CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, July 1994, p. 450–463
1071-412X/94/$04.00+0

Vol. 1, No. 4

Evaluation of an Animal Model System for Cryptosporidiosis: Therapeutic Efficacy of Paromomycin and Hyperimmune Bovine Colostrum-Immunoglobulin

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Received 20 December 1993/Returned for modification 28 March 1994/Accepted 29 April 1994

Several immunodeficient rodent models currently exist in which persistent, largely asymptomatic, *Cryptosporidium parvum* infections can be established. Piglets, in contrast, develop a self-limiting diarrheal illness. We have consequently developed an animal model system in which *scid* mice were used to screen drugs for inhibitory activity against *C. parvum*, after which the drugs' therapeutic potential was evaluated with piglets. Paromomycin and hyperimmune bovine colostrum-immunoglobulin were selected to evaluate this system. *C. parvum* infections in suckling *scid* mice tended to be associated with villus surfaces, while in weaned and in older *scid* mice infections were more commonly localized in abscessed crypts. Rates of oocyst shedding in suckling *scid* mice were 50 to 200 times higher than in weaned mice and therefore made suckling mice a considerably more sensitive model for drug testing. Paromomycin given in high doses over 9 to 10 days was not toxic to either *scid* mice (3,000 mg/kg of body weight per day) or piglets (500 mg/kg/day). Paromomycin treatment was very effective against villus surface infections in suckling mice and considerably less effective against infections in inaccessible sites such as abscessed crypts and stomach pits seen in weaned and adult *scid* mice. The therapeutic efficacy of paromomycin in piglets depended on the severity of the diarrheal illness. Mild to moderate diarrhea and infection were cleared after paromomycin treatment of piglets infected with one *C. parvum* isolate. However, paromomycin had no impact on severely affected piglets infected with a second isolate, presumably because of a rapid transit time through the gut. In contrast to paromomycin, hyperimmune bovine colostrum-immunoglobulin treatment reduced the rate of *C. parvum* infection moderately in *scid* mice and only slightly in piglets, again probably because of a rapid transit time through the gut and inactivation in the stomach. It was also clear that the impact of effective drugs against *C. parvum* can be detected within 5 days after the onset of treatment in either model.

Cryptosporidiosis is a common self-limiting protozoan infection of the gastrointestinal (GI) tract of vertebrates (5, 24, 28). In humans, *Cryptosporidium parvum* is responsible for sporadic cases of diarrhea, for occasional waterborne community outbreaks of diarrhea, and for outbreaks in day-care centers (1, 11). In patients with AIDS, infection is often fatal because of persistent diarrhea and wasting (4, 5, 14, 23). No consistently effective treatment is available. It is estimated that 10 to 15% of patients with AIDS in the United States and considerably higher percentages in Africa, South America, and Haiti (23) develop persistent diarrhea due to cryptosporidiosis.

Partially successful treatments of cryptosporidiosis in immunodeficient patients have been reported with hyperimmune bovine colostrum (HBC) (17, 31, 34), spiramycin (21, 37), and paromomycin (2, 12). Anti-*C. parvum* activity of HBC and paromomycin has also been demonstrated in cell culture (6, 13, 18, 22) and in animal models (3, 7, 6, 8–10, 15, 36).

Potential animal models that develop diarrheal symptoms—a key factor in the manifestation of the disease in humans—include calves and piglets (7, 15, 29, 35). Diarrhea models, which may be more appropriate for studies of the pathophysiology of diarrhea and therapeutic evaluation of drugs, are costly and labor intensive and develop a self-limiting diarrhea. Immunodeficient rodent models in contrast are cheaper and develop persistent infections but have no symptoms of diarrhea (16, 19, 26, 32, 38).

We have devised a two-stage model system in which preliminary screening of new therapies is performed in *scid* mice and subsequent evaluation of promising compounds is conducted in the gnotobiotic piglet diarrhea model. In this communication, we have used HBC and immunoglobulin (HBC-Ig) and paromomycin to evaluate this model system. Using paromomycin, we have also compared the sensitivity for drug screening of the neonatal *scid* mouse with that of the weaned *scid* mouse.

MATERIALS AND METHODS

Management and inoculation of animals. Litters of *scid* mice (CB17; Charles River Laboratories) kept in microisolators were inoculated at 4 to 6 days of age with 10^6 purified *C. parvum* oocysts. Mice were randomized into equal groups either at onset of treatment (neonates) or at weaning. Inoculated mice were monitored for oocyst shedding by modified acid-fast staining three times weekly and weighed once a week. At necropsy, sections for histology were taken from the pyloric

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TABLE 1. Summary of oocyst shedding, diarrhea, mucosal scores, and body weight gain among experimental piglets[a]

Group and pig no.	Oocyst level and presence of diarrhea at days after inoculation[b]:											Mucosal score[c]	Body wt gain (kg)[d]
	1	2	3	4	5	6	7	8	9	10	11		
Group 1, infected control													
1	0	0	2d	4d	3d	3d	4d					14	ND
2	0	0	1d	3d	4d	3d	4d	4d	4d			13	ND
3	0	0	1d	1d	3d	3d	4d	4d	4d			14	ND
4	0	0	1d	5d	4d	5d	5d	5d	5d	4d		11	0.45
5	0	0	1d	4d	4d	5d	5d	5d	5d	4d		14	0.45
6	0	0	2d	5d	5d	5d	5d	5d	4d	4d	3d	11	0.26
7	0	0	1d	5d	5d	4d	4d	4d	4d	3d	4d	12	0.41
Group 2, HBC-Ig													
8	0	0	2d	4d	3d	4d	3d					11	ND
9	0	0	3d	2d	3d	3d	3d					9	ND
10	0	0	1d	4d	5d	1d	4d	4d	5d	4d		12	0.40
11	0	0	1	4d	4d	4d	3d	3d	3d[e]			11	0.40
12	0	0	1d	4d	4d	5d	3d	4d	4d	1d		10	0.40
13	0	0	1d	4d	4d	3d	2d	4d	4d	3d		8	0.55
Group 3, paromomycin													
14	0	0	1d	5d	3d	3d	3d	2d	2d	2	1[f]	1	0.55
15	0	0d	1d	5d	2d	3d	3d	2d	1d	1	1	0	0.55
16	0	0	2d	4d	5d	2d	1d	1d	2d	1d	1[f]	0	0.66
17	0	0	0d	5d	4d	1d	2d	1d	1d	1d	1	0	0.65
Group 4, uninfected control													
18	0	0	0	0	0	0	0	0	0	0	0	0	0.35
19	0	0	0	0	0	0	0	0	0	0	0	0	0.40
20	0	0	0	0	0	0	0	0	0	0	0	0	0.55
21	0	0	0	0	0	0	0	0	0	0	0	0	0.45

[a] Piglets 1 to 17 were inoculated with 10^7 oocysts on day 2 after cesarean delivery; piglets 18 to 21 were uninfected control animals. Group 1 (piglets 1 to 7) received placebo treatment. Group 2 (piglets 8 to 13) was treated with 4 g of HBC-Ig given in three divided doses. Group 3 was given paromomycin in three divided doses of either 200 (piglets 14 and 15) or 500 (piglets 16 and 17) mg/kg. Group 4 was uninoculated and either treated with 500 mg of paromomycin per kg in three divided doses (piglets 18 and 19) or untreated (piglets 20 and 21).

[b] The oocyst level was scored as follows: 0, no oocysts detected in fecal smear; 1, ≤10 oocysts detected in 25 HPF; 2, ≤25 oocysts detected in 25 HPF; 3, ≤50 oocysts detected in 25 HPF; 4, ≤100 oocysts detected in 25 HPF; 5, ≥100 oocysts detected in 25 HPF. Treatment was begun on day 3. d, presence of diarrhea.

[c] The mean mucosal score (± SD) for each group was as follows: group 1, 12.7 ± 1.4; group 2, 10.2 ± 1.5; group 3, 0.25 ± 0.5; group 4, 0. Scores for five body sites are summed; see Table 3, footnote *b*.

[d] The mean body weight gain (± SD) for each group was as follows: group 1, 0.4 ± 0.1; group 2, 0.44 ± 0.08; group 3, 0.60 ± 0.06; group 4, 0.44 ± 0.1. ND, not done.

[e] Piglet euthanized because of poor health associated with diarrhea.

[f] One oocyst was found in 50 HPF.

region of the stomach, terminal ileum, cecum, proximal colon, and liver-gall bladder.

Gnotobiotic piglets were housed in microbiological isolators after cesarean delivery. They were fed twice daily with reconstituted evaporated cow's milk. After inoculation with *C. parvum* oocysts, piglets were monitored daily for oocyst shedding and weighed regularly. They were observed for symptoms of depression, anorexia, diarrhea, wasting, and dehydration three to four times per day during the acute phase of the illness. At necropsy, formalin-fixed sections were taken from the pyloric region of the stomach; from the proximal (duodenum), mid (jejunum), and distal (ileum) small intestine; and from the cecum and spiral colon. A section was also taken from the gall bladder.

