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Effects of Immune Colostrum and Orally Administered Antisporozoite Monoclonal Antibodies on the Outcome of *Cryptosporidium parvum* Infections in Neonatal Mice

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A neonatal BALB/c mouse model of cryptosporidiosis was used to examine the potential passive transfer of immunity via immune colostrum and oral treatment with anticryptosporidial monoclonal antibodies. Neonates suckled by dams that recovered from *Cryptosporidium parvum* infections were equally susceptible to infection as their control counterparts suckled by naive dams. Parasite loads among the control and immune colostrum-fed mice were indistinguishable. Neonates receiving orally administered antisporozoite monoclonal antibodies were equally susceptible to infections compared with the control and immune colostrum-fed mice. Parasite loads among the mice receiving daily oral treatment with monoclonal antibody mixtures exhibited significantly lower parasite loads compared with the control mice ($P < 0.05$).

Cryptosporidium parvum is a protozoan (coccidian) parasite that infects the intestinal epithelium of a variety of mammals. Infection is often accompanied by symptoms of gastroenteritis and diarrhea (6). In immunocompetent hosts the disease is self-limiting, and resolution is accompanied by antibody production to various life-cycle stages (2, 3, 15, 26, 28, 29). Immunodeficient humans (those with acquired immune deficiency syndrome and hypogammaglobulinemia) and mice (athymic) may exhibit persistent infections (6, 8, 10, 16, 18). Humoral and cell-mediated immune responses are apparently necessary for recovery from cryptosporidiosis.

Chemotherapeutic agents have proven unsuccessful in controlling cryptosporidial infections (6). Published reports regarding the transfer of passive immunity (resistance) to cryptosporidiosis via colostrum or breast-feeding have been contradictory (9, 13, 14, 19, 22, 30). It was recently reported that cryptosporidiosis in an immunodeficient (hypogammaglobulinemic) child was successfully treated with orally administered hyperimmune bovine colostrum (27). However, the child later developed dilatation of the bile duct accompanied by the presence of cryptosporidial oocysts. If found effective, oral immunotherapeutic measures might prove useful in the treatment of persistent or severe cases of cryptosporidiosis.

The study described here was initiated to determine whether colostrum from cryptosporidiosis-recovered murine dams confers resistance to infection in their offspring. Additionally, we sought to determine whether orally administered anti-*C. parvum* monoclonal antibodies (MAbs) could modulate infections in neonatal mice.

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MATERIALS AND METHODS

Mice. Inbred BALB/c mice were used for all experimental procedures. Mice were housed in stainless steel cages containing sterilized wood shavings and were provided with pelleted food and water ad libitum. Breeding pairs were established, and pups were weaned at 3 weeks of age (sires were removed prior to parturition). Experimentally treated mice were housed separately from the main mouse colony.

Cryptosporidial oocysts. Experimental murine cryptosporidial infections were established by using oocysts purified from experimentally infected calf feces (1). The *C. parvum* isolate we used was originally obtained from Harley Moon (U.S. Department of Agriculture, Ames, Iowa). Purified oocysts were stored in 2.5% potassium dichromate at 4°C. Before the mice were inoculated, the oocyst preparations (less than 5 months old) were washed with 0.025 M phosphate-buffered saline (pH 7.2) to remove the potassium dichromate and counted with a hemacytometer by phase-contrast microscopy. Oocyst inocula of 10^4 given per os produced detectable infections in >95% of control neonatal mice (see below). Experimental suckling and adult mice were subsequently infected per os with 10^4 oocysts in 5- to 10-μl and 100-μl volumes, respectively.

MAbs. The MAbs used in this study were derived as described previously (15). These MAbs were chosen because they reacted with surface components of sporozoites (the infectious stage of the parasite). MAb C6B6 (immunoglobulin G1 [IgG1]) and MAb C8C5 (IgG3) reacted with a 20-kilodalton (kDa) sporozoite membrane antigen on Western blots (immunoblots) (15). Isotyping, Western blot analysis, and antisporozoite immunofluorescence reactivity of MAb C4A1 were performed as described previously (15); results are reported below. Ascites tumors for each hybridoma were produced by intraperitoneal injection of 2×10^6 hybridoma cells per pristane-primed mouse (7). Ascites fluids were collected and delipified with silicon dioxide (Cab-O-Sil M-5 scintillation grade; Eastman Kodak Co., Rochester, N.Y.) (20). Ascites fluids were serially diluted and applied to air-dried sporozoites in indirect immunofluorescence assays to determine the antibody titer. For long-

term storage, delipified ascites fluids were dispensed in 5-ml volumes and frozen at −70°C.

