Effect of Spleen Cell Populations on Resolution of Cryptosporidium parvum Infection in SCID Mice

LANCE E. PERRYMAN,* PATRICIA H. MASON, AND CLARENCE E. CHRISP†
Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040

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Persistent infection was established in SCID mice given 107 Cryptosporidium parvum oocysts. Nine groups of infected SCID mice were inoculated with 106, 105, or 104 total spleen cells, CD8-depleted spleen cells, or CD4-depleted spleen cells from naive BALB/c donors. Infection was significantly reduced in all treatment groups. The most profound effect occurred with spleen cell preparations containing CD4 T lymphocytes but depleted of CD8 T lymphocytes.

Cryptosporidiosis is an enteric disease of humans and other animals caused by the protozoal agent Cryptosporidium parvum. The clinical course is determined by the immunocompetence of the infected host. Immunocompetent persons who ingest C. parvum oocysts experience 3 to 21 days of abdominal discomfort and diarrhea (12, 13, 15). Recovery is complete, and the vast majority of recovered individuals are resistant to reinfection. Immunodeficient patients, however, experience prolonged diarrhea associated with persistent infection of intestinal epithelial cells (10, 11, 15, 16). In the absence of effective immune responses to clear a cryptosporidial infection, diarrhea may persist for months (3, 15, 28).

Persistent life-threatening cryptosporidial infections are readily induced in C.B-17 SCID mice and young SCID host strains (5, 20, 25, 26, 31). In both animal SCID models, there is a profound deficiency of T and B lymphocytes, as well as a complete inability to produce antigen-specific immune responses, but natural killer cells are present (6, 21, 22, 24, 31, 36). These observations indicate that natural killer cells alone are insufficient to resolve infection and that B- and/or T-lymphocyte responses are necessary. Atypical nude mice have been used to analyze the role of T lymphocytes. In general, mice of immunocompetent strains can be infected with C. parvum only as neonates, and the infections are of short duration. In contrast, persistent cryptosporidial infections were established in both neonatal (18) and adult (34, 35) athymic nude mice. Since these mice lack mature CD4 and CD8 T lymphocytes but possess natural killer cells and make antibodies to thymus-independent antigens, these reports suggest that T lymphocytes are necessary for recovery. Three approaches are available to determine the relative contributions of CD4 and CD8 T lymphocytes to resistance against infection by C. parvum. One involves selective elimination of CD4 or CD8 T lymphocytes by in vivo treatment with relevant antibodies (7, 34, 35). A second strategy utilizes mice genetically deficient in CD4 T cells or CD8 T cells (1). A third approach involves reconstitution of SCID mice with lymphocyte preparations from which T-cell subsets have been selectively removed. Here we report experiments based on the third approach and show that spleen cell preparations containing CD4 T cells were significantly more effective than preparations depleted of CD4 T cells in mediating resistance to persistent cryptosporidial infection.

The Iowa isolate of C. parvum (obtained from H. Moon, Ames, Iowa) was used for all studies. The organism was propagated by passage in neonatal calves, and oocysts were purified from feces as previously described (4). Oocysts were treated with 3% peracetic acid for 10 min prior to administration to mice. Male SCID mice (C.B-17/Scid-Tac-scidGF), 4 to 5 weeks old, were obtained from Taconic, Germantown, N.Y., and maintained in microisolation cages. Adult BALB/c mice were obtained from a mouse hepatitis virus-free breeding colony maintained at Washington State University.

The following monoclonal antibodies (MAbs) were used in the preparation and/or analysis of cells administered to SCID mice and for analysis of spleen cells of recipient SCID mice following transplantation: GK 1.5 (anti-CD4) (14), 2.43 (anti-CD8) (32), B220 (anti-B cell) (9), and M1/70.15 (antimacrophage) (33). Rabbit complement (Low-Fox-M) was obtained from Accurate Chemical and Scientific Corporation, Westbury, N.Y.