***C. parvum* isolates.** Isolate GCH1 was derived from an AIDS patient with cryptosporidiosis and had been propagated 13 times in experimentally infected calves. In some piglet experiments, a UCP calf isolate (ImmuCell Corp.), also repeatedly propagated in calves, was used.

HBC-Ig. The HBC-Ig (ImmuCell Corp.) was prepared from *C. parvum* hyperimmune colostrum by a commercial-scale process described previously (6). This product (lot no. 40529) was approximately 50% by weight IgG, had a reference titer of 1,760,000 U/gram (dry weight), and had been shown to be effective prophylactically in vitro and in vivo (6). The titer was established by an enzyme-linked immunosorbent assay (ELISA) method measuring antibodies against *C. parvum* oocysts and sporozoite antigens, modified from the work of Ungar et al. (33). Nonimmune bovine colostrum-Ig (NBC-Ig) consisted of sham-immunized bovine colostrum-Ig (titer = 170,000 U/g). In one piglet experiment (Table 1, control piglets 2 and 3), a commercial bovine whey protein (BWP) isolate with no detectable *C. parvum* antibody (Bipro; Davisco, Le Sueur, Minn.) was used. Bovine gamma globulins were reconstituted in sodium bicarbonate (pH 8.4) in order to reduce the impact of mouse gastric acid on IgG1, which is the major constituent of bovine colostrum.

HBC-Ig treatment of *C. parvum*-infected weaned *scid* mice. Three litters of mice inoculated after birth with 10^6 GCH1 *C. parvum* oocysts were randomized at 25 days of age into three groups of six each. Treatment with either HBC-Ig or NBC-Ig started at age 30 days and lasted for 10 days. Mice were given 30 μl orally twice daily, containing 3.3 mg of either reconstituted HBC-Ig (group 1) in sodium bicarbonate (pH 8.4) or NBC-Ig (group 2), or were untreated (group 3). Mice from the three groups were euthanized 7 days after the end of treatment.

Paromomycin treatment of *C. parvum*-infected suckling *scid* mice. Three litters of CB17 *scid* mice were inoculated at 6 days of age with 10^6 oocysts of isolate GCH1. They were randomized at age 13 days and divided into three groups of evenly distributed body weights of 7 g per mouse. Smaller and weaker mice were eliminated. Group 1 (six mice) received orally 1,500 mg/kg of body weight per day in two separate doses of

paromomycin for 10 days. Group 2 (five mice) received double the above dose of 3,000 mg/kg/day for 10 days. Group 3 (five mice) received an equivalent volume in two separate doses of phosphate-buffered saline (PBS) per day. Mice were kept for an additional 11 days following treatment before they were euthanized and sections were taken for histology.

Paromomycin treatment of *C. parvum*-infected weaned *scid* mice. Two litters of *scid* mice were inoculated after birth as described above and then weaned and randomized into two groups of six each at age 21 days. They were monitored for oocyst shedding and were weighed regularly. At age 67 days (mice averaged approximately 20 g in weight), mice in group 2 each received orally 1,500 mg/kg/day in two separate doses for 10 days. Group 1 received an equivalent volume of 30 μl of PBS. After 10 days of treatment, mice were kept for 6 days before euthanasia.

HBC-Ig treatment of *C. parvum*-infected piglets. A total of 17 of 21 piglets derived from two litters were orally challenged with 10^7 oocysts of *C. parvum* GCH1 2 days after birth (Table 1). At onset of diarrhea and oocyst shedding in the feces, piglets in group 2 were commenced on a daily oral treatment of 4 g of HBC-Ig in three separate doses for 5 or 8 days. Of seven *C. parvum*-infected animals in group 1, some received an equivalent dose of NBC-Ig (piglet 1) or BWP (piglets 2 and 3). Group 4 served as the uninfected control. HBC-Ig, NBC-Ig, and the BWP were reconstituted in sterile water and were added to the milk diet.

Animals treated with either HBC-Ig, placebo, or control received 50 mg of cimetidine (SmithKline Beecham) orally 1 h before each treatment to reduce gastric acidity. To determine the impact of cimetidine on stomach acidity, piglets were treated with 50 mg of cimetidine 1 h before necropsy, and the pHs were compared with those for untreated animals.

To test the degree of HBC-Ig degradation in the GI tract, fecal samples were collected on two occasions during the course of treatment from 11 piglets which included 6 HBC-Ig-treated, 3 placebo (NCB-Ig and BWP)-treated, and 2 uninfected untreated piglets. Quantitative ELISA analysis of fecal supernatants used reference lot no. 40529 HBC-Ig to generate a standard curve from which unknown values were interpolated.

Paromomycin treatment of piglets infected with GCH1. At onset of diarrhea (3 days postinfection), *C. parvum*-infected group 3 (Table 1) was commenced on oral treatment of paromomycin. Two piglets received 200 mg/kg/day, and two others received 500 mg/kg/day, in three separate doses for 9 days. Two uninfected control piglets from group 5 received the higher dose of paromomycin for the same period.

Paromomycin treatment of piglets infected with UCP. A total of 21 piglets derived from two litters were devoted to this study. Preliminary experiments indicated that UCP is highly virulent for newborn piglets. Therefore, the oocyst dose was reduced to 10^6 and the age of inoculation was increased to 3 days. At onset of diarrhea (day 3 postinfection), paromomycin treatment of 335 or 500 mg/kg/day in three separate doses was administered orally to group 6 and group 7, respectively. Group 5, which was also infected, was the untreated control. All piglets in groups 5 to 7 became severely ill and either were euthanized within 1 to 6 days after onset of diarrhea or had died (Table 2).

In a subsequent experiment, groups 8 and 9 were challenged at age 5 days with 10^6 oocysts of UCP. At onset of diarrhea, 3 days after inoculation, group 9 was commenced on paromomycin treatment of 500 mg/kg/day in three separate doses for 8 days. Group 8 received placebo (PBS). Infected piglets were euthanized on day 10 after inoculation together with the control group 10 (Table 2).

Methods for oocyst counting and analysis. In mice, the number of oocysts in 25 high-power microscopic fields (HPF) of modified acid-fast-stained fecal smears per mouse was counted. Data are presented as the daily group mean ± standard error for each group. Oocysts in infected piglets were excreted in larger quantities than in mice and were therefore graded as outlined in the footnotes to Tables 1 and 2.

The methods for scoring the extent of mucosal infections for mice and piglets were similar (see footnotes to Table 3) except for the number of sites included for each system. In mice, the extent of infection was assessed in four sites (stomach, ileum, cecum, and colon), with a maximum score of 20. The hepatobiliary system, which was not consistently infected, was not included in the score. In piglets, five sites (proximal, mid, and distal small intestine; cecum; and colon) were assessed, with a maximum score of 25. The stomach was not consistently infected and therefore was not included in the total score.

Data analysis. Measures of outcome were weight, oocyst count, and mucosal infection, expressed as scores. In general, analysis of variance was used to analyze weight, oocyst scores for piglets, log oocyst count for mice, and mucosal infection scores. The Newman-Keuls test was used for post hoc analysis, where necessary. Statistical analyses were computed with Number Cruncher Statistical System, version 5.03, 1990 (Jerry L. Hintze, Kaysville, Utah).

RESULTS

Response of infected mice to treatment with HBC-Ig. The oocyst shedding patterns after treatment with either HBC-Ig (group 1), NBC-Ig (group 2), or bicarbonate (group 3) did not differ from each other. Despite the high HBC dose (6.6 mg/kg/day), two-way analysis of variance, with log of oocyst shedding as the dependent variable, showed no significant interaction between group and day after treatment ($P = 0.47$), and no significant main effects of group ($P = 0.21$) or day after treatment ($P = 0.32$). Moreover, there was no significant difference in the mean body weights among the three groups ($P = 0.28$). However, 7 days after the end of a 10-day course of treatment, the extent of mucosal infection, expressed as a total score of the four affected sites, averaged 11.8 (± standard deviation [SD] of 1.5) for the mice treated with HBC-Ig, 14.2 (± 1.5) for those treated with NBC-Ig, and 16.0 (± 0.7) for those treated with bicarbonate. These responses differed significantly from each other (analysis of variance, $P < 0.001$; Newman-Keuls test, <0.05). The 7-day time point was chosen to determine the long-term impact of HBC-Ig treatment. Oocyst shedding did not reflect any differences among the three groups in the manner in which the mucosal scoring did.

