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Acquired Resistance to *Giardia muris* in X-Linked Immunodeficient Mice

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A previous study from this laboratory (D. P. Snider, D. Skea, and B. J. Underdown, Infect. Immun. 56:2838–2842, 1988) indicated that immunodeficient mice expressing the *xid* gene develop prolonged infections with *Giardia muris*, unlike immunocompetent mice, which eliminate the intestinal protozoan parasite in 8 to 10 weeks. In this study, CBA/N (*xid*) and CBA/Ca mice were infected with *G. muris* cysts and at various times following this primary infection were cured by treatment with metronidazole. In contrast to the marked differences in the ability of *xid* and normal mice to eliminate a primary infection, mice of both strains were resistant to a secondary challenge of *G. muris* cysts. These data imply that the mechanism(s) responsible for elimination of a primary infection is not identical to those required to resist a secondary challenge infection. Splenocytes from immunocompetent CBA/Ca mice (but not immunodeficient CBA/N mice) could transfer the ability to eliminate a primary *G. muris* infection to irradiated mice of either strain. In contrast, splenocytes from previously infected CBA/Ca mice could not transfer resistance to a challenge infection, further supporting the hypothesis that there are differences between mechanisms required to eliminate a primary infection and those necessary to resist a second challenge infection.

Giardia muris is a protozoan parasite which infects the upper small intestine of mice (14). Mice become infected by ingesting infectious *G. muris* cysts. Conditions in the stomach, including low pH, cause excystation of cysts, which results in the release of trophozoites (3). Trophozoites reproduce by binary fission and attach to the mucosal epithelium in the upper small intestine by means of a ventral sucking disc (4). Encystation of trophozoites completes the life cycle of the parasite (6), the resulting cysts being excreted in the feces. Mice of most strains are able to eliminate a primary *G. muris* infection within 6 to 10 weeks, as evidenced by an absence of detectable cysts in the feces and trophozoites in the intestine (2). Following a natural infection with *G. muris*, mice of most strains are resistant to reinfection with the parasite (1, 15).

CBA/N mice bear an X-linked immunodeficiency gene, *xid*, the expression of which results in defective B-cell maturation with the consequent impairment of certain humoral immune responses (reviewed in reference 16). The *xid* gene is known to confer greater susceptibility to certain bacterial and parasitic infections in association with reduced or defective antibody responses (8, 9, 11, 12, 22). We have previously reported that mice expressing the *xid* gene fail to eliminate a primary infection with *G. muris*. Experiments in which mice expressing the *xid* gene were bred with normal mice indicated that prolonged infection was associated with the *xid* gene (19).

In this paper, we report that CBA/N mice that were drug cured of a primary *G. muris* infection were resistant to subsequent reinfection with the parasite. We interpret this result to indicate that the mechanisms responsible for elimination of a primary *G. muris* infection are different from the mechanism responsible for resistance to reinfection with this parasite.

MATERIALS AND METHODS

Mice. Male CBA/NJ and CBA/CaJ mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were between 6 and 10 weeks old at the beginning of the experiments. The mice were housed at the Central Animal Facility in the Health Sciences Centre at McMaster University (Hamilton, Ontario, Canada) and were maintained on standard rodent chow and water ad libitum. Female *nu/nu* (CD-1) BR (nude) mice were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). Nude mice were housed as described above, but in sterile cages with filter hoods, and were maintained on autoclaved bedding, chow, and water. *G. muris* was perpetuated in nude mice: the mice were infected when they were between 6 and 8 weeks of age, and the infection was transferred to new mice within 6 weeks.

Infection of mice with *G. muris* cysts. *G. muris* was originally obtained from I. C. Roberts-Thomson (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and was perpetuated as described above. To infect mice, *G. muris* cysts were isolated from the feces of infected, nude mice, as described below. A suspension of 5,000 cysts in 0.2 ml of saline was administered to each mouse, per os, with a blunt, curved, 16-gauge feeding needle (Popper and Sons, Inc., New Hyde Park, N.Y.).

