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# Monoclonal Antibody Immunotherapy in Nude Mice Persistently Infected with *Cryptosporidium parvum*

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**Three groups of congenitally athymic nude mice were persistently infected following oral administration of $2 \times 10^7$ *Cryptosporidium parvum* oocysts. Two groups were treated once daily for 10 days with either neutralizing monoclonal antibody (MAb) 17.41 or an isotype control MAb. The third group received no treatment. Intestinal-infection scores were significantly decreased in nude mice treated with MAb 17.41 compared with isotype control MAb-treated and nontreated control groups ($P < 0.005$). Biliary and pancreatic cryptosporidial-infection scores were similar for the MAb 17.41-treated and isotype control MAb-treated groups ($P > 0.05$).**

Cryptosporidiosis results from gastrointestinal infection by the coccidian *Cryptosporidium parvum* (5, 7, 12). In immunodeficient hosts, *C. parvum* sporozoites and merozoites cyclically infect intestinal epithelium, resulting in persistent disease characterized by severe, often life-threatening diarrhea (6, 12). *C. parvum* is not limited to the gastrointestinal tract of immunodeficient hosts but may also cause biliary and respiratory tract infections (5, 6, 8, 12, 17). Since no specific therapy is currently available, cryptosporidial infection often contributes to the death of patients with congenital immunodeficiency disorders or acquired immunodeficiency syndrome (6, 8).

Assessment of therapeutic protocols has been slowed by the absence of a convenient animal model of persistent cryptosporidiosis (5–7). Ungar et al. have recently demonstrated that persistent infection can be established in immunodeficient nude mice experimentally infected with *C. parvum* (24). Our hypothesis is that neutralizing antibodies to sporozoite and merozoite surface antigens will resolve persistent cryptosporidial infections in immunodeficient hosts. To test this hypothesis, nude mice persistently infected with *C. parvum* were treated orally with neutralizing monoclonal antibody (MAb) 17.41. This MAb neutralizes sporozoite and merozoite infectivity in suckling mice (2, 19, 20) and partially protects mice against oral challenge with *C. parvum* oocysts (19). In this report, we describe the diminution of persistent cryptosporidial infection in immunodeficient mice following oral treatment with MAb 17.41.

(These data were reported, in part, in an abstract [1b] and a thesis [1a].)

The *C. parvum* isolate used in these experiments was originally provided by H. Moon and D. Woodmansee (National Animal Disease Center, Ames, Iowa) and is infectious for humans, newborn mice, calves, and young horses with severe combined immunodeficiency (16, 21). The isolate was maintained by passage in neonatal calves. The purification of oocysts from calf feces was done as previously described (21) with minor modifications. Potassium dichromate was no longer used, and feces were processed within 36 h of collection. The extracted oocyst suspension was stored in Hanks' balanced salt solution containing 10,000 U of penicillin, 0.01 g of streptomycin, 0.05 mg of amphotericin B, and 500 U of nystatin per ml to prevent microbial overgrowth. The oocysts were treated with 3% peracetic acid at room temperature for 10 min to kill nonoocyst microbial agents prior to mouse inoculation.

Treatment reagents were ascites fluid collected from mice injected with the appropriate hybridoma cells producing either neutralizing MAb 17.41 or isotype control MAb 7.3.4 (both immunoglobulin M isotypes). The concentration of immunoglobulin in ascites was determined by radial immunodiffusion (Tago Inc., Burlingame, Calif.). MAb 17.41 and 7.3.4 ascites contained 0.9 and 1.0 mg of immunoglobulin M per ml, respectively. A previously described indirect immunofluorescence assay was used to ensure that the MAb 17.41 ascites retained specific reactivity to surface antigens (21). Ascites containing MAb 17.41 reacted with >95% of *C. parvum* sporozoites according to the indirect immunofluorescence assay. Sporozoites did not react with isotype control MAb 7.3.4 or the conjugate.

Western blot (immunoblot) analysis was performed to determine if sporozoites and merozoites shared similar antigens reactive with MAb 17.41. Sporozoites and merozoites were isolated as previously described (2, 21), adjusted to $5 \times 10^9$/ml, and boiled for 3 min in 65 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, and then the soluble fraction (containing $5 \times 10^7$ organisms) was electrophoresed in a 7.5% (wt/vol) polyacrylamide slab gel with a 4% stacking gel (15, 20). Sporozoite and merozoite antigens resolved by electrophoresis were transferred to nitrocellulose as previously described (15, 20) and probed with ascites containing MAb 17.41 or 7.3.4. The bound antisporozoite and antimerozoite MAb was detected with a peroxidase conjugated $F(ab')_2$ fragment of goat anti-mouse immunoglobulin M (mu chain-specific [Organon Teknika-Cappel, West Chester, Pa.]) and developed with 4-chloro-1-naphthol (15). On both sporozoites and merozoites, MAb 17.41 recognized two distinct antigens of approximately 75 and 215 kDa (Fig. 1). MAb 17.41 also reacted with two additional sporozoite antigens of approximately 150 and 175 kDa. The additional sporozoite antigens containing this epitope may be specific for this stage of the *C. parvum* life cycle.

