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Resistance of Severe Combined Immunodeficient Mice to Infection with *Cryptosporidium parvum*: the Importance of Intestinal Microflora

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***Cryptosporidium parvum* is a protozoan parasite which colonizes intestinal epithelium, causing transient diarrheal illness in immunocompetent hosts and severe chronic disease in immunocompromised hosts. We examined the resistance of severe combined immunodeficient mice, either bearing intestinal flora or germfree, to intestinal infection with *C. parvum*. Infection was not readily detected in flora-bearing adult severe combined immunodeficient mice until 5 to 7 weeks following oral challenge with *C. parvum*. In contrast, germfree adult severe combined immunodeficient mice were heavily infected 3 weeks following challenge. These data support the hypothesis that resistance of adult mice to *C. parvum* infection does not require a specific immune response but can be mediated by nonspecific mechanisms associated with the presence of intestinal flora.**

Cryptosporidium parvum is a protozoan parasite that is now recognized as an important cause of diarrheal disease in economically important livestock species and in immunocompetent and immunocompromised humans (5, 9, 18). While cryptosporidial disease is usually mild and self-limiting in immunocompetent humans, individuals with AIDS and various other immune disorders often have chronic, life-threatening infections (4). Management of disease in animals and humans has been hampered by the lack of antimicrobial agents or therapeutic regimens that are consistently effective for either prevention or treatment of cryptosporidiosis (10, 17).

Despite increased research in the last decade, mechanisms of resistance to and recovery from cryptosporidial infection are still not well understood (6). Studies with mice support the hypothesis that T-cell-mediated immune responses are important for recovery from *C. parvum* infection (12, 19, 20). However, the extreme resistance of normal adult mice to *C. parvum* infection, even in the absence of previous exposure (8, 15, 16), suggests that other factors may be important in resistance to initial infection with *C. parvum*. The increased susceptibility of germfree adult immunocompetent mice to *C. parvum* infection led us to speculate that the presence of intestinal microflora may influence resistance to this parasite (11). However, normal mice depleted of intestinal flora with antibiotics were still resistant to infection, suggesting that the intestinal flora indirectly affects resistance to *C. parvum* by activating some component of the immune system (11).

Severe combined immunodeficient (SCID) mice lack both T- and B-cell immunity because of a genetically inherited deficiency in the ability of T- and B-cell antigen receptor genes to rearrange properly (3). It has been reported that SCID mice can be chronically infected with *C. parvum* and may develop clinical symptoms similar to those seen in immunodeficient human patients (13). In the present study, we used SCID mice, either flora-bearing or germfree, to examine the effects of intestinal flora on resistance to *C. parvum* in a host unable to generate a specific immune response.

MATERIALS AND METHODS

Mice. Flora-bearing C.B-17/IcrTac-scid (SCID) and C.B-17/IcrTac (control) female mice maintained at the National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, were obtained from Taconic Farms, Inc. (Germantown, N.Y.). Flora-bearing SCID mice at the Trudeau Institute were originally obtained from Jackson Laboratory (Bar Harbor, Maine). Germfree SCID mice were obtained from the Trudeau Animal Breeding Facility (Saranac Lake, N.Y.) and the Gnotobiotic Laboratories at the University of Wisconsin Medical School (Madison). Flora-bearing mice were colonized with a defined cocktail of anaerobic bacteria (Altered Schaedler Flora). All mice were kept under barrier-sustained conditions in flexible-film isolator units.

Oral challenge inocula. Feces collected from calves experimentally inoculated with *C. parvum* were suspended in 2 volumes of 2.5% potassium dichromate solution. This suspension was passed through a graded series of sieves to remove large particles. Twelve milliliters of suspension was then overlaid onto 10 ml of 1:2 Sheather's sucrose solution (1)–0.025 M phosphate-buffered saline (PBS) in 50-ml centrifuge tubes. Tubes were centrifuged for 15 min at 450 × *g*. The interface layer, containing oocysts, was harvested with a Pasteur pipette, diluted with PBS, and rinsed twice by centrifugation in PBS. Oocysts were enumerated by direct count with a hemacytometer. Immediately before use, the oocysts were incubated at room temperature for 30 min in 2.5% peracetic acid to kill any contaminant bacteria and then washed three times with PBS. Oocysts were recounted and adjusted to the appropriate concentrations for inoculation of mice.

