Susceptibility of Germfree or Antibiotic-Treated Adult Mice to *Cryptosporidium parvum*

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Adult mice are more resistant than neonatal mice to intestinal colonization with the protozoan parasite *Cryptosporidium parvum*. Development of a mature intestinal flora may play a role in this resistance. We compared susceptibilities to colonization with *C. parvum* in adult conventional mice, adult germfree mice, and adult conventional mice treated with oral antibiotics to deplete the intestinal flora. Germfree mice of both CD1 and BALB/c strains were colonized at day 7 following inoculation with *C. parvum* oocysts isolated from the feces of an infected, diarrheic calf. Age-matched conventional mice of the same strains were comparatively resistant to colonization. Conventional mice treated with antibiotics remained resistant to colonization. These results suggest that the microflora in the intestine was not the sole determinant of resistance or susceptibility to colonization. The germfree adult mouse as an experimental model of cryptosporidiosis is discussed.

Cryptosporidium parvum was first described in the small intestines of laboratory mice (23). This organism, which appears to have little host specificity (25), parasitizes the intestinal mucosae of a number of mammals, including cattle, sheep, and humans (6, 24). Cryptosporidiosis in humans has recently received increased attention. In immunocompromised individuals, such as those with acquired immunodeficiency syndrome, colonization can result in a persistent, choleralike illness and death (1, 17). There are no safe, effective antimicrobic or chemotherapeutic regimens against this parasite in humans or animals (15, 17). Little is known about the mechanism of resistance or susceptibility to *C. parvum*. Infection can persist in immunocompromised individuals who have serum antibody against the organism (26), and passive transfer of lacteal antibody to suckling mice does not protect against experimental challenge (16). Immunity mediated by T lymphocytes may be important in recovery, since adult athymic (nude) mice colonized as infants are unable to clear cryptosporidia from the intestinal tract, although adult nude mice are resistant to primary challenge (9).

Infant mice are highly susceptible to colonization with *C. parvum* (4, 22). In contrast, while wild mice have been reported to be easily colonized with *C. parvum* as adults (13), laboratory mice develop relative resistance to colonization at about 3 weeks of age (9, 22). The development of resistance coincides with the development of a mature intestinal flora (2, 21). To examine the possibility that the intestinal flora plays a role in the resistance of adult mice to *C. parvum*, we challenged germfree, conventional, and antibiotic-treated adult mice with oocysts isolated from the feces of a diarrheic calf. Seven days postchallenge, *C. parvum* was found in the intestines of most of the germfree adult mice, although in lesser numbers than were found in the intestines of infant mice used as inoculum controls. *C. parvum* was infrequently seen in the intestines of conventional or antibiotic-treated conventional adult mice.

MATERIALS AND METHODS

Mice. CD1 germfree and conventional mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. BALB/c germfree mice were obtained from the University of Wisconsin–Madison Gnotobiotic Laboratory, Madison. Conventional BALB/c mice were from Harlan Sprague Dawley, Madison, Wis. Germfree mice of both sexes were used at 6 to 8 weeks of age. Conventional mice used as controls were age, strain, and sex matched. Conventional BALB/c mice used in antibiotic treatment experiments were 5 to 6 months of age. Infant mice used as inoculum controls were 1 week of age at challenge. Germfree and control mice were maintained in separate Trexler flexible-film isolator units. Mice were housed four to six per cage on autoclaved wood shavings and fed autoclaved food and water. Conventional mice receiving antibiotics were maintained three per cage in sterile plastic cages with paper filter tops for the first week of treatment and then transferred to isolator units; they were treated as germfree mice were.

Antibiotic treatment. Each cage of treated conventional mice received 50 ml of sterile drinking water containing vancomycin at 500 μg/ml, ampicillin at 1 mg/ml, and gentamicin at 100 μg/ml per day (11). The remaining water was discarded daily and replaced with fresh solutions for the first week. Following introduction into the isolators, the mice received 100 ml of the same antibiotic solution, which was changed every 2 days for the duration of the experiment. Treated germfree mice used as controls received the same regimen.

Oral challenge inoculum. Feces were collected from calves experimentally infected with *C. parvum*, and oocysts were isolated as previously described (16). Immediately before use, the oocysts were incubated for 30 min with 2.5% peracetic acid to kill any contaminant bacteria and then washed three times with phosphate-buffered saline. Numbers of oocysts were determined by direct counts with a hemacytometer. Adult mice received either 10⁵ or 10⁶ oocysts in 0.1 to 0.2 ml of phosphate-buffered saline per os, and infant mice received the same inoculum by gastric intubation. The level of challenge was determined by previously observed infectivity of the oocyst pool for infant mice.