Bone marrow cells were obtained from adult BALB/c mice by flushing the marrow plug from the femur with 2 ml of cold saline containing 10% fetal bovine serum. Clumps of cells were dispersed by passage through a 25-gauge needle. Spleens and thymuses were collected in Dulbecco modified Eagle medium with 100 U of penicillin per ml and 100 μg of streptomycin per ml. Cells were teased from the tissues, and erythrocytes were lysed from spleen cells with Tris-buffered NH4Cl (pH 7.2). The cells were washed and resuspended in cold saline with 10% fetal bovine serum for intravenous (i.v.) injection. Depletion of selected T-lymphocyte subsets was accomplished by incubation of spleen cell preparations with the relevant MAb and rabbit complement. Spleens were obtained from adult male BALB/c mice that were kept in an isolation room and that were not allowed contact with C. parvum. Spleens were minced and teased to yield single-cell preparations as described above. Two cycles of antibody and complement treatment were performed to deplete the intended cell population. Isolated spleen cells were adjusted to 106/ml and incubated on ice for 30 min with the appropriate dilution of MAb established in preliminary experiments. Residual MAb was washed away, and the cells were resuspended at 1.5 × 107/ml. Freshly reconstituted rabbit complement was incubated with the cells for 1 h at 37°C. After a repeat cycle of complement-mediated lysis, treated cells were centrifuged on Lymphocyte-rat medium.
(Cedarlane Laboratories, Limited) (density = 1.094 g/ml) to eliminate dead cells. Viable cells were recovered, washed, counted and adjusted to the appropriate concentration for use. The degree of cell depletion was assessed by flow cytometric analysis of treated spleen cell preparations. Cells were analyzed with a Becton Dickinson (Mountain View, Calif.) FACScan equipped with a Consort 32 computer and LYSYS II software.

At the completion of each experiment, tissues were collected from all mice and examined microscopically for the presence and number of *C. parvum* oocysts. Slides were coded so that evaluation was performed without knowledge of treatment groups. Tissue infection scores ranging from 0 to 3 were assigned to the pyloric region of the stomach, ileum, cecum, and colon as previously described (0, no organisms observed; 1, <33% of mucosal epithelium parasitized; 2, 33 to 66% of epithelium parasitized; 3, >66% of epithelium parasitized) (30). During some experiments, a second method for evaluating infection was also performed. Feces from individual mice were collected and analyzed for the presence and number of *C. parvum* oocysts as previously described (27).

Results were analyzed with the nonparametric Wilcoxon rank sum statistic to determine if differences between groups of mice were significant (2).

**Dose and time response of SCID mice to *C. parvum* oocysts.**
In preliminary experiments, two groups of 20 SCID mice each were exposed to *C. parvum* oocysts administered orally. One group received a single oral dose of $10^7$ oocysts. The other group received oral doses of $10^4$ oocysts on consecutive days for a total exposure of $2 \times 10^7$ oocysts. Two mice from each group were killed at weekly intervals, and their gastrointestinal tracts were scored for the presence of *C. parvum* organisms. Organisms were detected throughout the gastrointestinal tract but were concentrated in the pyloric region of the stomach, the distal ileum, and the proximal colon. For all further experiments, tissue infection scores were determined from four readings taken at the pylorus, distal ileum, cecum, and proximal colon. Infection scores for the two groups of mice were maximal 5 to 6 weeks postinfection, and the levels of infection were similar for the two groups. On the basis of these results, all further experiments were initiated with an infecting dose of $10^7$ oocysts and terminated 6 weeks later.

