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NOTES

Mouse T Lymphocytes That Express a $\gamma\delta$ T-Cell Antigen Receptor Contribute to Resistance to *Salmonella* Infection In Vivo

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Mice depleted of lymphocytes expressing the $\alpha\beta$ or the $\gamma\delta$ T-cell receptor for antigen (TCR) by antibody treatment were infected orally with *Salmonella enteritidis*. In both groups of treated mice, the 50% lethal dose decreased, suggesting that both the $\alpha\beta$ TCR$^+$ and the $\gamma\delta$ TCR$^+$ subsets contribute to resistance to oral infection. These data provide further evidence for the contribution of $\gamma\delta$ T cells in the response to bacterial infections.

T lymphocytes can be categorized into two subsets on the basis of their receptors for antigen. In mice, lymphocytes bearing a T-cell antigen receptor (TCR) composed of α and β chains are the predominant subset throughout the peripheral lymphoid system. In contrast, T lymphocytes bearing a TCR composed of γ and δ chains are relatively rare in the peripheral lymphoid tissues, 1 to 5% of the total, but are concentrated in various epithelia, including the intestinal lining, that are in contact with the external environment (1, 16). In most cases, the specificity of $\gamma\delta$ T-lymphocyte populations remains uncharacterized. However, a subset of mouse $\gamma\delta$ T cells has been shown to respond to the 65-kDa heat shock protein produced by *Mycobacterium tuberculosis*, and these T cells also are cross-reactive with the mouse homolog of this protein (2).

Until very recently, the importance of $\gamma\delta$ T cells in resistance to infection had not been demonstrated. Data from several experiments, however, indicate an important role for $\gamma\delta$ T cells, particularly in the response to *Listeria monocytogenes* (8, 14, 18, 21; reviewed in reference 11). To further investigate the role of $\gamma\delta$ T cells, we studied the survival of mice orally infected with a virulent strain of *Salmonella enteritidis*. The oral route of infection employed in these experiments is similar to the most common route in humans, via ingestion of contaminated food.

To study the role of the $\alpha\beta$ TCR$^+$ and $\gamma\delta$ TCR$^+$ T-cell subsets in resistance to oral infection with *S. enteritidis*, mice were treated with anti-TCR monoclonal antibodies (MAbs) in vivo. This has been shown to be an effective method to deplete TCR subsets and to inhibit T-cell responses in a variety of studies, including studies of autoimmune disease models (6), antitumor responses (3), and bacterial infections (15). By studying mice treated in this way, we confirm that both $\alpha\beta$ TCR$^+$ and $\gamma\delta$ TCR$^+$ subsets can be depleted following MAb treatment and that both subsets play an important role in the resistance to oral infection with *S. enteritidis*.

Specific-pathogen-free female BALB/c mice, 6 to 8 weeks of age, were purchased from Charles River, Inc. (Wilmington, Mass.), and housed at the University of California, Los Angeles, for at least 1 week before use. Hybridoma H57-597, which secretes an anti-$\alpha\beta$ TCR MAb (12), was a gift from Ralph Kubo (National Jewish Hospital, Denver, Colo.). Hybridoma UC7-13D5 (described in reference 9), which secretes an anti-$\gamma\delta$ TCR MAb, was a gift from Jeff Bluestone (Ben May Institute, Chicago, Ill.). Antibodies were purified by protein A gel chromatography (Sigma Chemicals, St. Louis, Mo.) or fast protein liquid chromatography with protein A (Pharmacia, Piscataway, N.J.) and dialyzed in phosphate-buffered saline (PBS), pH 7.4. Antibodies, biotinylated as previously described (13), or fluorochrome-conjugated anti-TCR MAbs, purchased from PharMingen (La Jolla, Calif.), were used. Biotinylated anti-B220, which detects a protein on the surface of B lymphocytes also known as CD45R, was detected by using immunoglobulin secreted by hybridoma 14.8. This antibody was a gift from W. Krall and J. Braun (Department of Pathology, University of California, Los Angeles). Fluorochrome-conjugated anti-CD4, anti-CD8, and streptavidin were purchased from Becton Dickinson (Mountain View, Calif.). Mouse-absorbed, anti-hamster immunoglobulin G fluorescein was purchased from Caltag (South San Francisco, Calif.).