Experimental design. Fecal pellets were collected from all mice before inoculation with *C. parvum*. Fecal smears were made, stained with carbol fuchsin, and examined for the presence of oocysts. All mice were negative for *C. parvum*

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prior to experimental challenge. Mice were given oral inoculations of oocysts suspended in 0.2 ml of PBS by using a feeding needle. Mice receiving 10^6 oocysts were given one dose, and mice receiving 2×10^7 oocysts were given two doses of 10^7 oocysts each on successive days.

In experiment 1, 8-week-old SCID and control mice were orally inoculated with 10^6 *C. parvum* oocysts. One week after this oral challenge, five SCID and five control mice were removed from the isolators. Mice were killed by CO_2 inhalation; fecal smears of colon contents were stained with carbol fuchsin and examined for oocysts. The ileum, cecum, and proximal colon were removed from each mouse and fixed in 10% formalin. Following fixation, tissue was embedded in paraffin. Histologic sections were cut at a thickness of 4 μm, stained with hematoxylin and eosin, and examined microscopically for *C. parvum*. At weeks 2, 3, 5, 7, and 9 postchallenge, three SCID and three control mice were killed and treated as described above.

In experiment 2, 8-week-old SCID and control mice were inoculated with 2×10^7 *C. parvum* oocysts. At weeks 2, 3, 5, 7, 9, 11, 12, 13, 14, 15, and 16 postchallenge, fecal pellets were collected from all mice, and smears were made, stained with carbol fuchsin, and examined for oocysts. At weeks 12 and 16, four mice from each group were killed, and intestinal tissues were prepared and examined for *C. parvum* as described above.

In experiments 3 and 4, 8-week-old germfree and flora-bearing SCID mice were inoculated with 2×10^7 *C. parvum* oocysts. Groups of two to five mice were killed at weeks 1 and 3 postchallenge. Cecal and colon contents were collected from all mice, stained with carbol fuchsin, and examined for oocysts. Intestinal tissues were prepared and examined for *C. parvum* as described above.

Data presentation. For scoring of fecal smears, 10 random high-power (500×) microscopic fields were examined per mouse. Data are presented as the percentage of microscopic fields examined that contained at least one *C. parvum* oocyst. Infectivity scores for histologic sections were determined (see Tables 1 and 2).

RESULTS

No *C. parvum* oocysts were seen in feces of flora-bearing SCID mice challenged with 10^6 oocysts until 7 weeks after challenge, when 1 oocyst was seen in 1 of the 30 total fields examined for three mice. At week 9, 11 of 30 fields contained oocysts. Ten of these fields contained one oocyst, and one field contained two oocysts. No *C. parvum* oocysts were detected in feces of control mice challenged with 10^6 oocysts.

Similar numbers of *C. parvum* oocysts were seen for the first 3 weeks after challenge in feces of flora-bearing SCID and control mice challenged with 2×10^7 oocysts. At 2 weeks, three oocysts were seen in a total of 80 fields for both groups. At 3 weeks, no oocysts were seen in 80 fields from control mice, and one oocyst was seen in 80 fields from SCID mice. From 5 weeks after challenge until the end of the experiment, 5 to 45% of fields in smears from flora-bearing SCID mice contained oocysts. Most positive fields in smears from flora-bearing SCID mice contained one or two oocysts.

Results of histological examination of tissues from mice challenged with 10^6 oocysts (experiment 1) are presented in Table 1. At 1 and 2 weeks after challenge, parasites were seen very rarely in ileum, cecum, and proximal colon tissues of flora-bearing SCID and control mice; usually they were seen over or near aggregates of lymphoid tissue. Subsequently, no parasites were seen in tissues from control mice. For the duration of experiment 1, increasing numbers of parasites were found in intestinal tissues from flora-bearing SCID mice. At 7 and 9 weeks after challenge, parasites were found mostly in cecum and proximal colon tissues; few were found in ileum tissue. Infection was localized, with evidence of necrosis and hyperplasia of crypt epithelium associated with the parasites. However, most areas of the mucosa remained normal, with no parasites present.