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TABLE 1. Ability of *C. parvum* to colonize the intestinal tracts of germfree and conventional adult mice

Expt no. and animals	No. of mice					
	Challenged	Colonized[a]	With *C. parvum* in:			
			Colonic contents	Ileum	Colon or cecum	Lower colon
1						
CD1 mice, germfree	12	12	6 (1.0)[b]	3 (1.0)	11 (1.0)	7 (1.1)
CD1 mice, conventional	12	1	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.0)
Infant mice, inoculum control	7	4	4 (1.0)	3 (1.7)	ND[c]	ND
2						
BALB/c mice, germfree	17	17	0 (0.0)	14 (1.0)	9 (1.0)	4 (1.0)
BALB/c mice, conventional	19	4	0 (0.0)	4 (1.0)	0 (0.0)	0 (0.0)
Infant mice, inoculum control	10	8	8 (1.6)	ND	ND	ND

[a] Number of mice in which *C. parvum* was found in at least one of the sites examined.

[b] The number in parentheses is the mean intensity score of all positive mice within a group. Results of examination of carbol-fuchsin-stained smears of colonic contents were scored as follows: 0, no oocysts found; 1, fewer than one oocyst per 500× field; 2, one to five oocysts per field. Histologic examinations of stained tissue were scored as follows: 0, no cryptosporidia found; 1, cryptosporidia found in two or three microscopic fields per histologic section; 2, cryptosporidia found in most microscopic fields of the histologic section.

[c] ND, Not done.

Experimental design. Before challenge with *C. parvum*, feces were collected from the mice and cultured for intestinal flora. For aerobic culture, fecal pellets were emulsified in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and plated to Trypticase soy agar plates with 5% bovine blood, MacConkey agar plates, and phenylethyl alcohol agar plates (BBL). Plates and tubes were incubated at 37°C in air and checked daily for 7 days. For anaerobic culture, fecal pellets were emulsified in peptone yeast glucose broth (10) and streaked to blood agar plates and multicarbohydrate agar roll tubes (I. Robinson, personal communication). Plates and tubes were incubated anaerobically at 37°C. Blood agar plates were checked at 48 h, and roll tubes were checked daily for 14 days. No bacteria were seen in cultures from germfree mice or antibiotic-treated conventional mice (feces were diluted sufficiently to eliminate effects of residual antibiotics). Normal intestinal floras were recovered from conventional mice not treated with antibiotics. Fecal smears were stained with carbol fuchsin (8) and examined for *Cryptosporidium* oocysts (none was ever found in prechallenge mice). Mice were challenged by oral inoculation with oocysts by using a 1-ml syringe and feeding tube. Extra inoculum was then removed from the isolator and used to challenge infant mice as an infectivity control. One week following challenge, fecal pellets from germfree mice and antibiotic-treated mice were again cultured for intestinal flora (no bacteria were found). All the mice were killed by ether anesthesia; fecal smears of colon contents were stained with carbol fuchsin and examined for oocysts. The intestinal tract was removed from each mouse and fixed in 10% Formalin-saline. Following fixation, tissue was embedded in paraffin. Histologic sections were cut at 4 μm, stained with hematoxylin and eosin, and examined microscopically for *C. parvum*.

Statistics. Differences between groups in the numbers of mice positive for cryptosporidia were evaluated for significance by χ^2 analysis.

RESULTS

In the first experiment, 12 germfree and 12 conventional CD1 adult female mice were challenged orally with 10^5 oocysts per mouse. Table 1, experiment 1, shows the results of examination for *C. parvum* in these mice 1 week following challenge exposure. Cryptosporidia were found in either the colonic contents or intestinal tissues of all 12 germfree adult mice. The greatest numbers were found in the upper colon and cecum. In contrast, *C. parvum* was found in the intestinal tract of only 1 of 12 conventional adult mice. The difference between the two groups in numbers of mice positive for cryptosporidia was statistically significant ($P <$ 0.01). Cryptosporidia were found in the ilea or colonic contents of four of seven infant mice challenged as infectivity controls. In this and all the following experiments, cryptosporidia colonized the intestines of infant mice more heavily than intestines of adult mice (Fig. 1).

To determine whether the susceptibility of adult germfree mice to *C. parvum* would extend to strains other than CD1, a similar study was performed with BALB/c germfree and conventional mice (Table 1, experiment 2). All 17 germfree BALB/c adult mice were found to be colonized with *C. parvum* 1 week after challenge with 10^6 oocysts per mouse. Cryptosporidia were commonly found in both the colon (10 of 17 mice) and ileum (14 of 17 mice). Cryptosporidia were found in only 4 of 19 conventional BALB/c adult mice. Again, the difference in numbers of mice colonized in these two groups was statistically significant ($P < 0.01$). Cryptosporidia were found in colonic contents of 8 of 10 infant mice.