Two methods of MAb purification were used. Ascites fluid (C6B6) was thawed and centrifuged at 22,000 × *g* for 10 min in 10-ml polypropylene tubes. The supernatant was mixed with an equal volume of the column starting buffer (16 mM Tris [pH 8.5]), filtered through a 0.8-μm-pore-size membrane syringe filter (Schleicher & Schuell, Inc., Keene, N.H.), and injected at 0.5 ml per high-pressure liquid chromatographic run. A high-pressure liquid chromatographic system (Bio-Rad Laboratories, Richmond, Calif.) was used with an anion-exchange column (TSK-DEAE-5-PW; Bio-Rad). A linear ionic gradient was formed with eluting buffer (20 mM Tris, 500 mM NaCl [pH 8.5]), and the peak(s) containing active MAb was collected and pooled.

Purification of MAbs C4A1 and C8C5 differed from that of C6B6 in the following manner. Antibody was partially purified from thawed ascites fluids by using polyethylene glycol precipitation (20). MAb preparations (dissolved in Veronal [Winthrop Laboratories, Div. Sterling Drug Co., New York, N.Y.]-buffered saline, prepared from complement fixation test diluent tablets [Oxoid U.S.A., Inc., Columbia, Md.]) were centrifuged at 22,000 × *g* in 10-ml polypropylene tubes for 20 min, decanted, filtered through a 0.8-μm-pore-size membrane syringe filter, and injected onto the high-pressure liquid chromatographic column in 1.5- to 2.0-ml volumes. The starting buffer in this instance was 10 mM sodium phosphate (pH 8.0), and the eluting buffer was 50 mM sodium phosphate–500 mM NaCl (pH 8.0). Active MAb fractions were collected and pooled.

The protein concentration of each purified MAb was measured by the BCA microassay (Pierce Chemical Co., Rockford, Ill.) and adjusted to approximately 2.5 to 3.0 mg/ml by concentrating it in phosphate-buffered saline on a stirred cell filtration apparatus equipped with a 30,000-molecular-weight cutoff membrane filter (PM30; Amicon Corp., Lexington, Mass.). Antibody concentrates were filter sterilized with 0.45-μm-pore-size membrane syringe filters. Their titers were determined by immunofluorescence, and they were stored at 4°C until they were administered to mice. Table 1 summarizes the characteristics of the MAbs used in the oral passive transfer mouse experiments.

Experimental groups for colostrum studies. Four experimental colostrum treatment groups (each with a minimum of nine litters) were established. Group BC was the control group, which consisted of 6- to 10-week-old naive parents (no known prior exposure to *C. parvum*); group BW was 6- to 10-week-old dams that were previously infected as neonates and that were fed oocysts again as adults (7 to 14 days prior to parturition); group BA was 6- to 10-week-old dams that were fed oocysts as adults (7 to 14 days prior to parturition); and group BI was 6- to 10-week-old dams that were previously infected as neonates. Neonatal BALB/c mice of the various experimental groups were infected per os with 10^4 purified oocysts at 4 days of age and were necropsied 4 days postinfection. Neonatally infected female mice (to be used as dams) were raised to adulthood in an isolation room apart from the stock mouse colony. Table 2 summarizes the treatment conditions for each of colostrum-treated mouse groups.

Experimental groups treated with antisporozoite MAbs. Experimental groups (each with a minimum of five litters) included those treated with ascites fluids, purified MAbs, mixtures of ascites fluids, and mixtures of purified MAbs. Neonatal BALB/c mice born of 6- to 10-week-old control (naive) parents were infected per os with 10^4 oocysts at 4 days of age. Mice treated with individual MAbs received a single oral dose of antibody approximately 1 h prior to oocyst inoculation. Mice treated with MAb mixtures received the primary antibody dose 1 h prior to oocyst infection and subsequent doses once daily until the mice were necropsied. Controls received no antibody treatment. Table 2 summarizes the MAb and ascites fluid treatment conditions for each of the MAb-treated mouse groups.