TABLE 1. Infectivity scores of intestinal *C. parvum* infection in SCID and control mice

Expt and wk	Infectivity score[a] (*n*) for mouse group	
	Control	SCID
1[b]		
1	0.5 ± 0.4 (5)	0.2 ± 0.2 (5)
2	0.3 ± 0.3 (3)	0.3 ± 0.3 (3)
3	0 (3)	0.4 ± 0.2 (3)
5	0 (3)	1.0 ± 0.5 (3)
7	0 (3)	1.1 ± 0.4 (3)
9	0 (3)	1.2 ± 0.2 (3)
2[c]		
12	0 (4)	0.8 ± 0.3 (4)
16	0 (4)	1.5 ± 0.2 (4)

[a] Mean ± standard deviation. Infectivity scores for histologic sections were determined as follows: 0, no *C. parvum* oocysts found; 1+, few *C. parvum* oocysts seen, but difficult to find; 2+, *C. parvum* oocysts seen, easy to find.
[b] Mice challenged with 10^6 oocysts per mouse.
[c] Mice challenged with 2×10^7 oocysts per mouse.

Results of histological examination of tissues taken from mice challenged with 2×10^7 oocysts (experiment 2) are also shown in Table 1. At 12 and 16 weeks after challenge, low to moderate numbers of parasites were seen in ileum, cecum, and proximal colon tissues of flora-bearing SCID mice; there was no evidence of necrosis or crypt hyperplasia. No parasites were seen in tissues from control mice.

Results of histological examination of tissues taken from germfree and flora-bearing SCID mice challenged with 2×10^7 oocysts (experiments 3 and 4) are shown in Table 2. No parasites were seen in histologic sections from flora-bearing SCID mice either 1 or 3 weeks after challenge. In histologic

TABLE 2. Infectivity scores of intestinal *C. parvum* infection in germfree and flora-bearing SCID mice

Expt and mouse group[a]	Infectivity score[b] (*n*) for mouse group at:	
	1 wk	3 wk
3		
Germfree, control	NE[c]	0 (3)
Germfree, infected	1.0 ± 0.0 (2)	2.0 ± 0.0 (5)
Flora-bearing, infected	0 (2)	0 (2)
4		
Germfree, control	NE	0 (2)
Germfree, infected	0.2 ± 0.3 (3)	2.0 ± 0.0 (4)
Flora-bearing, infected	0 (4)	0 (4)

[a] Germfree control SCID mice were not challenged with *C. parvum*. Germfree infected and flora-bearing infected SCID mice were challenged with 2×10^7 oocysts per mouse.
[b] Mean ± standard deviation. Infectivity scores for histologic sections were determined as follows: 0, no *C. parvum* oocysts found; 1+, few *C. parvum* oocysts seen, but difficult to find; 2+, *C. parvum* oocysts seen, easy to find.
[c] NE, not examined.

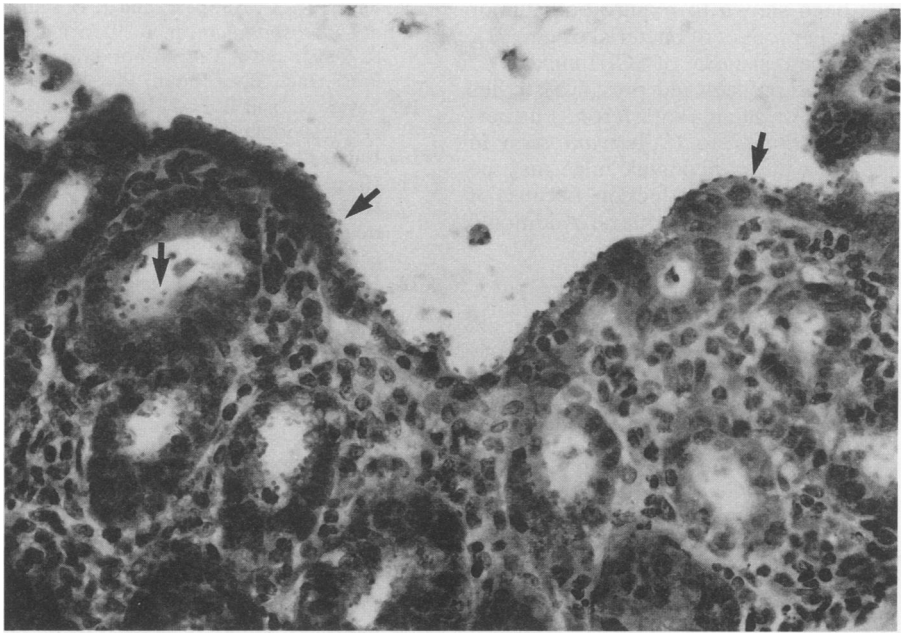

FIG. 1. Section of colon from adult germfree SCID mouse 3 weeks after challenge with *C. parvum*. Large areas of the intestinal epithelium were heavily colonized with cryptosporidia (arrows).