Isolation and counting of *G. muris* cysts. The course of *G. muris* infection in mice was monitored by measuring cyst output in feces, as previously described (15). Briefly, mice were placed in individual plastic containers, and eight fresh fecal pellets were collected. The pellets were incubated in saline for 30 min at room temperature, following which the pellets were broken up in the saline with wooden applicator sticks. The resulting suspensions were layered onto 1 M

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TABLE 1. Resistance of mice to a secondary challenge infection with *G. muris*: dependence on duration of primary infection

Duration (wks) of primary infection[a]	Mice	No. of secondary-infection cysts/8 fecal pellets[b] (range of SEM) at:		
		Wk 1	Wk 5	Wk 7
0	CBA/N	320,175 (282,100–363,470)	74,296 (53,991–103,554)	24,708 (21,438–28,481)
	CBA/Ca	262,802 (243,923–287,470)	13,296 (9,646–18,329)	5,385 (4,124–7,033)
3	CBA/N	2,650 (1,904–3,689)	4,304 (2,407–7,696)	8,259[c] (5,043–13,524)
	CBA/Ca	2,785 (2,073–3,741)	<550	884[d] (724–983)
6	CBA/N	<550	ND	ND
	CBA/Ca	<550	ND	ND
9	CBA/N	<550	ND	ND
	CBA/Ca	<550	ND	ND

[a] The primary infection was terminated by treatment with metronidazole (see Materials and Methods); the secondary challenge infection was given 1 week later.
[b] Geometric mean from five mice. ND, Not done.
[c] Five of five mice infected.
[d] One of five mice infected.

sucrose (BDH Chemicals, Toronto, Ontario, Canada) and centrifuged at 300 × *g* for 10 min at 4°C. The repelleted cysts were resuspended in 1 ml of saline and counted with a hemacytometer. The limit of detection was 600 cysts from eight fecal pellets. Cysts were collected with a pipette from the region at the interface between the aqueous layer and the 1 M sucrose layer.

Drug treatment of *G. muris*-infected mice. Mice were cured of *G. muris* infection by treatment with metronidazole (Poulenc Ltd., Montreal, Quebec, Canada). Mice were treated on 3 consecutive days with 0.4 ml of 12-mg/ml metronidazole in saline. The drug was administered, per os, with a blunt, curved, 16-gauge feeding needle. One week after the last treatment, feces from the treated mice were screened for the presence of cysts. Mice were considered to have cleared their infection when no cysts were detected in two fecal samples taken on days 3 and 7 following treatment with metronidazole (limit of detection, 600 cysts from eight fecal pellets).

Adoptive transfer of cells between mice. Spleen cell suspensions were prepared in RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). The cells were washed twice by centrifugation at 300 × *g* for 10 min at 4°C and were resuspended at a concentration of 2.5×10^8 cells per ml in RPMI medium. Recipient mice received a lethal dose of 1,000 rads of gamma radiation from a ^{137}Cs source prior to reconstitution. The immune systems of the lethally irradiated mice were reconstituted with 0.2 ml of a spleen cell suspension (50×10^6 cells per mouse), given intravenously in the tail vein. Mice were infected with *G. muris* 3 weeks after the reconstitution procedure.

RESULTS AND DISCUSSION

Resistance of CBA/N and CBA/Ca mice to reinfection with *G. muris* after drug cure of a primary infection. CBA/N mice fail to eliminate a primary infection with *G. muris*, while mice of the genetically related, non-*xid*-bearing strain, CBA/Ca, eliminate the parasite within 12 weeks (19) (see Fig. 1b). We studied the ability of CBA/N mice to develop resistance to *G. muris* by drug curing their primary infection and examining their ability to resist a subsequent challenge infection. Groups of CBA/N and CBA/Ca mice were infected with *G. muris* at various time points. These primary infections were terminated, simultaneously, by treatment of the mice with metronidazole. The durations of these primary infections were 3, 6, and 9 weeks. Seven days after termination of the primary infections, the mice were challenged by reinfection with *G. muris* cysts. The levels of *G. muris* cyst output by the challenged mice were compared with those of mice undergoing a primary infection. The results are shown in Table 1. Both CBA/N and CBA/Ca mice, whose primary infections were of 6 or 9 weeks' duration, were completely resistant to the secondary challenge infection. These mice had undetectable cyst output at week 1 of the challenge infection. Protection was confirmed by undetectable cyst output at weeks 2 and 3 of the challenge infection (data not shown).