Experiments were performed in which both germfree and conventional CD1 mice received antibiotics in drinking water daily for 3 weeks. After 10 days of treatment, no intestinal bacteria could be cultured either aerobically or anaerobically from any of the mice. On day 14, all the mice were challenged orally with 10^5 oocysts per mouse. At day 21, the mice were killed and intestinal tissue was examined as described above (Table 2, experiment 1). Seven of nine germfree mice were colonized with *C. parvum*, most commonly in the cecum. Only 4 of 12 conventional mice treated with antibiotics were colonized; the difference between the two groups was statistically significant ($P < 0.05$). Seven of seven infant mouse inoculum controls were colonized.

An additional experiment (Table 2, experiment 2) was performed with older (5- to 6-month-old) BALB/c mice. A total of 15 mice were treated with antibiotics as described in Materials and Methods, and 14 received sterile drinking
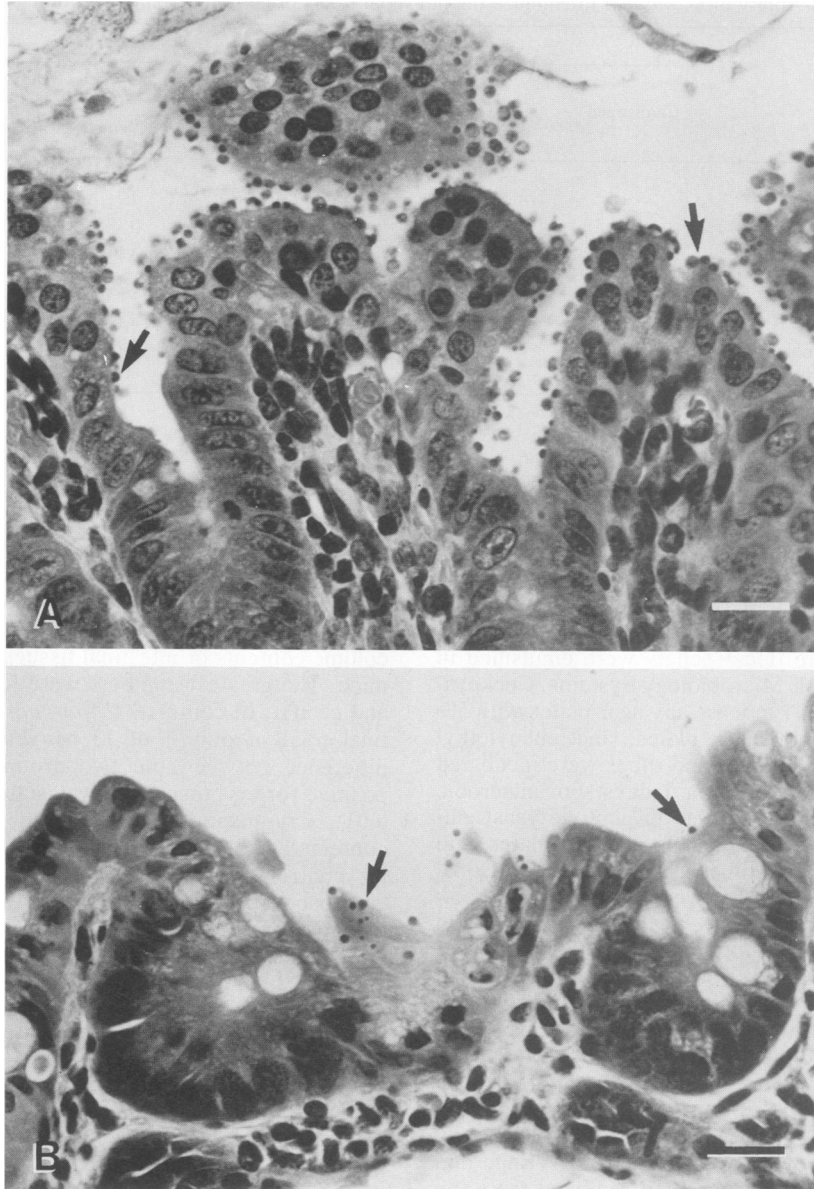
FIG. 1. Sections from large intestines of mice 7 days after challenge with *C. parvum*. Cryptosporidia (arrows) are seen on the lumenal surfaces of epithelial cells. Bars, 20 μm. (A) Conventional neonatal mouse. (B) Germfree adult mouse. Both sections were scored as 2+. Most microscopic fields in sections scored as 1+ did not contain *C. parvum*.

water without antibiotics. Antibiotic-treated mice were determined to be culture negative for intestinal flora before challenge with *C. parvum*. The mice were each challenged with 10⁵ oocysts and were killed 1 week later. None of the mice in either group had detectable cryptosporidia in colonic contents or intestinal tissue. All seven infant mice receiving oocysts as inoculum controls were colonized.