Paromomycin treatment of infected suckling *scid* mice. Paromomycin-treated groups 1 and 2 remained healthy throughout the experiment despite the high daily paromomycin dosage (Fig. 1). Three days after treatment began, there was a decline in oocyst shedding in both treated groups. Oocyst shedding dropped below detectable levels in mice receiving either dose within 6 days of treatment, while the placebo group continued shedding (Fig. 1). The two treated groups remained below detectable levels for at least 11 days after the end of treatment. Histological examination, however, revealed that residual infections were present in the mucosa of all 11 treated mice, but at a much reduced level compared with the placebo group. The extent of mucosal infection, expressed as a total score of the four affected sites, averaged 4.8 (± 2.4) for the

TABLE 2. Summary of oocyst shedding, diarrhea, mucosal scores, and body weight gain among experimental piglets[a]

Group and pig no.	Oocyst level and presence of diarrhea at days after inoculation[b]:										Mucosal score[c]	Body wt gain (kg)[d]
	1	2	3	4	5	6	7	8	9	10		
Group 5, infected control												
22	0	0	1	5d[e]							25	−0.15
23	0	0	1d	5d	5d[e]						24	−0.40
24	0	0	1d	5d							ND	−0.30
25	0	0	0d	3d	5d	5d[e]					25	−0.45
Group 6, paromomycin (335 mg)												
26	0	0	5d	5d	4d[e]						23	−0.475
27	0	0	2d	5d	5d[e]						25	−0.50
28	0	0	1d	5d	1d	3d					ND	−0.40
29	0	0d	1d	5d	4d	3d[e]					24	−0.20
Group 7, paromomycin (500 mg)												
30	0	0	1	5d	5d[e]						21	−0.60
31	0	0	1d	5d[e]							25	−0.45
32	0	0	1d	5d							ND	−0.25
33	0	0	4d	5d	2d	4d[e]					22	−0.30
34	0	0d	4d	5d	3d	2d	2d	4d	3d[e]		14	−0.40
Group 8, infected control												
35	0	0	0d	1d	3d	3d	3d	2d	2d	3d	14	+0.05
36	0	0	0d	4d	3d	4d	5d	4d	4d	3d	14	+0.20
37	0	0	0d	1d	3d	4d	5d	5d	5d	4d	14	+0.20
Group 9, paromomycin (500 mg)												
38	0	0	0[f]	0d	1d	1d	0d	0d	0d	0[f]	0	+0.65
39	0	0	1d	4d	1d	4d	3d	5d	4d	3d	13	+0.00
40	0	0	0d	1d	1d	1d	1[f]	1d	0d	0[f]	4	+0.30
Group 10, uninfected control												
41	0	0	0	0	0	0	0	0	0	0	0	+0.65
42	0	0	0	0	0	0	0	0	0	0	0	+0.50

[a] Piglets were inoculated with 10^6 UCP calf isolate oocysts. Of groups 5 to 7, which were challenged at age 2 days, group 6 (piglets 26 to 29) was infected and treated with 335 mg/kg/day in two divided doses, and group 7 (piglets 30 to 34) received 500 mg/kg/day in three divided doses. Of groups 8 and 9, which were challenged at age 5 days, group 8 (piglets 35 to 37) was the infected untreated control and group 9 (piglets 38 to 40) received 500 mg/kg/day in three divided doses. Group 10 (piglets 41 and 42) was the uninfected untreated control.

[b] Treatment was begun on the third day. The oocyst level was scored as follows: 0, no oocysts detected in fecal smear; 1, ≤10 oocysts detected in 25 HPF; 2, ≤25 oocysts detected in 25 HPF; 3, ≤50 oocysts detected in 25 HPF; 4, ≤100 oocysts detected in 25 HPF; 5, ≥100 oocysts detected in 25 HPF. d, presence of diarrhea.

[c] The mean mucosal score (± SD) for each group was as follows: group 5, 24.7 ± 0.6; group 6, 24.0 ± 1.0; group 7, 20.5 ± 4.7; group 8, 14; group 9, 5.7 ± 6.6; group 10, 0. ND, not done; piglet was dead. Scores are sums for five body sites; see Table 3, footnote *b*.

[d] The mean body weight gain (± SD) for each group was as follows: group 5, −0.33 ± 0.13; group 6, −0.39 ± 0.14; group 7, −0.4 ± 0.14; group 8, 0.15 ± 0.09; group 9, 0.32 ± 0.33; group 10, 0.58 ± 0.11.

[e] Piglet euthanized because of poor health associated with diarrhea.

[f] Loose feces.

mice treated daily with 1,500 mg/kg, 4.5 (± 1.0) for those treated with 3,000 mg/kg, and 16.6 (± 2.1) for the placebo-treated control animals. The two treated groups were statistically indistinguishable, but both differed significantly from the placebo group (analysis of variance, $P < 0.001$; Newman-Keuls test, <0.05).

The responses of the four sites to treatment were different; in the proximal colon (which in the untreated animals was the most extensively infected and histologically altered), parasite forms were present in much-reduced numbers in the two treated groups. In contrast, only a marginal reduction in parasite numbers was observed in the stomach sections of treated animals as compared with untreated controls (Fig. 2). Doubling the dose of paromomycin to 3,000 mg/kg/day did not result in the elimination or a significant reduction in the extent of mucosal infection compared with the lower dose of paromomycin (Fig. 3).

Paromomycin treatment of infected weaned *scid* mice. *C. parvum*-infected mice continued to gain weight until they reached an average weight of 19 to 20 g at age 55 to 60 days, after which weights in the treated and the control groups began to decline. After the onset of treatment with 1,500-mg/kg/day paromomycin, oocyst shedding in group 2 declined to below detectable levels in all six mice within 6 days. Shedding,

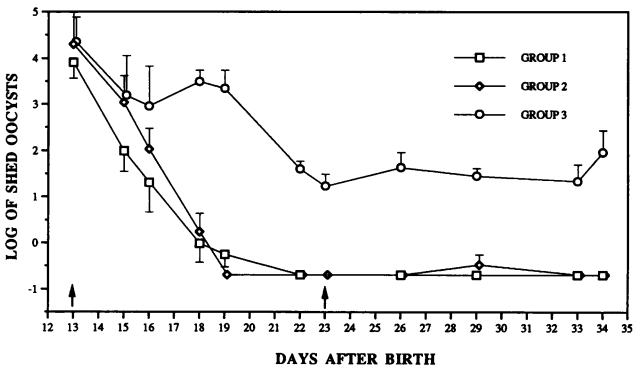


FIG. 1. Oocyst shedding in three *C. parvum*-infected groups of suckling *scid* mice, two of which were commenced on paromomycin treatment (left arrow) of either 1,500 mg/kg/day (group 1; six mice), or 3,000 mg/kg/day (group 2; five mice) for 10 days (right arrow). Group 3 (five mice) received a placebo. Two-way analysis of variance, with log of oocyst shedding as the dependent variable, and group and day after onset of treatment as factors, shows significant difference among the three groups ($P < 0.0001$). The Newman-Keuls test ($P < 0.05$) shows that responses to the treatments did not differ while both treatment groups differed from the placebo group.

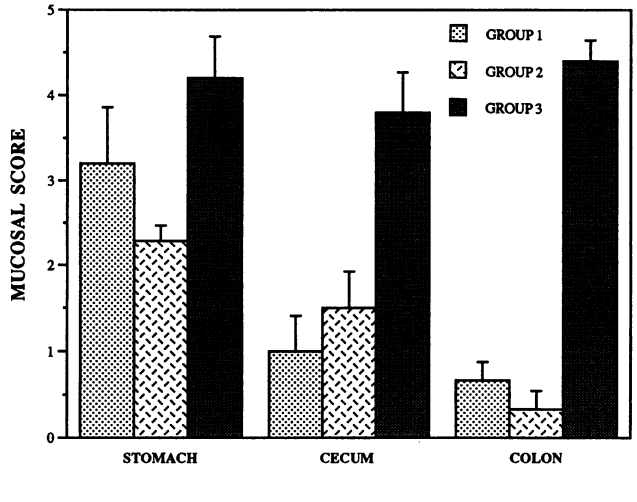


FIG. 2. The impact of paromomycin treatment on extent of mucosal infections in three selected GI sites of the three groups of suckling *scid* mice described in the legend to Fig. 1. Paromomycin had less impact on infection in the stomach pits than in the other two sites. Two-way analysis of variance, with mucosal infection expressed as a score as dependent variable, showed a significant interaction between group and site ($P < 0.05$), and both main effects, group and site, were significant ($P < 0.0001$).

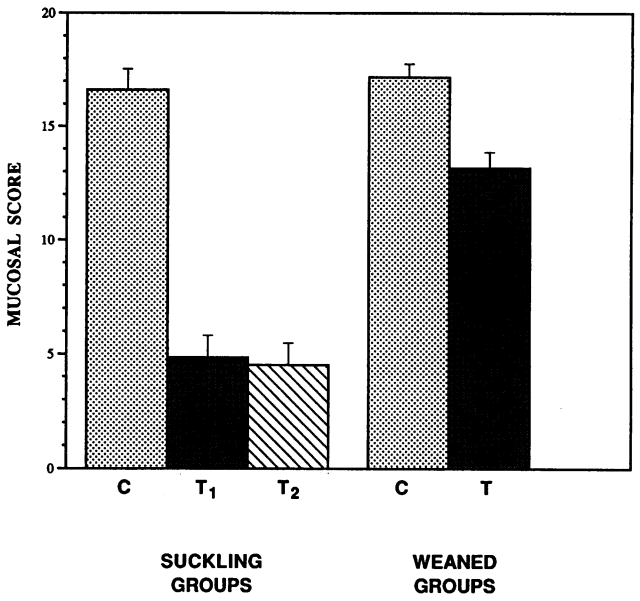


FIG. 3. Extent of mucosal infections in three suckling groups of *scid* mice (Fig. 1), compared with the two weaned *scid* groups (Fig. 2). One-way analysis of variance of mucosal infection, expressed as a score, shows significant differences among the five groups of animals ($P < 0.0001$). The Newman-Keuls test shows ($P < 0.05$) that neither the two control groups (C), nor the two treated suckling groups (T_1 and T_2), respectively, differed from one another. T_1 and T_2, however, differed from two control groups (C) and from the weaned treated groups (T), which differed from all others.

however, reemerged 5 days after the end of treatment and climbed quickly to the level of the control group (Fig. 4).