We conclude from the above data that CBA/N mice expressing the *xid* gene are capable of acquiring, through a primary infection, immune mechanisms sufficient to resist a secondary challenge of infective *G. muris* cysts. Moreover, additional experiments (not shown) indicated that the resistance acquired following primary infection was effective, even when the challenge dose of cysts was increased from 5×10^3 to 5×10^6 per mouse.

Kinetics of development of resistance to a primary infection. Following 3 weeks of a primary infection, we obtained evidence that CBA/N and CBA/Ca mice developed partial or complete resistance to a secondary challenge. In the experiment reported in Table 1, CBA/Ca mice developed a small parasite load following a secondary challenge, while in a subsequent experiment (Fig. 1b), CBA/Ca mice were totally resistant to a second infection. CBA/N mice displayed evidence of partial immunity to a secondary challenge following 3 weeks of a primary infection (Table 1 and Fig. 1a). Cysts were excreted 1 week after challenge in smaller numbers in CBA/N mice previously infected for 3 weeks than in CBA/N mice not previously infected. However, the small number of trophozoites which established themselves in partially resistant CBA/N mice continued to proliferate. Three-week-infected, drug-cured CBA/Ca mice which took on a small parasite burden following secondary challenge (Table 1) eliminated the parasite rapidly. We conclude that the progression of infection which occurred in CBA/N mice partially resistant to a secondary challenge was due to the