DISCUSSION

Both immunocompetent and athymic adult laboratory mice are resistant to intestinal colonization with *C. parvum* (9, 22). On the other hand, infant mice are highly susceptible to colonization (4). This susceptibility seems unaffected by the presence of anticryptosporidial immunoglobulin in the

milk of nursing dams (16) or adoptive transfer of lymphocytes from resistant adults (J. A. Harp et al., unpublished data). We noted that the onset of resistance to colonization with *C. parvum*, around 3 weeks of age, corresponded to the acquisition of a mature intestinal flora (2, 21). It has been demonstrated that gnotobiotic mice are more susceptible to colonization and dissemination by pathogenic bacteria (5, 27, 28). We therefore decided to examine the susceptibility of adult germfree mice to colonization by *C. parvum*.

Our first study demonstrated that adult germfree mice of both CD1 and BALB/c strains were colonized more readily by *C. parvum* than were age-matched mice with normal intestinal floras (Table 1). Based on numbers of mice in which cryptosporidia were demonstrated, there were clear differences between the groups. At least a few cryptosporidia were found in all germfree mice 1 week after oral

TABLE 2. Ability of C. parvum to colonize the intestinal tracts of antibiotic-treated conventional and germfree adult mice

Expt no. and animals	No. of mice					
	Challenged	Colonized[a]	With C. parvum in:			
			Colonic contents	Ileum	Colon or cecum	Lower colon
1						
CD1 mice, germfree, treated with antibiotics	9	7	0 (0.0)[b]	1 (1.0)	7 (1.3)	3 (1.0)
CD1 mice, conventional, treated with antibiotics	12	4	0 (0.0)	2 (1.0)	3 (1.0)	2 (1.0)
Infant mice, inoculum control	7	7	6 (1.2)	7 (1.7)	7 (1.7)	7 (1.7)
2						
BALB/c mice, conventional, treated with antibiotics	15	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
BALB/c mice, conventional, no antibiotics	14	0	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Infant mice, inoculum control	7	7	6 (1.5)	4 (2.0)	4 (1.8)	4 (1.8)

[a] Number of mice in which C. parvum was found in at least one of the sites examined.
[b] The number in parentheses is the mean intensity score of all positive mice within a group. See footnote b of Table 1.

challenge inoculation. In contrast, cryptosporidia were seldom seen in conventional adult mice.

There are several possible explanations for the increased susceptibility of adult germfree mice to C. parvum colonization. The physical presence of a flora in the intestine resulting in competition for receptor sites, production of anticryptosporidial agents, and stimulation of gut motility could all be involved in blocking colonization by the parasite. Alternatively (or perhaps additionally), the antigenic stimulation provided by the gut flora could be responsible for the activation of components of the immune system mediating resistance to C. parvum. If the latter were true, we hypothesized that, when depleted of intestinal bacteria by antibiotics, mice bearing normal floras should retain their resistance to colonization by C. parvum.

In experiments testing this hypothesis, we found that antibiotic-treated conventional adult CD1 mice were more resistant to colonization than were germfree adult mice receiving the same antibiotic treatment (Table 2). These results support the hypothesis and also indicate that the antibiotics used in this study had no effect on the ability of C. parvum to colonize the intestinal tract. The latter finding is in agreement with previous work that showed little or no efficacy of numerous antimicrobic agents against cryptosporidia (15, 17). In another experiment, we found that antibiotic-treated and untreated conventional adult BALB/c mice were equally resistant to colonization (Table 2).

It is likely that intestinal flora not culturable by our methods persisted in antibiotic-treated conventional mice. This residual flora may have had a role in resistance to colonization. However, in view of the increased susceptibility of laboratory mice to other pathogens following similar antibiotic treatment (3, 11, 12, 19) and the known contribution of the intestinal flora to the morphological and functional development of the immune system (7, 18), our results suggest that activation of the immune system by previous association with the intestinal flora contributed to the resistance of these mice to colonization by C. parvum. Further studies are needed to substantiate this suggestion.

The usefulness of the infant mouse as a model for studies of immune resistance to C. parvum and as a means for screening candidate anticryptosporidial drugs is limited by both the small size of the animal and the transient nature of colonization, since infant mice spontaneously clear oocysts by about 3 weeks of age, regardless of treatment (9). Several adult rodent models of cryptosporidiosis have been proposed (14, 20). We hoped that the germfree adult mouse

might prove to be a useful model, due to the larger size of the animal, possible chronicity of colonization, and defined status of the gastrointestinal milieu. Our results indicate that the usefulness of this system is limited, due to the comparatively low level of colonization seen in these animals following oral challenge. The number of cryptosporidia seen in the intestinal tissues of adult germfree mice never equaled that seen in infant mice following oral challenge (Fig. 1). Additionally, one must consider the difficulty and expense of maintaining germfree mice in evaluating the usefulness of this system.

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