The extent of mucosal infection, expressed as a total score of the four affected sites, averaged 13.2 (± 1.7) for the mice treated with a daily dose of 1,500 mg/kg and 17.2 (± 1.5) for the placebo-treated control group, a statistically significant difference (*t* test, $P = 0.002$). While paromomycin treatment decreased the extent of mucosal infection, the magnitude of the decrease was less than that of the decrease seen in the suckling *scid* mice (Fig. 3). In contrast, the mucosal architectural alterations in infected mice at age 67 days were much more extensive (Fig. 5 and 6) than in infected suckling mice (Fig. 7). This extensive mucosal damage may explain the loss of body weight in both weaned groups. Even in the paromomycin-treated group, which had a significantly milder infection compared with the infected untreated group, extensive mucosal damage was still apparent. As in the suckling mice, the severity of infection in the stomach sections (Fig. 8) was comparable in both groups.

Figure 3 compares the extent of mucosal infections of the two paromomycin-treated suckling groups with the treated weaned group. One-way analysis of variance of extent of mucosal infection, expressed as a score, showed significant differences among the five groups of mice ($P < 0.0001$). The Newman-Keuls test shows ($P < 0.05$) that the two control groups did not differ from each other, nor did the two suckling treatment groups, which differed significantly from the two control groups, differ from each other. The weaned treatment group differed significantly from all other groups.

HBC-Ig treatment of *C. parvum*-infected piglets. Treatment with HBC had a statistically significant effect on *C. parvum*-infected piglets (group 2) in terms of oocyst shedding as compared with infected untreated animals of group 1 (Fig. 9). However, the extent of mucosal infections, expressed as scores, of the HBC-Ig-treated group 2, which averaged 10.2 (± 1.5), did not significantly differ from that for the control group 1, with a score of 12.7 (± 1.4) (Table 1).

On the basis of the administration of 7.04×10^6 U/day ($4 \times 1{,}760{,}000$ U of HBC-Ig per g), the expected specific antibody activity in approximately 200 ml of feces per piglet per day is 35,000 U/ml. The actual residual *C. parvum* antibody concentration in feces was 1.3% (0.2 to 9% in range or 468 ± 387 U/g)

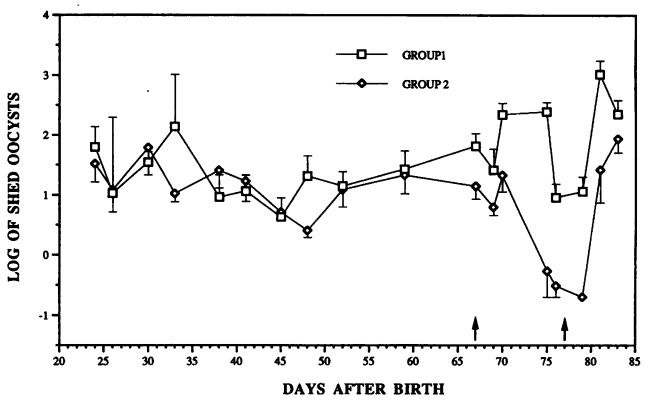


FIG. 4. Oocyst shedding in two *C. parvum*-infected groups of weaned *scid* mice of which group 2 (six mice) was commenced at age 67 days (left arrow) on paromomycin treatment of 1,500 mg/kg/day for 10 days (right arrow). Group 1 (six mice) received a placebo. Two-way analysis of variance, with log of oocyst shedding as the dependent variable, and group and day after onset of treatment as factors, shows significant difference between the groups ($P < 0.0001$).

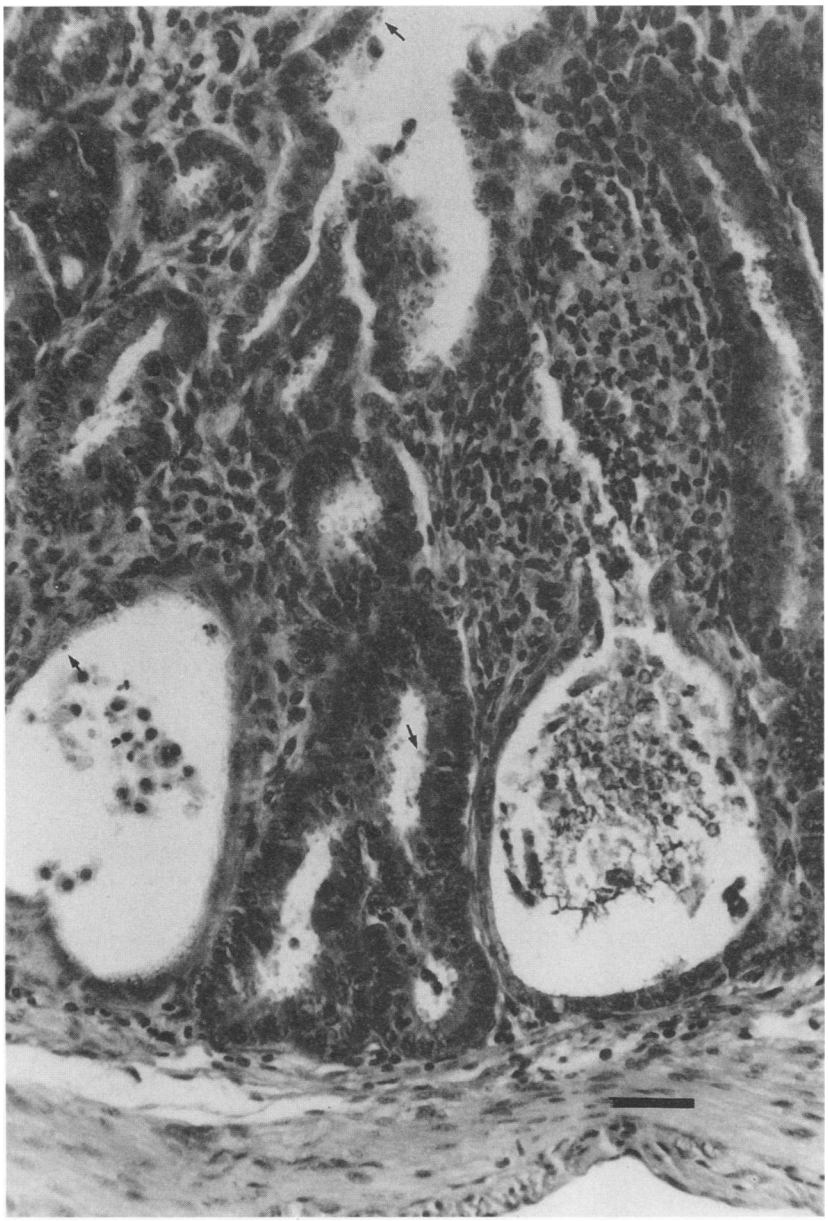


FIG. 5. A colonic section from an untreated weaned *scid* mouse showing *C. parvum* forms (arrows) infecting mucosal surface and crypts, with heavy cellular infiltration of the lamina propria and crypt abscessation (Fig. 4) (hematoxylin and eosin stain). Bar, 40 μm.

in piglets fed HBC. This indicated that a considerable proportion of the *C. parvum* antibody activity in the HBC was lost during transit through the GI tract.

One possible cause of this profound loss in activity was the gastric acidity of the stomach, which could have inactivated the IgG in the HBC-Ig. Treatment with cimetidine 1 h before necropsy increased the stomach pH only marginally, from 1.80 (two non-cimetidine-treated piglets) to 3.13 (SD, ± 0.3) in seven cimetidine-treated piglets. This indicated that cimetidine did not appreciably suppress stomach acidity. Additional losses of Ig activity may have also resulted from GI tract proteolysis.

Paromomycin treatment of piglets infected with GCH1. Treatment of piglets infected with GCH1, with 200 or 500 mg of paromomycin per kg/day, either eliminated or markedly reduced oocyst shedding and the extent of mucosal infection compared with HBC-Ig-treated piglets of group 2 or untreated group 1 (Table 1; Fig. 9). Remarkably, diarrhea also ceased in the four treated piglets compared with the two control surviving animals of group 1 (piglets 6 and 7 [Table 1]). In three of four piglets, no infection was detected in the five sites examined. The body weight gain data indicated insignificant differences among the four groups (Table 1).

The pattern of oocyst shedding, among the HBC-Ig-treated group 2, the paromomycin-treated group 3, and the untreated control group 1 is illustrated and analyzed in Fig. 9. The extent of mucosal infection, expressed as a score, averaged 12.7 (± 1.4) for the infected control pigs, compared with 0.25 (± 0.7) for the paromomycin-treated piglets. The two groups of animals differed statistically (analysis of variance, $P < 0.0001$).