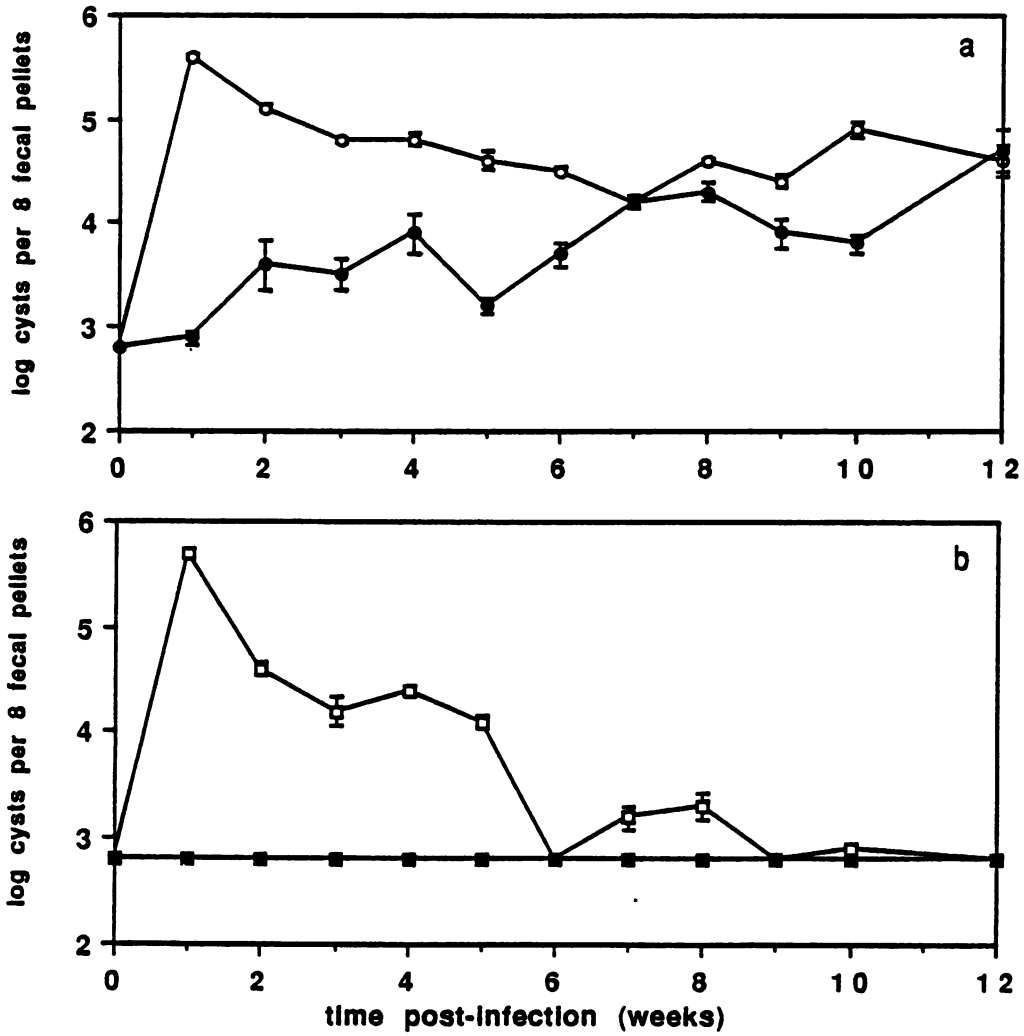

FIG. 1. The ability of mice to develop resistance to *G. muris* was tested by drug curing the mice of a primary infection with the parasite and then challenging the mice by peroral administration of *G. muris* cysts at week 0. The course of the challenge infection was monitored by measuring cyst output in feces. (a) CBA/N mice that were previously infected for 3 weeks (●) or not previously infected (○); (b) CBA/Ca mice that were previously infected for 3 weeks (■) or not previously infected (□). Each point represents the mean value for five mice; error bars represent the standard errors of the mean. The limit of detection occurred when log cysts per eight fecal pellets equaled 2.8, which corresponds to 550 cysts per eight fecal pellets.

absence in such mice of the mechanisms normally responsible for eliminating a primary infection. Clearance in CBA/Ca mice partially resistant to a secondary challenge infection may have been mediated either by the cooperation of the two types of mechanisms or by an enhanced level of mechanisms responsible for primary elimination induced by previous infection.

Adoptive transfer of spleen cells from uninfected and immune mice. The transfer of spleen cells from uninfected CBA/Ca mice to lethally irradiated CBA/Ca or CBA/N recipient mice was sufficient to allow the recipient mice to eliminate a primary infection with *G. muris* (Fig. 2). In contrast, spleen cells from uninfected CBA/N mice, when transferred to lethally irradiated recipient mice, failed to promote elimination of a primary infection with *G. muris* in either CBA/Ca or CBA/N mice. These results support the hypothesis that elimination of *G. muris* is immune mediated and that the defect of CBA/N mice that renders them susceptible to chronic giardiasis is a defect in their immune response to the parasite.

The transfer of spleen cells from mice resistant to a secondary challenge infection (that is, mice that had undergone a primary infection of greater than 6 weeks' duration) failed to transfer to recipient mice the ability to resist a challenge infection with *G. muris* (Fig. 3). The peak of mean cyst output by the reconstituted mice was equivalent to the peak of mean cyst output by mice undergoing a primary *G. muris* infection. However, the lethally irradiated recipients of spleen cells from *G. muris*-immune CBA/Ca mice eliminated the parasite more rapidly than did intact, naive CBA/Ca mice (Fig. 1) or lethally irradiated recipients of spleen cells from uninfected CBA/Ca mice (Fig. 2). The lethally irradiated recipients of spleen cells from *G. muris*-immune CBA/N mice failed to eliminate *G. muris*. The course of infection in these mice was similar to the course of infection in intact, naive CBA/N mice (Fig. 1) and that in

