falcon) which had been coated with the antimacrophage monoclonal antibodies F4/80 and Mac-1 (13). Nonadherent cells were removed by gentle rinsing with fresh medium and were incubated for 30 min at 37°C on plates coated with 20 µg of goat anti-mouse immunoglobulin G (Cappel Laboratories, Cochranville, Pa.) per ml to allow the adherence of immunoglobulin-bearing cells. The T cell-enriched nonadherent cells were rinsed off, washed in cold complete medium, and resuspended in 0.01 M phosphate-buffered saline containing 1% fetal calf serum, and standardized to a concentration of $2 \times 10^6$ viable cells per ml (15).

The splenic T cells were infused into sublethally irradiated (500 rads of gamma radiation given 24 h earlier) syngeneic recipient mice at one-spleen equivalent dose. In some experiments, antibiotic-treated donor mice were also pretreated with 150 mg of CY (Cytoxan; Mead-Johnson, Evansville, Ind.) per kg of body weight by intraperitoneal injection 1 day prior to the transfer (10). Other mice were pretreated intravenously with 500 µg of anti-L3T4 (GK1.5) and/or anti-Lyt2 (TIB 210) monoclonal antibodies 1 day prior to transfer. In vivo T cell subset depletion by this method was preferred because it utilized the mouse’s complement system to lyse the relevant antigen-bearing T cell subsets prior to each transfer. Recipient mice were challenged intravenously 1 day later with $10^6$ CFU of M. tuberculosis. A fluorescence-activated cell sorter (FACS) was used to analyze the T cell subsets present in the recipient mice 2 days after the T cell transfer to confirm the depletion of the appropriate T cell subsets (9).

Delayed-type hypersensitivity (DTH). M. tuberculosis-infected mice were injected in the left hind footpad with 0.025 ml of saline containing 10 µg of purified protein derivative (PPD) (Evans Medical Ltd., Langhurst, England). Saline alone was injected into the contralateral footpad, and the footpad thickness was measured with dial-gauge calipers at 3, 24, and 48 h (3).

Statistics. Significant differences between test mice and controls, both in the number of viable M. tuberculosis per organ and in the footpad swelling following injection with PPD, were determined by Student’s t test using a Statview 512 program and a Macintosh SE computer.

RESULTS

Growth of M. tuberculosis in susceptible or resistant mice. In order to determine whether the innately susceptible C57Bl/6 and resistant B6D2 mice varied significantly in their abilities to control the growth of a fully virulent mycobacterial challenge, groups of normal mice were infected intravenously with approximately $10^6$ CFU of M. tuberculosis Erdman, and the number of viable organisms in the spleens and lungs was determined over an 8-week period (Fig. 1). Logarithmic growth of the inoculum occurred in the spleens of mice of both strains during the first 2 weeks of the infection, and in C57Bl/6 mice, this was followed by a decline in the number of viable bacilli recovered from the spleens over the next 6 weeks of infection. The number of organisms in the spleens of B6D2 mice continued to increase for 2 weeks further before beginning a gradual decline. The infection increased in the lungs of both mouse strains over the entire 8-week period. Both strains of mice developed substantial tuberculin reactivity (4 weeks), but this reactivity had declined to nonsignificant levels by 8 weeks (Fig. 1).

Groups of C57Bl/6 and B6D2 mice which had been infected 8 weeks previously were used as donors of “immune” T cells, which were infused into irradiated syngeneic recipients. Control mice were infused with an equivalent number of normal T cells. Acquired resistance was measured as a reduction in the growth of M. tuberculosis in the recipients of immune T cells compared with growth in recipients of normal T cells (it should be noted that the infection develops more rapidly in the irradiated mice). The recipients of immune T cells from either test strain showed substantial resistance 2 weeks postinfecution (Fig. 1). The cells also transferred a barely significant level of tuberculin hypersensitivity to the recipient host, measured 1 week postinfection, compared with the recipients of normal T cells.

Transfer of acquired resistance to M. tuberculosis by memory T cells. Equal numbers of T cells were transferred from both mouse strains after a 6-week regimen of antibiotic therapy which effectively eliminated the immunizing infection (Fig. 2 and 3). Memory T cells acquired from the spleens of these treated donor mice were transferred into syngeneic irradiated recipients, and they induced substantial protection
against a fully virulent challenge given to both recipient mouse strains, compared with growth in the corresponding control mice (Fig. 2 and 3). However, the memory immune T cells harvested from the C57BL/6 donors conferred less (though still significant) acquired resistance to the recipient mice (Fig. 2) than the B6D2 memory T cells did (Fig. 3). The resistance indices for the lungs were always significantly lower than those for the spleens at 4 weeks postinfection.