Paromomycin treatment of piglets infected with UCP. All

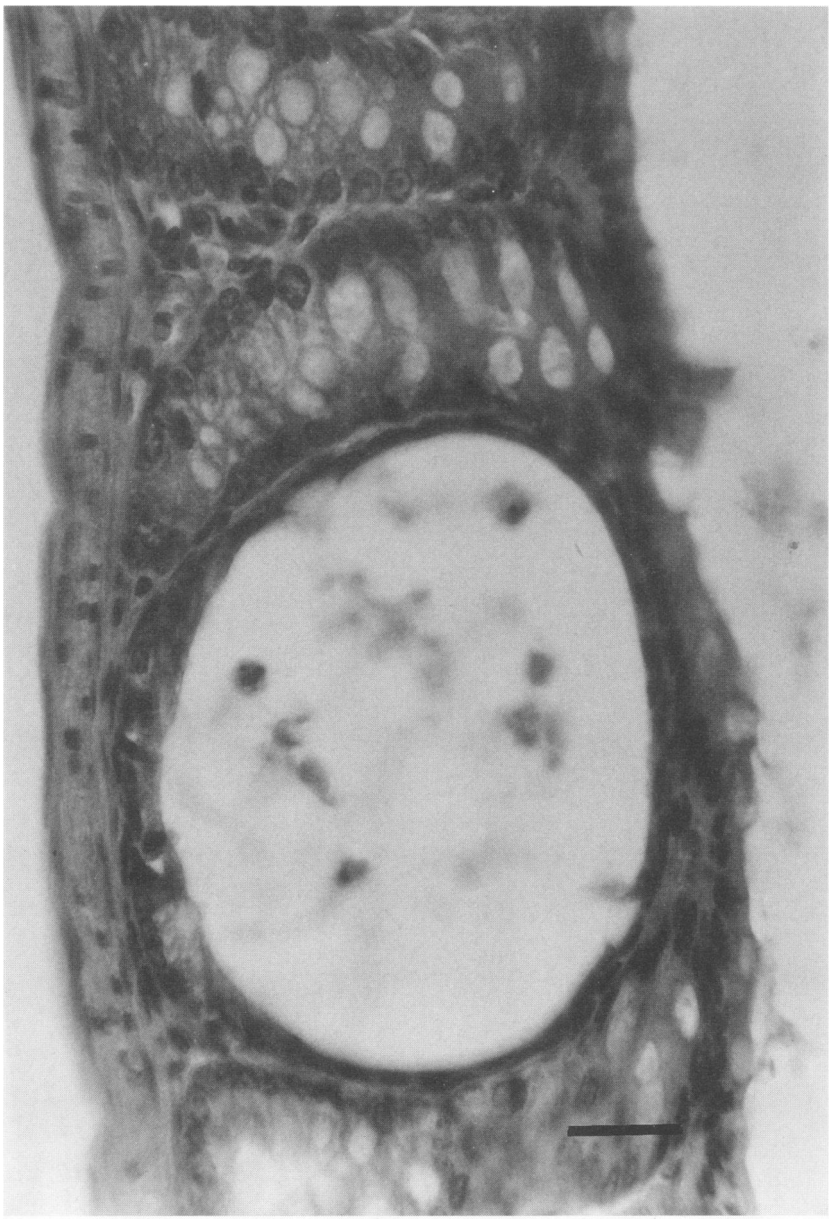


FIG. 6. A cecal mucosal section from a *C. parvum*-infected weaned *scid* mouse taken after paromomycin treatment (Fig. 4). Note abscessed crypt in otherwise almost normal mucosa. Bar, 40 μm.

piglets infected with *C. parvum* UCP 3 days after birth developed severe illness, and within 2 days of the onset of diarrhea, they developed anorexia, dehydration, and wasting. They had to be euthanized because of the severe illness. This was despite the reduced dose of parasites (10^6 UCP oocysts instead of 10^7 GCH1 oocysts) and older age in the UCP group (3 days versus 2 days, UCP versus GCH1 piglets, respectively). Treatment with either 335 mg (group 6) or 500 mg (group 7) of paromomycin per kg/day had no impact on the course or the outcome of illness (Table 2). Treated piglet 34 (group 7), which survived 7 days, also failed to recover. In these piglets, the entire small and large intestinal mucosa was infected (Table 3) and severely damaged, with villus contraction and little or absent epithelial surface layer (Fig. 10). There was also a profound weight loss due to severe dehydration and wasting (Table 2).

In the experiment in which groups 8 and 9 were inoculated at age 5 days with the UCP isolate, animals were less severely affected. They developed diarrhea within 3 days but continued to drink their milk and gained some weight. In the three animals in group 9, which received 500 mg (750 mg per piglet) of paromomycin per kg/day for 8 days, a spectrum of clinical responses was seen. Piglet 38 had a delayed onset of diarrhea and a rapid response to treatment (Table 2). This piglet shed a few oocysts for 2 days only, and at necropsy, the five mucosal sites examined were free of infection and with almost intact morphology. Piglet 38 also gained the most weight (Table 2). In contrast, piglet 40 shed more oocysts and for a longer period (4 days), there were still residual infections in the stomach and duodenum, and the mucosa was moderately altered. Piglet 39, whose onset of diarrhea and shedding was the earliest in the

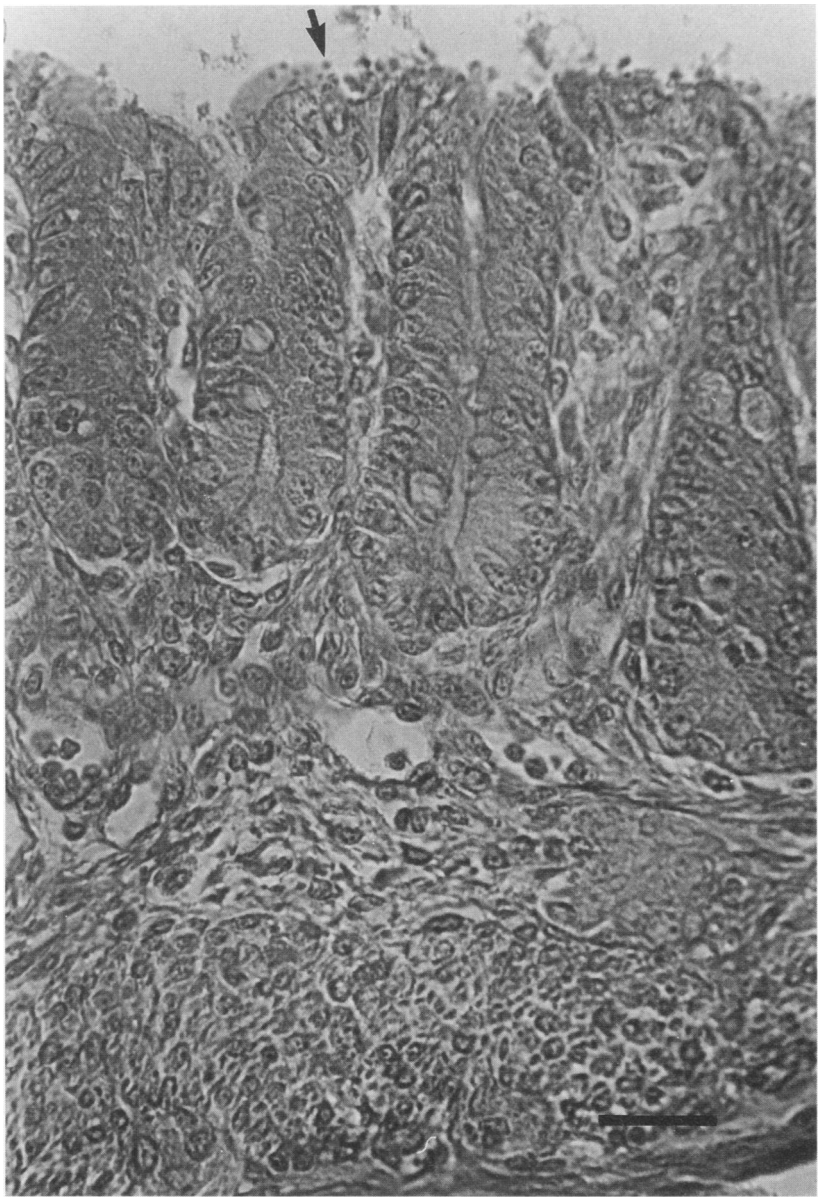


FIG. 7. A colonic mucosal section from an untreated suckling mouse showing infection with *C. parvum* forms confined to mucosal surface (arrow) with no crypt involvement (Fig. 1). Bar, 40 μm.

group, developed diarrhea and shed oocysts in the same order as the untreated group 8 (Table 2). Mucosal infection in this animal was also as extensive as for the untreated piglets in group 8.