TABLE 1. Antisporozoite MAb characteristics and treatment volumes used in oral MAb passive transfer treatments of *C. parvum*-infected neonatal BALB/c mice

Treatment	Subclass	Treatment vol (μl)	MAb quantity (μg)	Anti-sporozoite titer by IFA[a]
Purified MAb				
C6B6	IgG1	10	25	≥1,024
C4A1	IgM	5	29	≥256
C8C5	IgG3	5	30	≥128
Ascites fluid				
C6B6	IgG1	10	ND[b]	≥2,048
C4A1	IgM	10	ND	≥512
C8C5	IgG3	10	ND	≥512
Purified MAb mix (C6B6 [10 μl], C4A1 [5 μl], C8C5 [5 μl])		20	ND	ND
Ascites fluid mix C6B6, C4A1, C8C5 (all 10 μl)		30	ND	ND

[a] IFA, Immunofluorescence assay.
[b] ND, Not determined.

MAb dosages for experimental groups. Antibody dosage was based on the dosage reported for the prophylactic treatment of calves against enterotoxigenic *Escherichia coli* infections (23). Mice in the treatment groups described above received 10 μl of ascites fluid, a dosage approximately 100 times greater than that used for calves (comparing antibody volume with body weight). Mice treated with purified MAbs received 25 to 30 μg of antibody protein in 5- to 10-μl volumes of phosphate-buffered saline. Mice treated with the ascites fluid mixture received a total volume of 30 μl per dose (10 μl of each MAb). Mice treated with the purified MAb mixture received a total volume of 20 μl per dose (10 μl of C6B6, 5 μl of C4A1, and 5 μl of C8C5). Tables 1 and 2 summarize the MAb and ascites fluid treatment conditions for each of the MAb-treated mouse groups.

Examination of mice within experimental groups. Parasite infections were assessed in all of the necropsied mice following cervical dislocation and removal of the terminal ileum. A small portion of the ileum was spread onto a microscope slide (wet mount) for immediate observation of parasite life-cycle stages by phase-contrast microscopy. The remaining, larger portion of the terminal ileum was fixed in 10% buffered Formalin, embedded in paraffin, longitudinally sectioned with a microtome, and stained with hematoxylin and eosin. These longitudinal sections were examined for parasite life-cycle stages along villus surfaces. Infection rates were assessed by recording a score of plus or minus for the presence of parasites in the mucosal scrapings and in the entire histologic ileum tissue sections for each of the mice.

Parasite load quantitation in experimental mouse groups. The control group and colostrum treatment groups were each represented by nine replicate experimental litters (in

TABLE 2. Experimental group identification, treatment conditions, and infection rates for colostrum and oral MAb passive transfer-treated, *C. parvum*-infected BALB/c mice

Treatment group	Immunologic status of dam	MAb	MAb form	Treatment time day(s)[a]	No. of litters	Total no. of mice	No. of infected mice	% Infected[b]
BC	Naive	None	NA[c]	NA	13	72	71	98.6
BW	Infected as neonate and adult	None	NA	NA	11	75	70	93.3
BA	Infected as adult only	None	NA	NA	9	69	67	97.1
BI	Infected as neonate only	None	NA	NA	10	65	63	96.9
BSP	Naive	C6B6	Purified	0	5	22	21	95.5
BSA	Naive	C6B6	Ascites	0	6	26	26	100.0
BFP	Naive	C4A1	Purified	0	5	21	18	85.7
BFA	Naive	C4A1	Ascites	0	6	31	31	100.0
BEP	Naive	C8C5	Purified	0	5	23	23	100.0
BEA	Naive	C8C5	Ascites	0	6	28	28	100.0
BM	Naive	C6B6, C4A1, C8C5	Purified	0, 1, 2, 3	5	20	19	95.0
BU	Naive	C6B6, C4A1, C8C5	Ascites	0, 1, 2, 3	5	15	14	93.3

[a] Day 0 indicates mice that received antibody approximately 1 h prior to infection.
[b] The mean value was 96.6.
[c] NA, Not applicable.

groups containing more than nine litters, a subset of nine litters was randomly selected for quantitation studies). MAb treatment groups were each represented by five replicate experimental litters (in groups containing more than five litters, a subset of five litters was randomly selected for quantitation studies). Three randomly chosen mice from each litter were selected for parasite load quantitation. Two randomly chosen high-power (×400) fields were selected for each tissue section (one per mouse). While each field was randomly chosen, the tissue section was positioned to fill the field with as much villous epithelium as possible. Total parasite numbers were counted in each field, and these raw data were transformed by the square-root method (i.e., $x = [y + 0.5]^{0.5}$) to make variances independent from means before an analysis of variance was performed.