To test the efficacy of the anti-TCR antibody treatment, T-cell numbers and phenotypes were first determined for antibody-treated, uninfected mice. To more accurately reflect the experimental setup used subsequently for infected mice, these control mice were orally intubated with PBS. The first antibody injection was 3 days prior to oral intubation (day −3), and the second antibody injection was on the day of oral intubation (day 0). Mice were injected with either 250 μg of anti-$\alpha\beta$ TCR MAb or 180 μg of anti-$\gamma\delta$ TCR MAb in the peritoneal cavity (i.p.) in 0.1 ml of PBS per injection. On day 5, cell suspensions from lymphoid tissues of treated mice were pooled and then treated with directly conjugated antibodies at

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TABLE 1. Specific depletion of TCR T-cell subsets in vivo by MAb treatment

MAb treatment	% Positively staining lymph node cells[a]						
	αβ TCR^+	γδ TCR^+	$B220^+$ (B cells)	$CD4^+$ $CD8^-$	$CD4^+$ $CD8^+$	$CD4^-$ $CD8^-$	$CD4^-$ $CD8^+$
None	84.0	1.8	7.9	67.4	0.5	11.7	20.5
Anti-αβ TCR	24.6	6.8	44.1	11.4	3.5	67.1	18.0
Anti-γδ TCR	69.4	0.9	11.9	60.8	0.4	24.2	14.7

[a] After subtraction of background. Data were obtained with pooled cells from groups of three mice each; representative data from one of two experiments are presented.

TABLE 2. Specific depletion of γδ TCR^+ IEL in vivo by anti-γδ TCR MAb treatment

MAb treatment (n)	Mean % of total TCR^+ cells[a]		CD8/CD4 ratio[b]
	αβ TCR^+	γδ TCR^+	
None (3)	74.1	25.9	24.4
Anti-γδ TCR (2)	96.7	3.3	23.6

[a] Expressed as the mean percentage of total (αβ and γδ) TCR^+ IEL, as determined by two-color flow cytometry analysis. A small percentage of TCR^- cells which are present in the IEL preparations were excluded from the calculation.

[b] Ratio of $CD4^-$ $CD8^+$ to $CD4^+$ $CD8^-$ cells as determined by two-color flow cytometry analysis. The small percentage of $CD4^+$ $CD8^+$ and $CD4^-$ $CD8^-$ cells found in IEL preparations were excluded from the calculation.

4°C in PBS containing 2% (wt/vol) sodium azide (Sigma) for 0.5 h. Incubation with biotinylated anti-TCR MAbs was followed by incubation with streptavidin-phycoerythrin. Cells were fixed in PBS containing 1% (wt/vol) paraformaldehyde (Sigma) and stored at 4°C until analysis with a Becton Dickinson FACScan 440 (Jonsson Cancer Center Flow Cytometry Core Facility, University of California, Los Angeles).