Table 3 summarizes the extent and distribution of mucosal infections of piglets challenged with UCP, measured in five intestinal sites. The entire small and large intestines were heavily infected in groups challenged on day 3 and killed 4 to 7 days thereafter, irrespective of whether they were treated with paromomycin (groups 6 and 7) or with placebo (group 5). In groups 8 and 9, which were challenged on day 5 and had survived for at least 10 days, mucosal infections had moved with time, from the proximal to the distal part of the GI tract.

DISCUSSION

We have selected paromomycin and HBC to evaluate an animal model system, with the *scid* mouse to screen drugs for inhibitory activity and the gnotobiotic piglet for their therapeutic evaluation. Mice treated with high levels of oral paromomycin (3,000 mg/kg/day) showed no signs of toxicity. Doses of 500 mg/kg/day (750 mg per animal) were also well tolerated by piglets. Of the compounds tested so far in our system (elbendazole, azythromycin, colchicine, and combined paromomycin-azythromycin), paromomycin was the most effective and least toxic in the mouse and in the piglet models. HBC-Ig significantly reduced the rate of infection in treated mice, as reported by others (9, 10, 35), but was considerably less effective than paromomycin.

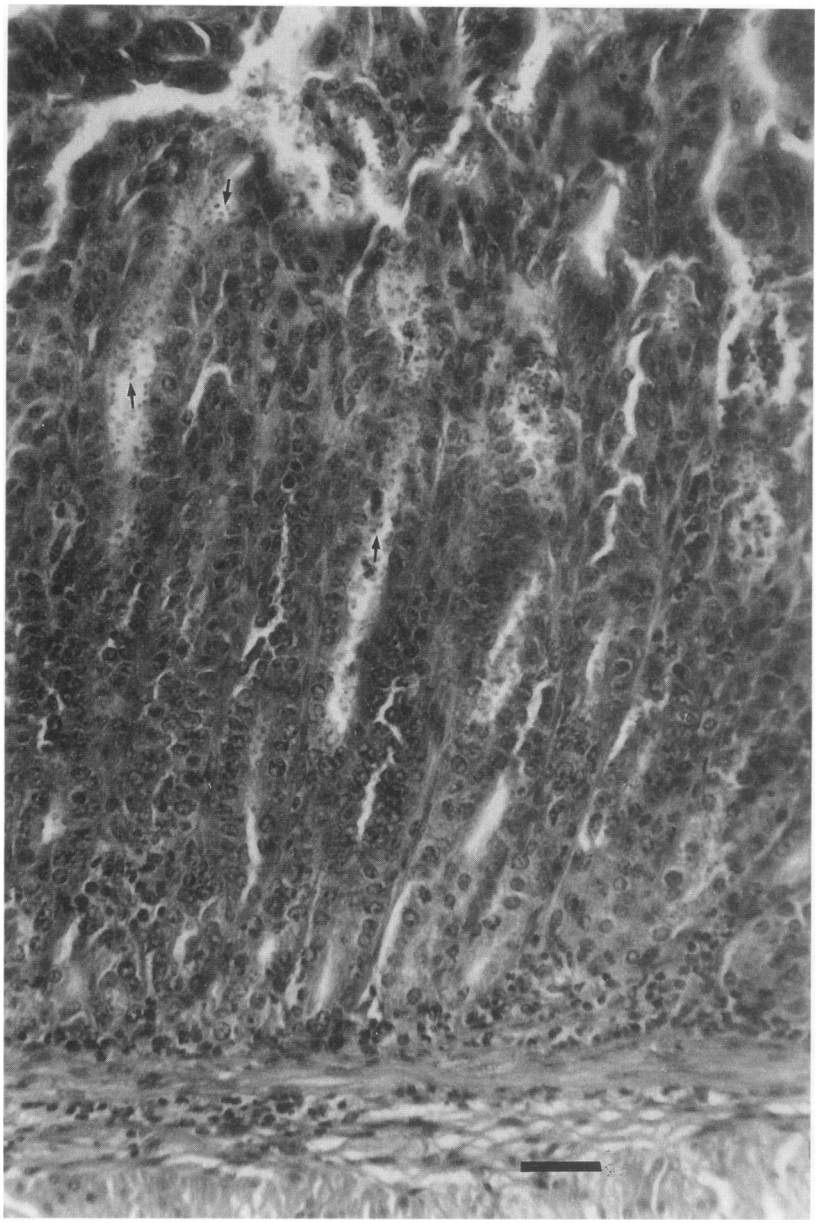


FIG. 8. A mucosal section from the pyloric region of the stomach of a *C. parvum*-infected weaned *scid* mouse showing parasite forms (arrows) deep within pits. Bar, 40 μm.

In general, we found the suckling *scid* mouse model to be superior to the weaned mouse model. Infected suckling *scid* mice began shedding oocysts shortly after inoculation. Shedding remained high between 10 and 20 days of age and continued at reduced levels for up to 65 days of age after which it gradually increased with age. This pattern of infection has now been observed in over 12 studies and was found to be independent of strain and origin of *scid* mice or the origin of the *C. parvum* (data not shown). The response to paromomycin was a good measure of sensitivity and simplicity in the suckling *scid* mouse.

Oocyst shedding was a less sensitive indicator of infection in mice than mucosal infection, as was evident from HBC-Ig and paromomycin experiments. Oocysts were not detectable in feces after paromomycin treatment despite evidence of mucosal infection. Oocyst shedding was not a sensitive indicator of slight variation in the degree of infections, nor was it useful in determining whether infections were eliminated as a consequence of treatment. Indeed, 11 days after the end of a course of paromomycin treatment in the suckling groups, oocysts were still undetectable in feces although all were infected as observed histologically.

In contrast, the scoring system used accurately reflected small changes in the extent of infections within individuals or groups. This was well reflected in the extent of mucosal infections in mice treated with HBC-Ig, NBC-Ig, and placebo. In suckling mice, paromomycin cleared the infection from all luminal epithelial surfaces, which were the predominant location of infection. Paromomycin in weaned mice was considerably less effective presumably because of the poor accessibility

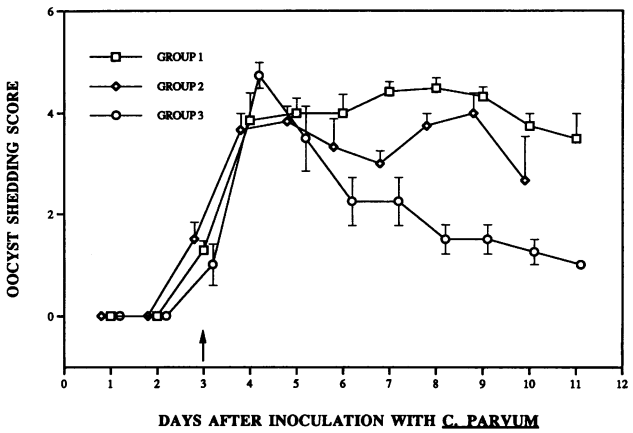


FIG. 9. Oocyst shedding of three *C. parvum*-infected groups of piglets with moderate diarrheal illness treated (arrow indicates onset) with either HBC (group 2; six piglets) or paromomycin (group 3; four piglets) or not treated (group 1; seven piglets). Two-way analysis of variance, with oocyst shedding score as the dependent variable, and group and day after treatment as factors, shows significant difference among the three groups ($P < 0.0001$). The Newman-Keuls test ($P < 0.05$) shows that the response of each of the groups of animals differs from the responses of the other two groups.

of areas such as the pits of the pyloric region of the stomach, the cecum, and most of the infected crypts that were abscessed or distorted. Doubling the dose of paromomycin to 3,000 mg/kg/day had no significant effect on clearing infections from all sites. The morphology of the distal ileum in the treated suckling groups was restored remarkably well after treatment; less well restored was that of the proximal colon. Importantly, weight gain or loss in mice was not a consistent measure of infection or the impact of treatment.

It was interesting that the stomach, in which paromomycin was in the highest concentration, remained heavily infected and was severely altered in all treated groups. Aminoglycosides, to which paromomycin belongs, are reported to be considerably less active in low pH (20) and readily form complexes with divalent cations such as calcium which are present in abundance in the milk diet. This may explain the persistence of infections in the stomachs of paromomycin-treated mice and in some piglets. It is possible that the inclusion of H_2 blockers such as cimetidine with paromomycin treatment may help eradicate the infection from the stomach.

Suckling *scid* mice became infected within a short time (5 to 6 days) and developed predominantly villus surface infections, which were more responsive and sensitive to luminal drug testing. In contrast, in chronically infected weaned *scid* mice parasite forms were confined largely to inaccessible abscessed crypts and were therefore less sensitive to drug testing. The level of oocyst shedding was some 50- to 200-fold higher in suckling mice than in weaned mice. While the mean mucosal infection scores for the untreated suckling and weaned groups were similar (approximately 17), the same dose of paromomycin reduced the mean scores in the suckling groups to 4 to 5, compared with only 13 in the weaned mice.