Data analysis of parasite loads in experimental groups. A model II nested analysis of variance (24) accommodating unequal sample sizes was applied to square-root-transformed numeric data collected on parasite numbers along villus surfaces, in an effort to determine the significance of treatments on parasite infections. Multiple comparisons among the treatment group means (T method [24]) were performed to identify specific treatment groups with parasite load variances significantly different from those of the control group. For illustrative purposes, the transformed means and 95% confidence limits were squared again, allowing graphic scaling to represent original parasite numbers.

RESULTS

MAbs. The Western blot (immunoblot) reactivities of MAbs C6B6, C8C5, and C4A1 (an IgM) for solubilized *C. parvum* sporozoite antigens are presented in Fig. 1. As previously reported, both MAbs C6B6 and C8C5 reacted with a 20-kDa antigen (15). The reactivity of MAb C4A1 was apparent to several antigenic bands ranging from 25 to 200 kDa. MAbs C6B6 and C8C5 showed uniform surface reactivities for air-dried sporozoites in indirect immunofluorescence assays (Fig. 2A). A pronounced polar reactivity for air-dried sporozoites was displayed by MAb C4A1 (Fig. 2B). Additionally, all three MAbs cross-reacted with antigens on merozoites in immunofluorescence assays, and results of preliminary immunoelectron microscopic studies suggest that these antigens are present in or on tissue stages of *C. parvum* (data not shown).

Cryptosporidial infection rates among mice in different treatment groups. Infection rates for mice in all of the experimental groups are summarized in Table 2. Infections were confirmed by identifying parasites in mucosal scrapings, the presence of parasites in histologic sections of the terminal ilea of all of the experimentally infected mice, or both. Infection rates varied from a low of 85.7% in group BFP to 100% in groups BSA, BFA, BEP, and BEA (see Table 2 for a description of the treatment groups). The mean infection rate for all treatment groups was 96.6 ± 4.2% (standard deviation).

Analysis of variance of treatment group parasite loads. Based on the analysis of variance, the parasite load variance between the treatment groups was significant ($P < 0.05$). The variance component representing the error (i.e., the degree to which the two counts made on each tissue section differed from one another, and thus contributed to the overall variance) was only 6.5%, which was far less than the variance contributed by differences between individual mice (33.6%), differences between litters (48.8%), or differences between treatment groups (11.1%). Indeed, preliminary counts of up to 10 high-power tissue fields per mouse showed no evidence

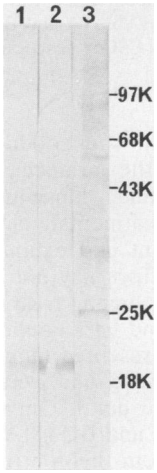

FIG. 1. Comparative Western blot (immunoblot) analysis of detergent-solubilized sporozoite antigens with MAbs C6B6 (lane 1), C8C5 (lane 2), and C4A1 (lane 3). K, Kilodaltons.