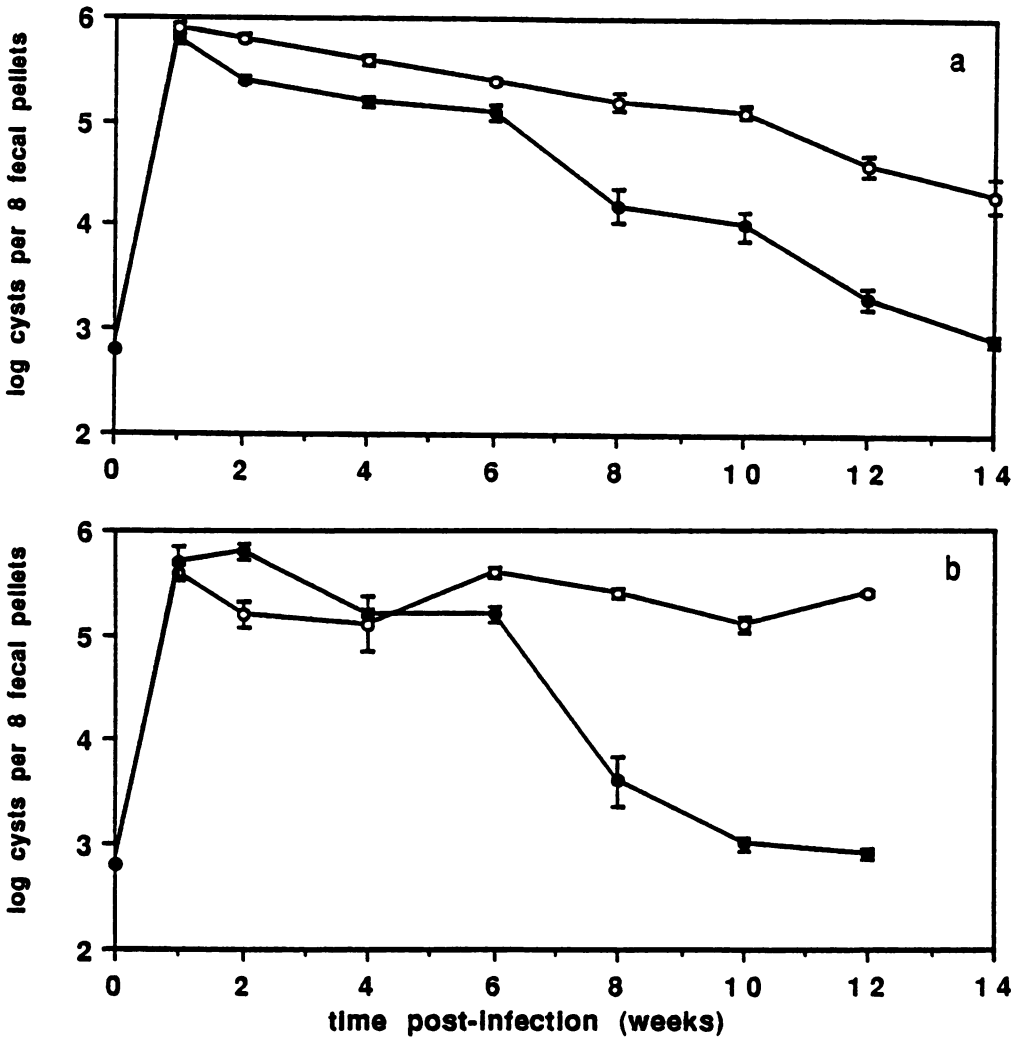

FIG. 2. The ability of spleen cells from CBA/N and CBA/Ca mice to transfer resistance to infection with *G. muris* was tested by reconstituting the immune systems of lethally irradiated recipient mice with 50×10^6 donor spleen cells and then challenging the recipient mice by peroral administration of *G. muris* cysts at week 0. The course of infection was monitored by measuring cyst output. (a) Recipient CBA/N mice receiving spleen cells from CBA/N (○) or CBA/Ca (●) mice that were not previously infected with *G. muris*; (b) recipient CBA/Ca mice receiving spleen cells from CBA/N (○) or CBA/Ca (●) mice that were not previously infected with *G. muris*. Each point represents the mean value for five mice; error bars represent the standard errors of the mean. The limit of detection occurred when log cysts per eight fecal pellets equaled 2.8, which corresponds to 550 cysts per eight fecal pellets.