Treatment of piglets with high doses of HBC-Ig for 8 days led to a statistically significant reduction in oocyst shedding, but the impact was less significant in terms of manifestation of diarrhea, and extent of mucosal infections and damage, compared with infected untreated animals. These results are not unlike the results obtained from clinical trials conducted recently with AIDS patients with persistent diarrhea with the same or similar products (16a). The underlying reasons for this failure are not clear. We have examined the likely impact of stomach acidity and whether sufficient antigenic variation existed between the *C. parvum* isolate used in the challenge experiments and the immunizing bovine isolate. We have used high doses of cimetidine (also used in the human clinical trials), which only marginally increased the stomach pH in the piglet. This probably was insufficient to prevent inactivation of some or most of the Igs in the HBC-Ig or may have predisposed the antibody to extensive proteolysis. This was confirmed by the low residual antibody activity in the feces of piglets fed large quantities of HBC-Ig. Western blot (immunoblot) analysis demonstrated no significant differences between the immunizing and the challenging *C. parvum* isolates (data not shown). The therapeutic value of HBC-Ig against *C. parvum* remains uncertain, as successes and failures in human patients with chronic cryptosporidiosis (17, 27, 31, 34) and in experimentally infected calves (7, 15) and mice (3, 9, 10) have been reported. The method of HBC-Ig production (use of intramammary boosters to generate higher *C. parvum*-specific

TABLE 3. Distribution of mucosal infections[a]

Group and pig no.	Score for distribution					Mucosal score[b]
	Small intestine			Large intestine		
	Proximal	Mid	Distal	Cecum	Colon	
Group 5						
22–25	5.0 (± 0.0)	4.7 (± 0.6)	4.7 (± 0.6)	5.0 (± 0.0)	5.0 (± 0.0)	24.7 (± 0.6)
Group 6						
26–29	5.0 (± 0.0)	4.7 (± 0.6)	4.7 (± 0.6)	4.7 (± 0.6)	5.0 (± 0.0)	24.0 (± 1.0)
Group 7						
30–34	4.5 (± 1.0)	4.0 (± 1.4)	4.5 (± 1.0)	3.7 (± 1.3)	3.7 (± 1.3)	20.5 (± 4.7)
Group 8						
35–37	1.0 (± 1.0)	1.3 (± 0.6)	2.6 (± 0.6)	4.7 (± 0.6)	5.0 (± 0.0)	14.0 (± 0.0)
Group 9						
38–40	1.3 (± 2.3)	0.7 (± 1.1)	0.3 (± 0.6)	1.7 (± 2.9)	1.7 (± 2.9)	5.7 (± 6.6)

[a] The distribution of mucosal infections is expressed as scores (means ± SDs) for five intestinal sites in piglets infected with *C. parvum* UCP (Table 2). Groups 5 to 7, which were infected at age 2 days, developed severe diarrhea, anorexia, wasting, and recumbency whether treated with paromomycin (groups 6 and 7) or untreated (group 5). Groups 8 and 9, which were infected at age 5 days, in contrast developed moderate diarrhea, and two of three treated piglets (group 9) responded well to paromomycin treatment compared with untreated piglets in group 8.

[b] The mucosal score was as follows: 0, no infection detected; 1, 1 to 20% of epithelial surface is infected; 2, 21 to 40% is infected; 3, 41 to 60% is infected; 4, 61 to 80% is infected; 5, 81 to 100% is infected. Scores for the five body sites are summed.

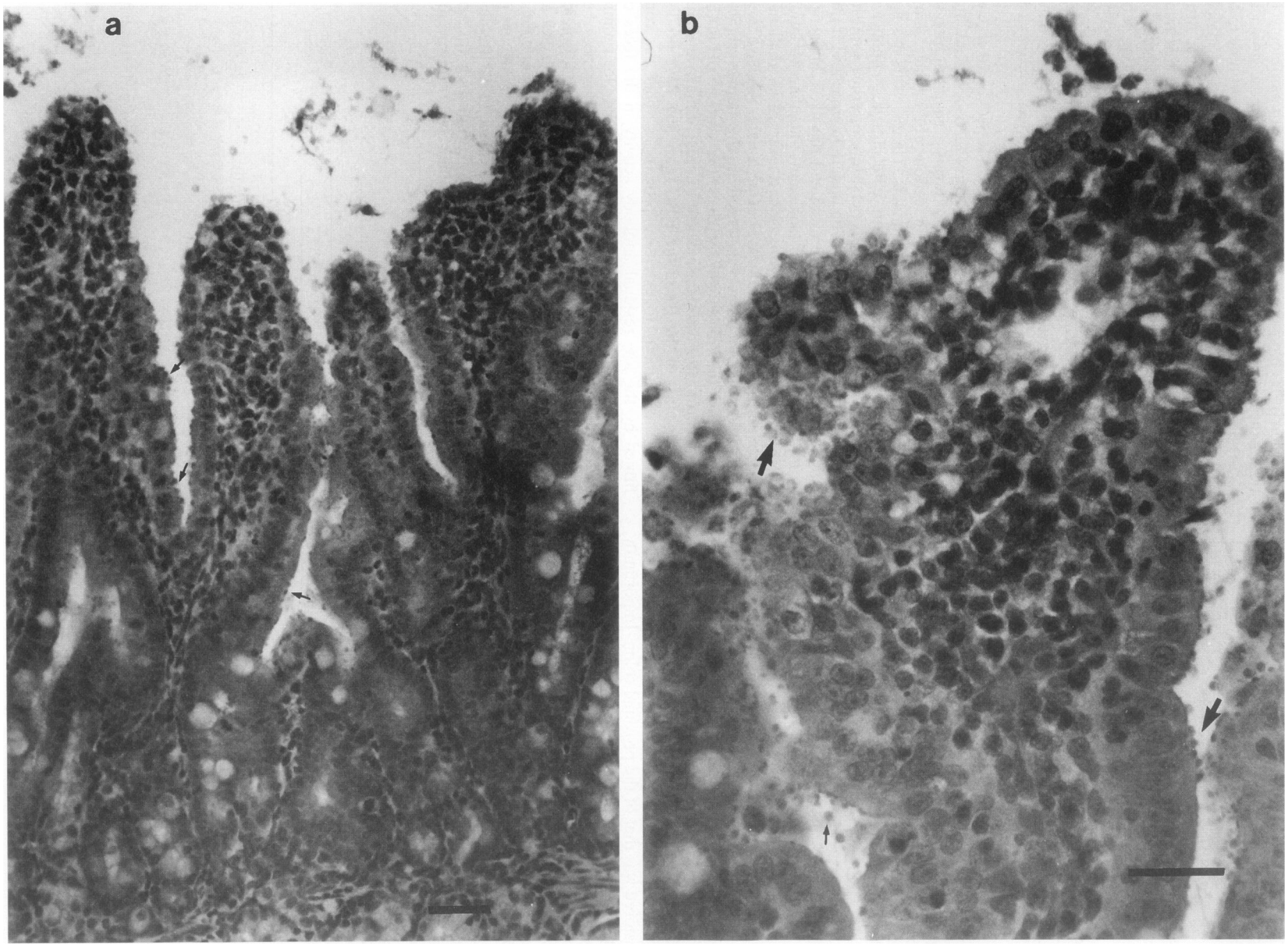
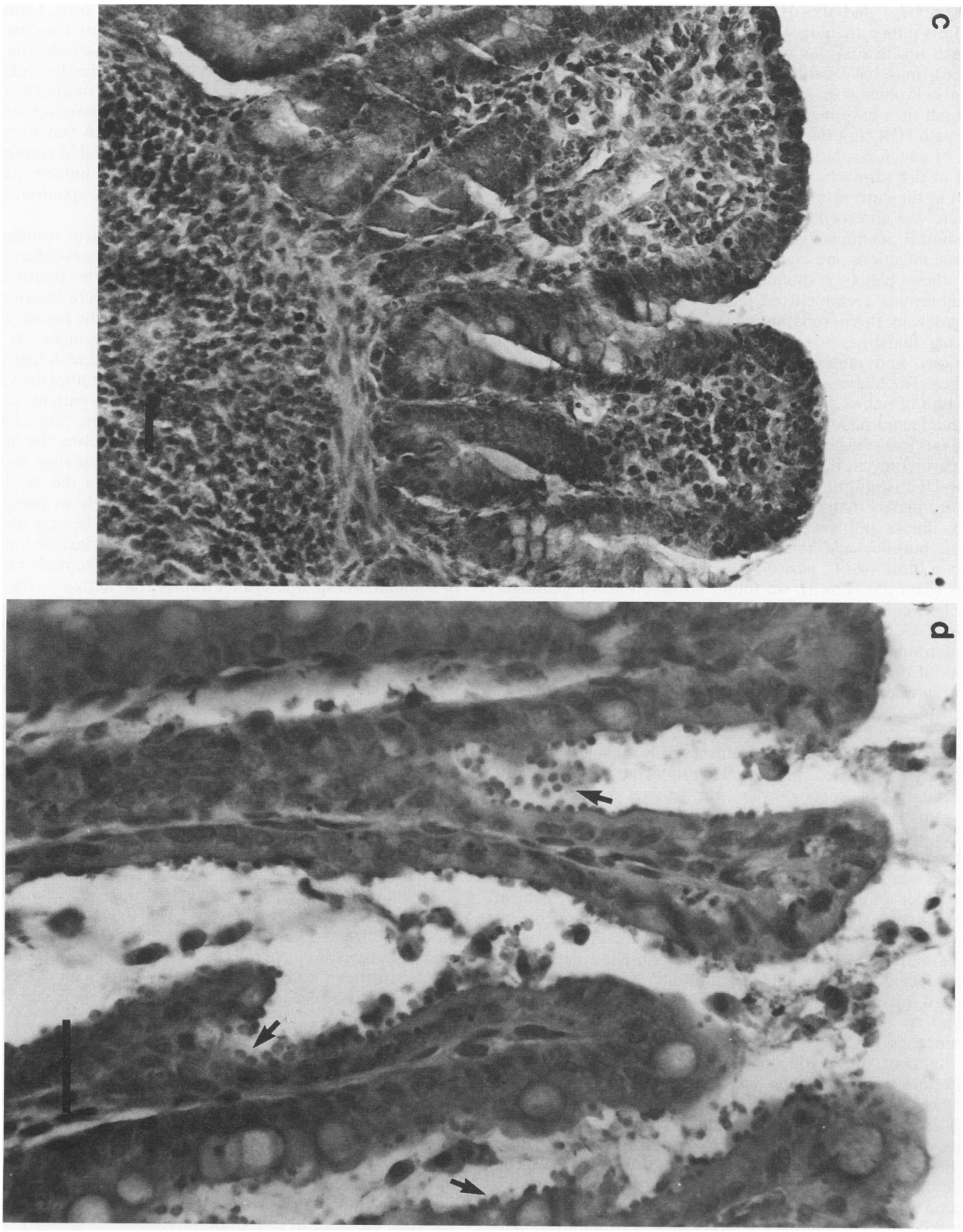