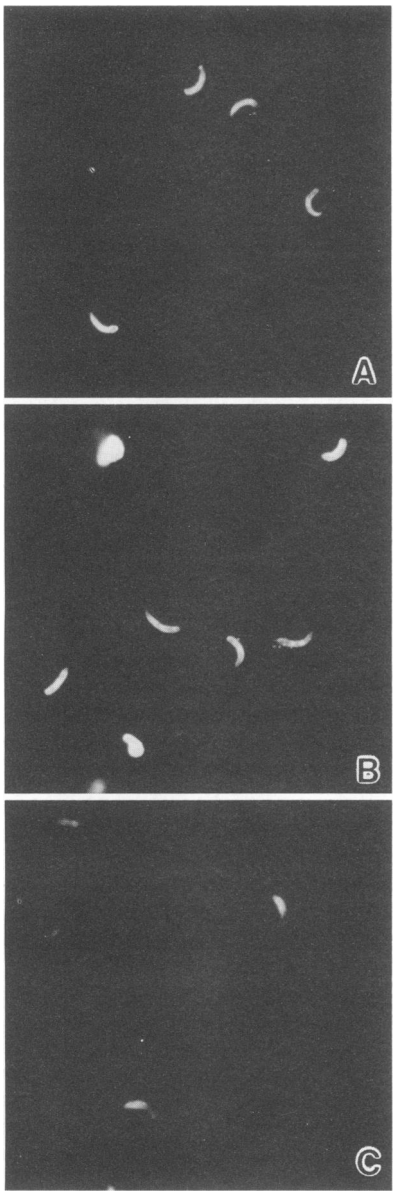

FIG. 2. Immunofluorescent appearance of *C. parvum* air-dried sporozoites labeled with MAbs. (A and B) Uniform surface reactivities typical of C6B6 and C8C5, respectively. (C) Polar reactivity typical of C4A1.

of improved results following goodness-of-fit analysis (data not shown). To reduce the variance components contributed by litters and mice (and, consequently, to improve the distinction between treatments), larger numbers of litters would require treatment and examination. The results to date suggest such an effort may not be warranted.

Multiple comparisons among treatment group means. The mean parasite loads (and 95% confidence limits) for the 12 treatment groups are presented numerically in Table 3 and graphically in Fig. 3 (these data were recalculated from the square-root-transformed data). Only treatment groups BM (purified MAb mixture) and BU (MAb ascites fluid mixture) differed significantly from the control group (BC); i.e., the 95% confidence limits for groups BM and BU did not overlap those for group BC (see Table 2 for a description of the treatment groups).

TABLE 3. Parasite loads for the control, colostrum, and MAb passive transfer treatmentgroups of *C. parvum*-infected neonatal BALB/c mice[a]

Treatment group[b]	No. of litters[c]	$\bar{x}$	95% C.L.		N^d
			+95% C.L.	−95% C.L.	
BC	9	237.53	123.43	98.70	54
BW	9	213.98	119.78	90.88	54
BA	9	170.59	106.57	80.83	54
BI	9	139.29	97.53	71.80	54
BSP	5	123.43	130.11	83.78	30
BSA	5	173.63	150.01	103.67	30
BFP	5	87.68	113.31	66.97	30
BFA	5	206.76	161.57	115.25	30
BEP	5	134.77	134.91	88.58	30
BEA	5	142.44	138.06	91.72	30
BM	5	43.35	86.54	40.21	30
BU	5	20.03	66.26	19.92	30

[a] Means and 95% confidence limits (C.L.) were recalculated from square-root-transformed parasite counts after analysis of variance.
[b] See Table 2 for treatment group definitions.
[c] Three mice per litter were randomly selected for parasite load quantitation.
[d] Number of counts per group (two counts per mouse tissue).

DISCUSSION

In the present study, mice receiving colostrum from dams with either a prior or recent history of exposure to *C. parvum* were not protected from infection. Neonatal mice orally treated with MAbs were equally susceptible to infection. The experimental treatment conditions did not abrogate parasite infections in mice. Our results confirm the observation that colostrum from mice recovered from cryptosporidiosis fails to protect neonatal mice from *C. parvum* infection (19).

Parasite loads were significantly reduced ($P < 0.05$) 4 days postinfection in two neonatal mouse treatment groups: BM (mice treated with purified MAb mixtures on a daily basis) and BU (mice treated with ascites fluid mixtures on a daily basis) as compared with the control mouse group. These treatments used MAbs that were reactive with sporozoite

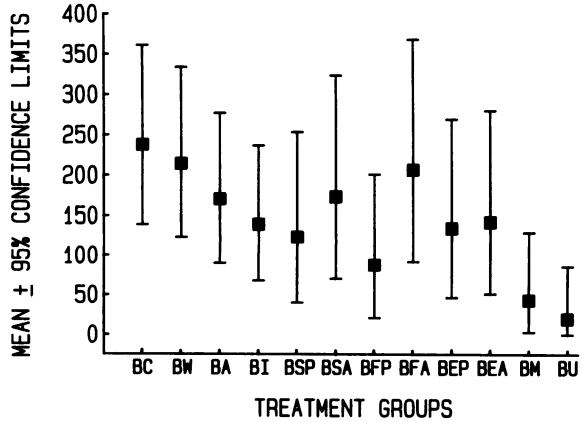

FIG. 3. Multiple comparison of the mean parasite loads ± 95% confidence limits for the control, colostrum, and MAb passive transfer treatment groups of *C. parvum*-infected neonatal BALB/c mice. Means and 95% confidence limits were recalculated from square-root-transformed parasite counts after analysis of variance. See Tables 2 and 3 for group definitions and numeric data, respectively.