When the donors were pretreated with CY 1 day prior to the transfer, this resistance was completely ablated in the C57BL/6 recipients (Fig. 2) but not in the B6D2 mice (Fig. 3). The effect of CY treatment of the C57BL/6 mice was shown to be dose dependent, but even the highest dose of CY was unable to ablate the memory T cell response in the B6D2 mice (Table 1). This differentiated effect was observed in repeated experiments and indicates a clear difference in the CY sensitivity of memory T cells present in the NS and NR hosts.

Transfer of tuberculin hypersensitivity by memory immune T cells from both B6D2 and C57BL/6 mice was also highly susceptible to CY treatment (Fig. 2 and 3). Treatment of immune donor mice with CY ablated the ability of the recipient mice to elicit a DTH response to PPD, whereas the recipients of untreated memory immune T cells exhibited a significant level of tuberculin hypersensitivity 1 week post-transfer.

Effect of T cell subset depletion on the transfer of immunity. The phenotype of the memory T cells was determined by in vivo depletion of L3T4+ and/or Lyt2+ T cell subsets in the donor mice prior to the transfer of T cells into syngeneic recipients. There was no substantial reduction exhibited in the level of acquired resistance after depletion of either T cell subset (Table 2). However, when the donor mice were treated with both anti-L3T4 and anti-Lyt2 antibodies, resistance was reduced to near control levels. A FACS analysis carried out on the T cell populations in the recipient mice 2 days after the adoptive transfer indicated a 90 to 95% depletion of L3T4+ T cells and a 89 to 94% depletion of the Lyt2+ T cell subsets, compared with animals in undepleted immune recipients (Table 2). Depletion of both L3T4+ and Lyt2+ T cells markedly reduced the number of Thy1.2+ cells detected in recipient mice (87% depletion), whereas recipients of control cells showed a normal distribution of T cell subsets (data not shown).

T cell depletion also affected the ability of the recipient mice to express a tuberculin response. The DTH response in the recipient mice was substantially reduced by depletion of L3T4+ T cells in the immune donor before transfer to the recipient animals (Table 3). On the other hand, depletion of donor Lyt2+ cells slightly enhanced the DTH response seen in the B6D2 mice (Table 3). As expected, treatment of the donor mice with both anti-L3T4 and anti-Lyt2 antibodies eliminated the ability of the recipient mice to express tuberculin responsiveness (Table 3).

**DISCUSSION**

The present study indicates that both C57BL/6 and B6D2 mice are capable of developing memory T cell populations able to mediate effective antituberculous resistance to a subsequent virulent challenge. Thus, when memory immune T cells were transferred to naive syngeneic recipients, both test groups resisted the rapid growth of the M. tuberculosis
challenge which normally would lead to the death of nonimmune animals. The difference in growth of *M. tuberculosis* in recipients infused with immune versus normal T cells was especially evident during the first 2 weeks of the infection (Fig. 2 and 3). Of particular interest was the finding that the innate susceptible C57BL/6 mice were still able to generate a memory T cell population which effectively stopped the growth of the virulent challenge in a manner little different from that of the B6D2 mice. Thus, the innate susceptibility of the C57BL/6 mice to BCG infection (6) does not negate the ability of this host to develop a protective memory immune response against *M. tuberculosis*, provided that the host is appropriately vaccinated.

The genetic differences at the *Bcg* gene locus, which segregates the mouse strains into NR and NS categories, were based on early proliferation of BCG in the tissues, which in turn depends upon the ability of the macrophages to limit the growth of the intracellular mycobacteria (1). These macrophage functions also appear to be important in the expression of resistance to other acute infections caused by rapidly proliferating intracellular organisms such as salmonellae or listeriae (17, 18). Although the infected macrophage is ultimately the effector cell in the innate immune response to *M. tuberculosis* infection, this response requires a T cell-mediated response in order to fully protect the host (4). This is also true for the memory cell response, which has been clearly shown to depend on T cells bearing the Thy1.2 surface marker (10). The present study indicates that both L3T4+ and Lyt2+ T cells may be involved in this memory immune response, a finding which is consistent with earlier data obtained with *M. tuberculosis*-infected CBA and B6D2 (NR) mice (8, 9), and indicates a role for L3T4+ and Lyt2+ T cells in protection against virulent challenge. Recently, Orme reported that memory T cells which were generated in B6D2 mice in response to an *M. tuberculosis* infection were L3T4+ and that the Lyt2+ T cells were not capable of transferring resistance to irradiated recipient mice (11). This difference from the present results (which show that both T cell subsets were involved in the expression of memory) may be due to technical differences in the methodologies, such as the length and type of the chemotherapy (42 versus 90 days and isoniazid combined with rifampin versus isoniazid alone), as well as the time interval between infusion of the recipient mice and the challenge (1 day versus 1 h) and the mode of T cell subset depletion (in vivo versus in vitro monoclonal antibody treatment). However, depletion of Thy1.2+ T cells (11) and depletion of both L3T4+ and Lyt2+ T cells (Fig. 2 and 3) both ablated the ability of these cells to transfer T cell-mediated memory immunity.