FIG. 10. Intestinal sites (a to d) of piglets infected with *C. parvum* (hematoxylin and eosin stain). Bar, 40 μm. (a) Heavily infected (arrows) villus surface of the duodenum, with cellular infiltration and damaged or highly modified and irregular epithelial surface layer. (b) Higher magnification of the ileum showing heavy villus surface infection. (c) Ileal section taken 9 days after treatment with paromomycin (Fig. 3; group 3), showing partial villus atrophy and cellular lamina propria covered with irregular surface epithelium which is free of parasite forms. (d) Mucosal section from the colon showing heavy surface and crypt colonization by parasite forms, resulting in extensive damage to colonocytes.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IgA antibody) and the delivery into the host of whole colostrum—rather than Ig—via nasoduodenal tubes to bypass stomach inactivation, as was reported by some investigators (30, 34), may have contributed to the successes in selected patients. Problems in the delivery of active antibody to the intestinal sites have been encountered in some recent clinical trials with HBC-Ig (16a). These findings have led to development of new formulations that will eliminate antibody breakdown in the stomach. The new HBC-Ig formulations will be tested in the current model system to evaluate efficacy.

There was an excellent correlation among oocyst shedding, the clinical manifestation of diarrhea, and the extent of mucosal infections and injury in GCH1-infected piglets. Moreover, these piglets responded very well to treatment with paromomycin. Treatment of piglets with either 200 or 500 mg/kg/day in three separate doses was equally effective in reducing diarrhea, oocyst shedding, the extent of mucosal infections, and mucosal damage in piglets with moderate diarrhea. The higher dose of paromomycin led to a more rapid reduction of oocyst shedding in feces. The high dose given to two uninfected piglets caused neither ill effects nor diarrhea, which has been suggested as one of the side effects associated with paromomycin treatment in human patients.

The UCP strain of *C. parvum* was far more pathogenic for newborn piglets than the GCH1 strain, leading to much more severe illness and death in age- and dose-matched piglets. Piglets, but not *scid* mice, were capable of differentiating between these two *C. parvum* isolates. In other piglet experiments (data not shown), we found no difference in pathogenicity between the GCH1 (a human isolate) and a second calf isolate, GCC1, indicating that the remarkable difference between strains UCP and GCH1 in piglets was not due to the species of origin. Differences in pathogenicity among *C. parvum* isolates obtained from different sources were recently also demonstrated in calves (25).

Paromomycin, which was effective in piglets infected with the GCH1 isolate, was ineffective in severely ill piglets infected with the UCP isolate. Only one UCP-infected piglet, treated with 500 mg of paromomycin per kg/day, survived for 9 days—albeit with severe and continuous diarrhea and weight loss; on necropsy, the gut was heavily infected. In the treated and the infected control UCP piglets, the mucosa of the small intestine was extensively infected and profoundly damaged. When older animals were inoculated with UCP, their clinical responses were similar to those of younger animals inoculated with GCH1. In sharp contrast, UCP-infected *scid* mice treated with paromomycin exhibited a similar response to treatment as those infected with the GCH1 isolate (data not shown), which indicated that the failure by some piglets infected with UCP to respond to paromomycin treatment was not due to drug resistance.

In humans, watery diarrhea and wasting are the central manifestation of *C. parvum* infection, symptoms which were clearly observed in infected piglets. Clinically affected species, which include humans, calves, and experimentally infected piglets, share as a characteristic the extensive villus surface infections involving most of the small intestine and part or all of the large intestine. As our study with piglets showed, infection was most extensive on villus surfaces and hence more likely accessible to luminal chemotherapy. Paromomycin efficacy in piglets depended on the severity of the diarrhea. The drug was effective against moderate cryptosporidiosis in piglets because of a more accessible villus surface infection in this model. However, paromomycin was less effective when diarrhea was profound, presumably because the rapid gut transit of fluids led to the rapid elimination of luminal paromomycin, thus minimizing the therapeutic impact of the drug. Generally, the success of treatment in piglets depended on the onset and severity of diarrhea; indeed, the earlier the onset and the more severe the illness at onset of treatment, the less effective paromomycin was in reducing either infection or diarrhea. This was clearly illustrated in the response of paromomycin-treated piglets of group 9 (Tables 2 and 3), in which two recovered completely and one failed to respond. A variable response to paromomycin treatment—also observed in human patients with cryptosporidiosis—was considerably less apparent in the mouse model.

Our observations may explain the inconsistent responses to paromomycin treatment reported for *C. parvum*-infected patients (12). Poor responses to paromomycin treatment in humans may also be associated with more severe diarrhea and rapid transit time, localization of infection in the hepato-biliary system, and/or extensive crypt and pit involvement. As these studies showed that no toxicity was associated with high doses of paromomycin, including in neonates, higher total daily doses than the currently used 2 g/day for adult AIDS patients (2) may prove more effective against luminal infection. Since chronic cryptosporidiosis in humans occasionally involves the hepatobiliary system and presumably other inaccessible sites, we think that orally administered therapies that act in the gut lumen (e.g., paromomycin and HBC-Ig) are unlikely to completely eliminate such infections. However, they may be very useful in early stages of cryptosporidiosis or for prophylaxis in high-risk individuals or may help combat outbreaks. Chemotherapeutic or immunotherapeutic agents transported across to the luminal epithelial surface are likely to be more effective in reaching such sites. Systemically transported antimicrobial agents that are luminally effective in the model system described may subsequently be tested in the adult chronic *scid* mouse model, which exhibits involvement of the hepato-biliary system and infections in other inaccessible sites.

It is clear that the impact of effective therapy against *C. parvum* can be detected within 5 days in either model. This was also evident from successful treatments of *C. parvum*-infected immunodeficient human patients with HBC (30). This is not surprising considering that the parasite's life cycle and gut epithelial cell turnover are completed within 2 to 3 days. This means that diarrhea models in which symptoms last 10 to 12 days are adequate to evaluate drug efficacy against *C. parvum*. This study highlighted the role of diarrhea in evaluating the therapeutic efficacy of drugs and the inadequacy of rodent models. Since the gnotobiotic piglet model is expensive and labor intensive, we have selected only HBC-Ig and paromomycin for further therapeutic evaluation in this model, both of which showed some inhibitory activity and low toxicity in the mouse model.

In summary, this study has demonstrated the following. Paromomycin showed low toxicity and relatively moderate therapeutic efficacy at high doses in both models. HBC-Ig, though it significantly reduced the extent of infection in the mouse, was less effective in the piglet diarrhea model. Neonatal *scid* mice were superior to weaned *scid* mice for screening drugs, as oocyst shedding was 50 to 200 times higher and the infection was mostly on villus surfaces and hence more accessible to the action of paromomycin. Oocyst shedding was a less sensitive measure of low-grade infections compared with mucosal infection, as assessed histologically. Piglets were superior to mice because of good correlation among diarrhea, oocyst shedding, the extent of mucosal infection, and the therapeutic efficacy of drugs. Piglets also allowed the demonstration of *C. parvum* isolate differences in pathogenicity, which were not detectable in the mouse. The response of piglets to paromo-

mycin was related to the extent of diarrhea, a parameter which is absent in rodent models, and may have particular significance, therefore, in predicting therapeutic responses in humans.

ACKNOWLEDGMENTS

This work was supported by Public Health Service contract NO1-AI-25143 from the National Institute of Allergy and Infectious Diseases, Division of AIDS.

The technical assistance of Melissa Paris and Keith Johnson is greatly appreciated.

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