surface antigens. The 20-kDa antigen identified by MAbs C6B6 and C8C5 has been identified as an immunodominant antigen recognized by immune sera from humans, calves, and horses (15). A 23-kDa immunodominant antigen of sonicated oocysts identified in a previous study (28) probably corresponds to the 20-kDa antigen of sporozoites in our study. The third MAb used in these studies (MAb C4A1) recognized multiple antigens that were localized at the sporozoite anterior region.

It could not be determined whether the reduced parasite loads in the mice from the BM and BU treatment groups correlated with a difference in clinical symptoms. While a significant reduction in parasite load was observed in these mice at 4 days postinfection, additional studies are required to address the impact of long-term treatment on cryptosporidial infections, especially regarding parasite load and severity of clinical symptoms.

An oral immunotherapeutic approach to treatment with MAbs was first attempted against enterotoxigenic *E. coli* infections in calves (23). Significant prophylaxis was demonstrated when an antipilus MAb was used. This MAb apparently prevented bacterial adhesion to the intestinal mucosa, thus averting severe disease. The significantly reduced parasite loads in mice treated with sporozoite-specific MAbs compares favorably with in vitro studies in which constant antibody exposure was necessary to inhibit eimerian sporozoite penetration of cultured host cells (4). Whereas prophylaxis against *E. coli* in calves was afforded by a single oral inoculum of MAb (23), parasite load reduction in neonatal mice occurred only after daily treatment with parasite-specific MAb mixtures at 100 times the volumetric dose for calves (comparing volume with body weight). Antibody-mediated parasite life-cycle modulation may require high titers of parasite-specific antibody in the lumen. In this regard, Fig. 3 can be interpreted as showing a biologic trend of reduced parasite loads corresponding with increased levels of lumenal antisporozoite antibodies.

In vitro exposure of apicomplexan sporozoites to stage-specific antibodies has been shown to inhibit their ability to infect cells in culture (4, 12). *C. parvum* sporozoites treated in vitro with hyperimmune bovine sera were unable to infect neonatal mice on intraanal inoculation (21). Experiments conducted in vitro may not necessarily correlate with results of experiments conducted in vivo. For example, MAb agglutinated *Eimeria tenella* sporozoites in vitro, but it also induced capping of immune complexes on the sporozoites (25). Capping mechanisms may enable sporozoites to escape antibody neutralization in the gut. In support of this idea, eimerian sporozoites were observed to be fully capable of infecting the mucosal epithelia of their immune hosts (11, 17). Effective immune responses may not prevent invasion, but rather, they may operate on infected host cells.

Reports of colostrum-mediated protection from cryptosporidiosis among other mammals are contradictory and inconclusive (6, 9, 13, 14, 22, 30). Ruminants, like calves, appear to be quite susceptible to infection even after they receive colostrum containing antiparasite antibodies (6). Recent studies suggest that hyperimmune bovine colostrum with high antisporozoite antibody titers reduced the severity of disease in experimentally infected neonatal calves (R. Fayer, C. Andrews, B. L. P. Ungar, and B. Blagburn, submitted for publication) and mice (5), but it did not prevent infection. These results are consistent with those described in the present study and suggest that antibody may not be the strongest or best mediator of protection from cryptosporidial infections. The colostrum-treated neonatal mice suckled regularly during the 4-day experiment, and as a result they should have been exposed continually to lacteal immune factors (e.g., antibody). Since we did not attempt to analyze the antibody component of the mouse colostra in our experiments, we can only conjecture that the colostra contained lower levels of antiparasite antibodies, contributing to the failure to protect (even partially) the neonatal mice. The clinical immunotherapeutic value of orally administered antiparasite antibody preparations must await answers of additional studies.

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