Immune T cells harvested from both B6D2 and C57BL/6 mice transferred similar levels of tuberculin hypersensitivity to the recipient mice, a finding which is in contrast to previous reports that only the innately susceptible mice developed tuberculin responsiveness following infection with BCG (6). This difference may be due to the use of a virulent strain which resulted in substantial growth in the spleens of mice of both strains (Fig. 1). This hypersensitivity was ablated by CY treatment of the donor mice (Fig. 2 and 3), suggesting that the T cells responsible for this response (L3T4+ [Table 3]) were different from those transferring memory T cell immunity (13, 20). The fact that CY treatment of the immune mice had different effects on the memory T cell populations in the two mouse strains was unexpected (Fig. 2 and 3) and may indicate that the memory response in the C57BL/6 mice does not enter a quiescent state (21), possibly because of the presence of a nuidus of infection (or bacterial products) in the innately susceptible strain which serves as a T cell-stimulatory antigen long after the antibiotic treatment has apparently eliminated the infection (7). Another possibility is that the C57BL/6 mice are more susceptible to the toxic action of CY than are the more tolerant B6D2 mice (Table 1). Further evaluation of the memory T cell populations in these genetically distinct mouse strains in terms of their relative resistance to CY cytotoxicity would seem to be appropriate.

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**TABLE 2. Percent T cell subset depletion and \( \log_{10} \) CFU per spleen in immune T cell recipient mice 2 and 4 weeks postinfection with *M. tuberculosis* a**

<table>
<thead>
<tr>
<th>T cell treatment</th>
<th>C57BL6</th>
<th>B6D2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Depletion(^b)</td>
<td>CFU/spleen at wk:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Normal</td>
<td>5.9 ± 0.3</td>
<td>6.3 ± 0.1</td>
</tr>
<tr>
<td>Immune</td>
<td>5.2 ± 0.3(^**)</td>
<td>4.9 ± 0.1(^**)</td>
</tr>
<tr>
<td>LYT2+-depleted immune</td>
<td>90</td>
<td>5.1 ± 0.1(^*)</td>
</tr>
<tr>
<td>L3T4+-depleted immune</td>
<td>91</td>
<td>5.3 ± 0.1(^*)</td>
</tr>
<tr>
<td>LYT2+ and L3T4+-depleted immune</td>
<td>91, 90</td>
<td>5.5 ± 0.3</td>
</tr>
</tbody>
</table>

\(^a\) Asterisks indicate a statistically significant difference between the mean CFU per ml in the spleens of immune T cell recipient mice versus that in the spleens of normal T cell recipient mice. \(*, P < 0.05; **, P < 0.01 (n = 5).\)

\(^b\) Percent depletion of L3T4+ and/or Lyt2+ T cells in recipient mice 2 days after the adoptive transfer of immune T cells from untreated donors or from donors depleted in vivo of specific T cell subsets 1 day before the adoptive transfer.

\(^*\) Mean CFU per spleen ± standard error of the mean.

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**TABLE 3. DTH in *M. tuberculosis*-infected mice 1 week after the adoptive transfer of normal, immune, or subset-depleted immune T cells into recipient mice a**

<table>
<thead>
<tr>
<th>T cell treatment</th>
<th>C57BL6</th>
<th>B6D2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0 ± 0.6</td>
<td>1.0 ± 0</td>
</tr>
<tr>
<td>Normal</td>
<td>2.5 ± 0.6(^*)</td>
<td>2.5 ± 0(^*)</td>
</tr>
<tr>
<td>Immune</td>
<td>2.5 ± 0.6(^*)</td>
<td>2.5 ± 0.5(^*)</td>
</tr>
<tr>
<td>LYT2+-depleted immune</td>
<td>1.5 ± 0.5(^*)</td>
<td>1.0 ± 0</td>
</tr>
<tr>
<td>L3T4+ and Lyt2+ depleted immune</td>
<td>1.5 ± 0.5</td>
<td>1.0 ± 0.8</td>
</tr>
</tbody>
</table>

\(^a\) DTH values are expressed as 0.1-mm differences between the thicknesses of hind footpads injected with 10 \(\mu\)g of PPD and contralateral footpads injected with diluent (pyrogen-free saline) alone. \(^*\) statistically significant increase in footpad thickness (\(P < 0.05\)) (n = 5). All values are expressed as mean ± standard error of the mean.
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