**Reduction of infection scores following administration of bone marrow cells, thymocytes, or spleen cells.** SCID mice were infected with $10^7$ *C. parvum* oocysts administered orally. Two weeks postinfection, mice were inoculated i.v. with $10^6$ BALB/c bone marrow cells, thymocytes, or spleen cells (15 mice per group in experiment 1 and 12 mice per group in experiment 2). The mice were killed 6 weeks postinfection (4 weeks after transplantation) and scored for the presence and number of *C. parvum* organisms. The results of the two experiments are presented in Fig. 1. Administration of all cell types significantly reduced infection scores in both experiments ($P < 0.001$). On the basis of these results, spleen cells were selected for use in future experiments because of the relative ease with which T-cell subset depletion could be achieved.

**Reduction of infection scores following administration of spleen cells depleted of CD8 T cells or CD4 T cells.** SCID mice (10 per group) were infected with $10^7$ *C. parvum* oocysts. Two weeks postinfection, they were inoculated i.v. with $10^6$, $10^5$, or $10^4$ cells from one of the following preparations: total spleen cells, CD8 T-cell-depleted spleen cells, or CD4 T-cell-depleted spleen cells. The results from this experiment are shown in Table 1. For all of the cell preparations, the excretion of *C. parvum* oocysts by SCID mice was significantly reduced ($P < 0.001$). Tissue infection scores of SCID mice that were inoculated with $>10^4$ cells were also significantly reduced ($P < 0.05$).

The experiment was repeated and expanded to assess the characteristics of spleen cells in recipient SCID mice 6 weeks postinfection (Table 2). Significant reductions in fecal oocysts and tissue infection scores were again noted with SCID mice receiving injections of spleen cell preparations. Although the values of infection parameters were strikingly reduced by the previous i.v. administration of spleen cell preparations, the number and type of cells in the spleens of recipient SCID mice were only moderately affected. The most consistent observations involved CD4 T-cell numbers. They were significantly increased in those mice that received cell preparations enriched for CD4 T cells.

**CD8-depleted and CD4-depleted spleen cells were compared for their abilities to reduce *C. parvum* infection scores in recipient SCID mice (Table 2). No significant difference in**

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**TABLE 1** Cryptosporidial infection parameters of SCID mice treated with spleen cells

<table>
<thead>
<tr>
<th>Spleen cell type</th>
<th>No. of cells injected</th>
<th>Infection score (mean ± SD)</th>
<th>Oocyst/ing of feces (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>3.78 ± 2.17</td>
<td>16.57 ± 4.04</td>
</tr>
<tr>
<td>Total</td>
<td>$10^7$</td>
<td>1.50 ± 0.85$^a$</td>
<td>0.24 ± 0.41$^a$</td>
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<td>$10^6$</td>
<td>1.40 ± 0.97$^b$</td>
<td>0.03 ± 0.07$^b$</td>
</tr>
<tr>
<td></td>
<td>$10^5$</td>
<td>1.60 ± 0.84$^b$</td>
<td>0.23 ± 0.36$^b$</td>
</tr>
<tr>
<td></td>
<td>$10^4$</td>
<td>1.80 ± 1.23$^b$</td>
<td>0.50 ± 0.50$^b$</td>
</tr>
<tr>
<td></td>
<td>$10^3$</td>
<td>1.10 ± 0.88$^c$</td>
<td>0.40 ± 0.23$^c$</td>
</tr>
<tr>
<td></td>
<td>$10^2$</td>
<td>2.80 ± 1.23$^c$</td>
<td>0.55 ± 0.25$^c$</td>
</tr>
<tr>
<td></td>
<td>$10^1$</td>
<td>0.80 ± 1.14$^d$</td>
<td>2.16 ± 1.57$^d$</td>
</tr>
<tr>
<td></td>
<td>$10^0$</td>
<td>1.20 ± 1.23$^d$</td>
<td>2.54 ± 1.27$^d$</td>
</tr>
<tr>
<td></td>
<td>$10^{-1}$</td>
<td>2.40 ± 1.58$^d$</td>
<td>1.79 ± 1.07$^d$</td>
</tr>
</tbody>
</table>