lethally irradiated recipients of spleen cells from uninfected CBA/N mice (Fig. 2). The fact that the recipients of spleen cells from immune CBA/Ca mice showed a more rapid elimination of the challenge infection supports the hypothesis that the mechanism of immunity which operates to eliminate an established parasite load is inducible by a primary infection. The fact that the recipients of spleen cells from immune CBA/N mice showed a course of infection that was similar to that of a primary chronic infection in naive CBA/N mice indicates that no form of immunity was transferred to these mice.

The ability of chronically infected C3H/He mice to resist a challenge infection of *G. muris* was reported previously by one of us (20) and strengthens our hypothesis that the mechanisms involved in elimination of a primary *G. muris* infection are not identical to those involved in mediating resistance to a secondary challenge. The exact nature of the immune elements that constitute these respective mechanisms is not yet fully understood. T-cell-dependent humoral immunity appears to be important for elimination of *G. muris*, since nude mice, L3T4$^+$ T-cell-depleted mice, and B-cell-depleted mice all develop chronic *G. muris* infection (7, 13, 18). Cytotoxic T cells do not appear to play a role in the elimination of a primary *G. muris* infection, since depletion of Lyt 2$^+$ T cells in mice does not affect their ability to eliminate the parasite (7). Nonetheless, the possible involvement of other regulatory or effector immune elements has not been ruled out. The reasons that CBA/N mice are incapable of eliminating an established primary infection are not clear. CBA/N mice were shown previously to synthesize lower levels of serum immunoglobulin G (IgG) anti-*G. muris* antibody levels (50% of normal levels), but transfer of immune CBA/Ca sera to CBA/N mice does not provide CBA/N mice with the ability to eliminate a primary infection