sections taken from germfree SCID mice, moderate colonization of the terminal ileum and large intestine was seen 1 week following challenge. At 3 weeks, very large numbers of parasites were seen, sometimes forming a continuous layer over the crypts and surface epithelium of the terminal ileum and large intestine (Fig. 1). The intestinal tissues of infected germfree SCID mice did not show necrosis or crypt hyperplasia and appeared histologically similar to those of flora-bearing SCID mice.

DISCUSSION

In a recent publication describing several potential models of cryptosporidial infection, it was reported that adult SCID mice became chronically infected with *C. parvum* and that some of these mice exhibited clinical signs of disease (13). We have replicated those experiments and have confirmed several of the findings. In the present study, infections in flora-bearing SCID mice were very light for the first several weeks after challenge. One to two months after challenge, chronic infection was seen in most mice. These findings agree with those previously reported (13).

While we have confirmed that adult SCID mice can become chronically infected with *C. parvum*, we were most impressed with the ability of these severely immunocompromised mice to resist initial colonization following challenge with large numbers of parasites. In both the present and previous (13) studies, SCID mice challenged with 10^6 or 2×10^7 oocysts were able to resist infection with *C. parvum* for at least 3 weeks, neither shedding oocysts nor showing histologic evidence of any significant colonization in the intestinal tract. The resistance seen in these SCID mice was much greater than that seen in immunocompetent neonatal mice (15), infant monkeys (14), and calves (10a), because neonatal animals exhibited diarrheal disease and/or fecal shedding of parasites 1 week after challenge with only 10^1 to 10^3 oocysts. The resistance of SCID mice to initial colonization with *C. parvum* is unlikely to be mediated by specific immunity, since these mice are deficient in both T- and B-cell functions (3).

We have previously proposed that mechanisms other than specific immunity may be important in resistance to cryptosporidial infection, since germfree immunocompetent mice are more readily colonized with *C. parvum* than are flora-bearing controls (11). In the present study, histologic sections taken from germfree SCID mice 1 week after challenge with *C. parvum* showed considerable colonization with the parasite. At 3 weeks following challenge, colonization in germfree SCID mice was quite heavy, comparable to that seen in infected neonatal mice (11).

Thus, while recovery of mice from *C. parvum* infection appears to require a specific immune response, probably involving T helper cells (12, 19, 20), data from the present study suggest that resistance to initial infection can be mediated by a nonspecific mechanism which is present in SCID mice. Furthermore, the expression of this resistance is affected by the presence and absence of intestinal flora.

Previous studies have also indicated that intestinal flora is important in resistance to *C. parvum*. Germfree adult BALB/c and CD1 mice were more susceptible to infection than flora-bearing controls (11), and the age-related resistance of infant mice to *C. parvum* coincides with the acquisition of mature intestinal flora (7, 11). The present results in germfree SCID mice suggest a possible mechanism for this resistance. Ungar et al. (20) reported that gamma interferon is involved in protective immunity to *C. parvum* in BALB/c mice treated with various antibodies to T-cell subsets and cytokines. These studies further suggested that a non-T cell was the source of the gamma interferon. It is known that SCID mice are able to produce gamma interferon through a non-T-cell pathway, and this pathway has been shown to be important in the resistance of SCID mice to several intracellular pathogens (for a review, see reference 2). Recently, production of gamma interferon by this path-

way in SCID mice has been shown to require both tumor necrosis factor and a soluble product of bacterial origin (21). Thus, it seems likely that the resistance of SCID mice to *C. parvum* infection seen in the present and previous studies may be due to the presence of gamma interferon. Furthermore, the increased susceptibility to *C. parvum* seen in various adult germfree, as well as neonatal, mice may be related to deficient gamma interferon production, because of a lack of stimulation by normal intestinal flora. Additional studies are needed to verify these hypotheses.

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