* SCID mice (10 per group) were infected at 5 to 6 weeks of age by oral administration of $10^7$ *C. parvum* oocysts. Spleen cells were injected i.v. 2 weeks postinfection.
* Total spleen cell preparations contained 28% CD4 T cells, 12% CD8 T cells, and 20% B cells.
* $P < 0.05$ compared with mice receiving no injections of spleen cells.
* $P < 0.001$ compared with mice receiving no injections of spleen cells.
* CD4-depleted spleen cell preparations contained 36% CD4 T cells, 19% CD8 T cells, and 22% B cells.
* CD4-depleted spleen cell preparations contained 8.9% CD4 T cells, 19% CD8 T cells, and 44% B cells.
scores was noted in experiment 1. In experiment 2, however, infection scores were significantly lower in those SCID mice that received cell preparations enriched for CD4 T lymphocytes by depletion of CD8 cells. The quality of cell depletion was better in experiment 2, which may explain the highly significant differences noted.

The results of this study indicate that T lymphocytes mediate recovery from persistent C. parvum infection in SCID mice and that CD4 T cells are more important than CD8 T cells in this process. Infection was significantly reduced by reconstitution with bone marrow cells, spleen cells, or thymocytes, confirming and extending the results of Mead et al. (25), who showed that a combination of all three cell types resolved infection in SCID mice. The results are consistent with those of McDonald et al., who demonstrated that injection of SCID mice with spleen cells from naive BALB/c donors protected the mice against infection with a coccidial isolate of C. parvum (23). The ability of thymocytes alone to reconstitute resistance in SCID mice demonstrates that T cells play a central role. However, the mechanisms by which T lymphocytes mediate recovery are not known. Possibilities include (i) cytotoxic activity mediated by major histocompatibility complex (MHC) class I-restricted CD8 cells, (ii) cytotoxicity mediated by MHC class II-restricted CD4 cells, (iii) provision of T-cell help by CD4 cells for production of antibodies which clear infection by neutralization or other mechanisms, and (iv) production of gamma interferon and other cytokines that contribute to clearance of infection (7, 8, 35).

Selective depletion of T-cell subsets from spleen cell preparations allowed us to test the relative contributions of CD4 and CD8 T cells. The results demonstrate that CD4 T cells are more effective than CD8 T cells in resolving persistent cRYPTosporidial infection in SCID mice. Two other lines of evidence support the hypothesis that CD4 T cells are required for resistance to persistent infection by C. parvum. The first was derived from experiments in which lymphocytes were depleted in vivo by administration of a MAb (34, 35). The second line of evidence was derived from C57BL/6J mice genetically deficient in β2-microglobulin or MHC class II antigens. These mice are selectively deficient in CD8 and CD4 T lymphocytes, respectively (17, 19, 37). CD8 T-cell-deficient C57BL/6J mice recovered from infection, while CD4 T-cell-deficient mice remained persistently infected following exposure to C. parvum (1).

In the present study, passive transfer of spleen cells enriched for CD4 T cells resulted in a significant increase in the number of CD4 T cells in spleens of recipient SCID mice. No evidence for splenic CD8 T-cell engraftment was observed. These observations are similar to results obtained when purified CD4 T cells or purified CD8 T cells were injected into SCID mice (29). Even though splenic engraftment was apparently limited to CD4 T cells, all spleen cell preparations tested in the present study resulted in significant reductions in one or both measures of C. parvum infection. Perhaps critical effector cells homed preferentially to the gastrointestinal tract and were not reflected in the splenic compartments of recipient mice.

The availability of mice with defined immune deficiencies has facilitated definition of the role of CD4 T cells in recovery from C. parvum infection. The opportunity to define the role of CD4 Th1 and Th2 cells and their respective cytokine products in the pathogenesis of cryptosporidiosis now exists. This information will facilitate development of immunotherapeutic approaches to persistent cryptosporidial infection in immuno-deficient hosts.

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