The antibody treatment was effective at depleting the target T-cell subset. Lymphocytes from pooled lymph nodes of control mice (no MAb) were 84% positive for the αβ TCR (Table 1). In contrast, lymph node cells from mice treated with the anti-αβ TCR antibody were only 24.6% positive for the αβ TCR. In the treated mice, the percentage of γδ T cells and B lymphocytes in the lymph nodes increased. This reflects the decreased number of total lymph node cells in these mice, as opposed to an increase in the absolute numbers of these types of cells (data not shown). The reduction in total cell number suggests that the decrease in αβ TCR^+ lymphocytes was probably not due to masking of the αβ TCR by residual anti-TCR MAb. The percentage of cells that express other T-cell markers, such as CD4 and CD8, also was reduced (Table 1), providing further evidence for a real αβ T-cell reduction. The mechanism for the reduction in T-cell number is not known, although hypothetically it could be due to complement-mediated lysis, antibody-dependent cellular cytotoxicity, or programmed cell death mediated by cross-linking the TCR (19, 20). We have shown previously that a similar anti-αβ TCR treatment is effective at depleting αβ TCR^+ cells in other sites including the intestinal epithelium (13). Mice treated with a γδ TCR MAb showed a decrease in the percentage of γδ TCR^+ lymph node cells from 1.8 to 0.9%, while αβ TCR^+ cells and B lymphocytes ($B220^+$) were not greatly affected. The relatively small decrease in the percentage of αβ TCR^+ lymph node cells in mice treated with an γδ TCR MAb is not likely to be significant, because there is considerable variation in this percentage even in untreated mice. A series of six untreated BALB/c mice that were analyzed in a separate experiment had 66.7% ± 11.8% (mean ± standard deviation) αβ TCR^+ lymph node cells, not significantly different from the data obtained with mice treated with γδ TCR MAb shown in Table 1. Given the small percentage of γδ TCR^+ cells in the lymph nodes and spleen, it is difficult to be certain if the observed reduction is significant. In a separate experiment, we therefore analyzed intestinal intraepithelial lymphocytes (IEL), a population that contains many γδ T cells. In two treated mice, the γδ TCR^+ IEL were nearly eliminated by this treatment (Table 2). Between 2.9 and 3.6% of the TCR^+ cells expressed a γδ TCR (mean = 3.3%) following anti-γδ TCR MAb treatment, and these remaining γδ TCR^+ IEL had very low levels of surface TCR expression (data not shown). Treatment with αβ TCR MAb was as effective in the depletion of αβ TCR^+ IEL as it was in the depletion of αβ TCR^+ lymph node T cells. While the IEL of control mice were 74.1% αβ TCR^+, in three treated mice an average of only 24.4% of the IEL still expressed an αβ TCR.

Resistance to *Salmonella* infection was tested with a streptomycin-resistant derivative of a clinical isolate of *S. enteritidis* (CDC5) obtained from the Centers for Disease Control and Prevention (Atlanta, Ga.). This isolate is designated CDC5-str. Strain CDC5-str was grown in 10 ml of Luria-Bertani (LB) broth at 37°C with aeration and then washed in PBS. Serial dilutions of bacteria in PBS were carried out, assuming that the culture contained 10^9 CFU. An exact determination of the number of CFU in 0.1 ml of each serial dilution was performed by plating out further dilutions of these suspensions on LB plates containing 50 μg of streptomycin per ml (LB-str) and counting the bacterial colonies after overnight incubation at 37°C. Mice were injected twice with antibodies i.p., on day −3 and on day 0. Mice received either no MAb, 250 μg of pooled hamster serum immunoglobulin G (Rockland, Gilbertsville, Pa.), 250 μg of anti-αβ TCR MAb, or 180 μg of anti-γδ TCR MAb in 0.1 ml of PBS in both injections. On day 0 mice were anesthetized and given 0.1 ml of PBS containing bacteria by oral intubation with 23G tubing (Intermedic). Groups of five mice were given doses of *S. enteritidis* CDC5-str; 10 doses were tested. The mice were then monitored at least twice daily for 14 days, and the number of surviving mice in each group was noted. To ensure that the mice died of infection by *S. enteritidis*, the spleens, livers, and Peyer's patches of dead mice were removed and homogenized. Cell suspensions from these organs were diluted in PBS and then plated on LB-str plates. All tissues from these infected mice yielded streptomycin-resistant CFU. At the end of the 14-day course, the 50% lethal dose (LD_{50}) was calculated by the method of Reed and Meunch (17).

Results from the LD_{50} determination indicated that both T-cell subsets contribute to the host defense against oral *Salmonella* infection. The LD_{50} for oral infection with *S. enteritidis* CDC5-str was calculated to be 4.5×10^6 CFU. Anti-αβ TCR MAb treatment severely impaired the ability of the host to respond to oral infection, lowering the LD_{50} more than 4 log units from that of the PBS-treated mice, to 4.2×10^2 CFU. The anti-γδ TCR MAb treatment also impaired the ability of the mice to resist *Salmonella* infection, lowering the LD_{50} (by approximately 300-fold) to 1.6×10^4 CFU. Treatment with pooled hamster immunoglobulin G did not greatly affect the LD_{50} for oral infection (LD_{50} = 4.3×10^6 CFU), indicating that the effect observed for anti-TCR MAb-treated mice was likely to be a consequence of T-cell depletion and/or inhibition.