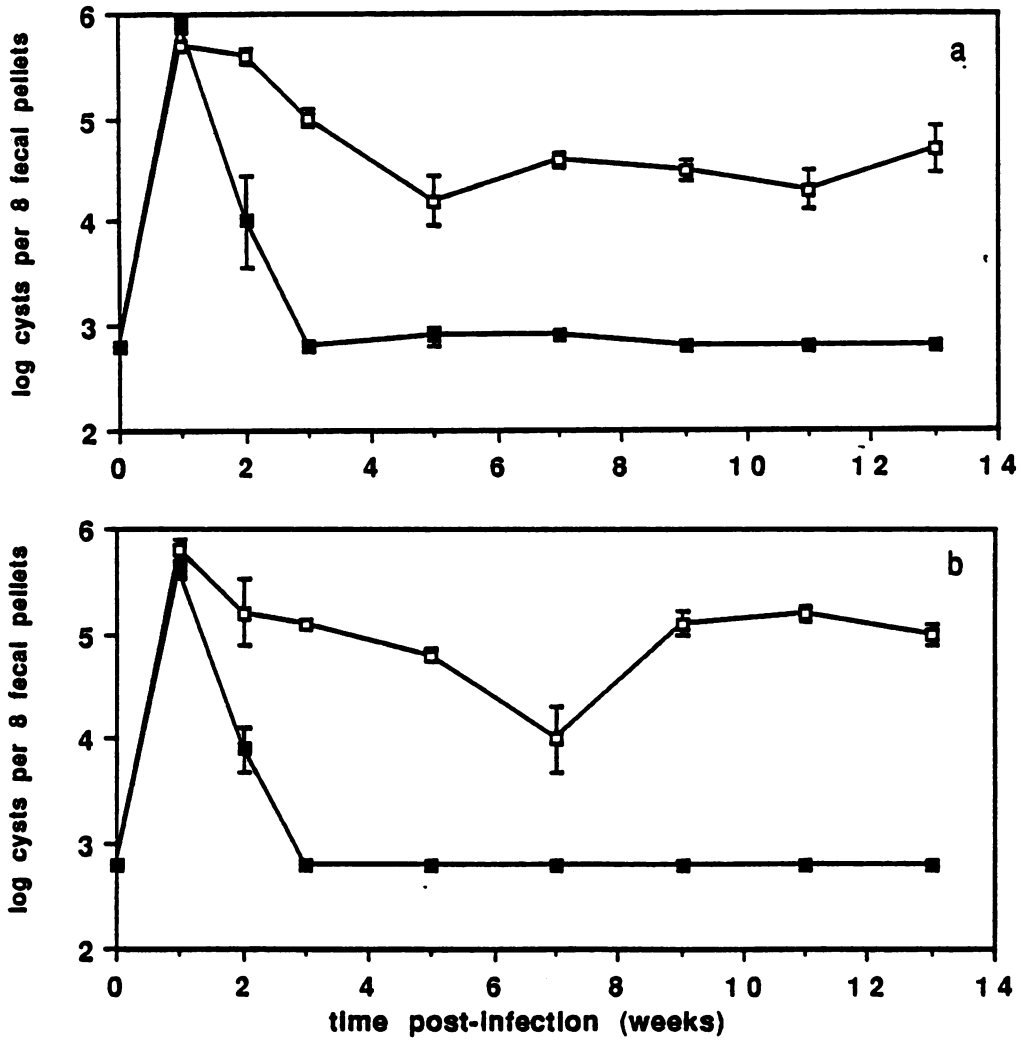

FIG. 3. The ability of spleen cells from *G. muris*-immune CBA/N and CBA/Ca mice to transfer resistance to infection with *G. muris* was tested by reconstituting the immune systems of lethally irradiated recipient mice with 50×10^6 donor spleen cells and then challenging the recipient mice by peroral administration of *G. muris* cysts at week 0. The course of infection was monitored by measuring cyst output. (a) Recipient CBA/N mice receiving spleen cells from CBA/N (□) or CBA/Ca (■) mice that were shown to be immune to *G. muris*; (b) recipient CBA/Ca mice receiving spleen cells from CBA/N (□) or CBA/Ca (■) mice that were shown to be immune to *G. muris*. Each point represents the mean value for five mice; error bars represent the standard errors of the mean. The limit of detection occurred when log cysts per eight fecal pellets equaled 2.8, which corresponds to 550 cysts per eight fecal pellets.

(17). Our previous data indicated that the IgA antibody levels were greater in CBA/N mice than in normal controls (19), presumably reflecting the prolonged infection in such mice. Despite the high level of intestinal IgA antibody directed to whole trophozoites, it is possible that a deficiency in anticarbohydrate antibodies accounts for the inability of *xid* mice to eliminate a primary infection. Recent data from our laboratory indicate that CBA/N mice have markedly reduced anti-*G. muris* lipid antibody levels. In this regard, the surface of *G. muris* is known to contain *N*-acetylglucosamine (5), which in the form of a glycolipid surface coat may be an important parasite antigen in terms of the generation of immune-mediated elimination of a primary infection. Current experiments are under way to investigate this possibility.

To be effective, the mechanism for resistance to reinfection with *G. muris* (small numbers of trophozoites that are not yet established on the intestinal epithelium) likely must be present or rapidly inducible in the small intestine following the inoculation of mice with cysts. The most plausible candidate for this mechanism is secretory IgA antibody, which may prevent the establishment of trophozoites by blocking their adherence to the small intestinal epithelium. In support of this hypothesis, it has been shown that immune mouse milk containing anti-*G. muris* IgA antibody reduced adherence of *G. muris* trophozoites to isolated intestinal loops (10). Also, *G. muris* trophozoites preincubated with gut-derived polymeric anti-*G. muris* antibody were less infectious when transferred by laparotomy to the intestines of naive mice than were trophozoites that were preincubated with similar immunoglobulin fractions from nonimmune mice (21). Moreover, it has been shown that CBA/N mice make quantitatively normal or elevated intestinal IgA antibody responses to *G. muris* infection

(19), an observation in keeping with our hypothesis, since CBA/N mice develop normal resistance to reinfection with *G. muris*.

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