The number of γδ TCR^+ lymphocytes was increased in the

intestinal epithelium following oral infection, consistent with a possible role for γδ TCR IEL in resistance. Mice were infected with 2×10^6 CFU of *S. enteritidis*, a dose below the LD_{50}, and at day 5 following infection, IEL and lymphocytes from the peritoneal cavities were prepared and analyzed by flow cytometry. Lymphocytes in the IEL preparations from infected mice consisted nearly entirely of $CD8^+$ T cells (data not shown), confirming that the preparations from these mice were not heavily contaminated with lymphocytes from the lamina propria or some other sites. Infected mice had an average of 17.5 $\times 10^6$ total IEL (± 0.19, $n = 4$), compared with 4.1×10^6 IEL (± 1.4, $n = 3$) for control mice. This more than fourfold difference in total IEL number is statistically significant ($P < 0.001$). The percentage of γδ TCR^+ IEL in infected mice was 33.8% of the total number of lymphocytes, while only 25.9% were γδ TCR^+ in the controls. Therefore, although the absolute numbers of both αβ TCR^+ and γδ TCR^+ IEL increased in infected mice, the increase in γδ TCR^+ IEL was somewhat more pronounced.

Previous work has demonstrated a predominant role for $CD4^+$ T cells in the resistance of mice infected i.p. with *Salmonella typhimurium* and a smaller role for $CD8^+$ T cells (15). The CD4 molecule identifies the helper subset of T cells that recognizes antigen in association with class II histocompatibility molecules. Because $CD4^+$ T cells expressing the γδ TCR are extremely rare (10), the protective $CD4^+$ T cells presumably express an αβ TCR. However, increases in the number of γδ T lymphocytes in the blood samples of patients recovering from *Salmonella* infection (7) and in the peritoneal cavities of BALB/c mice infected with an avirulent strain of *Salmonella choleraesuis* (4) have been reported. Furthermore, in vitro culture of mouse peritoneal lymphocytes with *S. choleraesuis*-infected macrophages leads to an increase in the number of γδ T cells (4). The increase in γδ T cells in response to *S. choleraesuis* is related to the presence of the *Ity*^{r} allele, which affects resistance to *Salmonella* infection (5). However, these studies did not establish the protective role of the increased γδ T cells, and they did not examine the oral route of infection, which is the prevalent one in nature.

The experiments presented herein demonstrate that γδ T cells function in protection against *Salmonella* infection. The treatment of infected mice with the anti-γδ TCR MAb depleted the number of γδ T cells, and it resulted in an approximately 300-fold reduction in the LD_{50} compared with the LD_{50} for the control mice. Although this is less dramatic than the reduction following anti-αβ TCR MAb treatment, it is still a considerable effect. The experimental results further suggest that γδ T cells located in the epithelium of the intestine could play an important role following oral infection, because of the marked increase in γδ TCR^+ IEL following infection. It is noteworthy that we did not find an increase in γδ TCR^+ lymphocytes in the peritoneal cavities of orally intubated mice (data not shown). γδ T cells were barely detectable in the peritoneums of both control and infected mice, although an increase in peritoneal γδ T cells in mice infected i.p. with *S. choleraesuis* was reported previously (4). This suggests that a local accumulation of γδ T cells occurs, at the site of infection, although the different *Salmonella* strains used also could be responsible for the differing results.

Our data are consistent with those from several recent studies demonstrating a protective role for mouse γδ T cells in the host response to either i.p. or oral infection with another bacterium, *L. monocytogenes* (8, 14, 18, 21). In one previous study, a role for γδ TCR^+ cells in the intestinal epithelium in response to oral infection was suggested. γδ TCR^+ cells were the major IEL population responsible for gamma interferon secretion following oral infection with *L. monocytogenes*, although an expansion of γδ TCR^+ IEL following oral infection was not reported in this case (21). In summary, data from this and other recent studies suggest that γδ T cells could play a broad protective role in the host response to a variety of bacterial infections, and they further suggest that IEL could be important following oral infection with bacteria.

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