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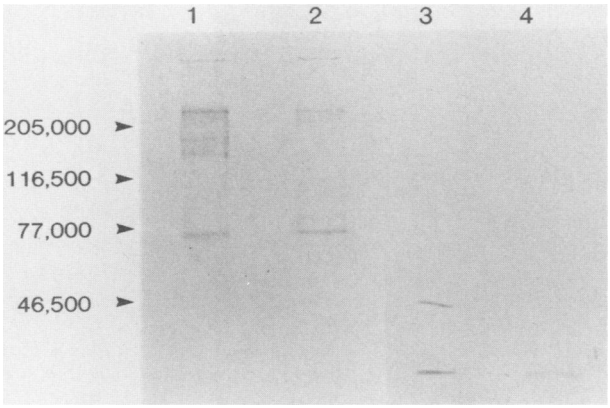


FIG. 1. Western blot detection of sporozoite (lane 1) and merozoite (lane 2) antigens recognized by MAb 17.41. Lanes 3 and 4 contain sporozoites and merozoites, respectively, analyzed with isotype control MAb 7.3.4. Molecular weights of standards are indicated on the left.

Thirty-eight congenitally athymic nude (*nu/nu*) female mice, 6 to 7 weeks old (Simonsen Laboratories, Inc., Gilroy, Calif.), were randomly assigned to one of three groups. These included a MAb 17.41-treated group (16 mice), an isotype control MAb 7.3.4-treated group (16 mice), and a nontreated group (6 mice). The nude mice were inoculated orally via a 22-gauge feeding needle with $10^7$ *C. parvum* oocysts in 250 μl of 0.02 M phosphate-buffered saline (pH 7.4). To ensure that infection was established, the mice were given a second dose of $10^7$ oocysts 24 h later. Clinical signs of cryptosporidiosis were absent during the first 5 weeks of infection, and no deaths occurred. Occasional loose stools were noted for mice in all three groups, but diarrhea was not observed.

Treatment was initiated 5 weeks postinfection and lasted 10 days. The mice in the MAb-treated groups received a daily oral dosage of 0.5 ml of ascites fluid (containing either 0.45 mg of MAb 17.41 or 0.50 mg of isotype control MAb 7.3.4) administered via a 22-gauge feeding needle. The mice were lightly anesthetized with methoxyflurane (Pitman-Moore, Inc., Washington Crossing, N.J.)-soaked gauze sponges in a sterile glass jar to facilitate oral dosing. Three mice from the MAb 17.41-treated group and three from the isotype control MAb-treated group died during the treatment period because of aspiration of the ascites fluid. All of these six mice had histologic evidence of *Cryptosporidium* infection, i.e., numerous endogenous stages within the intestinal and biliary mucosal epithelium, but no gross changes were present. These mice were excluded from the final analysis of data.

The presence of *C. parvum* in feces was determined by using a modified Kinyoun acid-fast stain (13). All nude mice were negative for *Cryptosporidium* spp. in the preinfection samples. At 5 weeks postinfection (and prior to treatment), all 38 mice were shedding *C. parvum* oocysts. All but four mice were shedding *C. parvum* oocysts at the end of the treatment period in at least one of two fecal samples obtained on day 10 posttherapy and at necropsy. The four mice that were negative on fecal exam (two from the MAb 17.41-treated group and two from the isotype control MAb 7.3.4-treated group) had histologic evidence of *Cryptosporidium* infection.

The mice were necropsied 24 h after the final treatment.

TABLE 1. MAb 17.41 treatment of persistent cryptosporidiosis in nude mice

| Treatment reagent | No. of nude mice | Mean infection score ± SD for: | |
|---|---|---|---|
| | | Intestine | Biliary system and pancreatic ducts |
| MAb 17.41 | 13 | 3.2 ± 1.5[a] | 1.5 ± 2.1[b] |
| MAb 7.3.4 | 13 | 5.5 ± 1.5[c] | 2.5 ± 3.0[c] |
| None | 6 | 6.3 ± 1.5 | 4.5 ± 2.3 |

[a] Significantly less than isotype control MAb 7.3.4-treated and nontreated mice ($P < 0.005$).
[b] No significant difference from isotype control MAb 7.3.4-treated mice ($P > 0.05$).
[c] No significant difference from nontreated mice ($P > 0.05$).

Gross and histologic changes observed in all groups were similar to those previously described for persistent cryptosporidiosis in nude mice (16, 24). The predominant gross findings included icterus, multifocal areas of hepatic necrosis, diffuse hepatic congestion, hypertrophy and thickening of the gallbladder and common bile duct, and intestinal distention in seven mice (5 of 13 isotype control MAb-treated and 2 of 6 nontreated mice). Sections of stomach, intestine, liver (including gallbladder and common bile duct), and pancreas were scored for intensity of infection by histologic examination (21). Individual scores were assigned to distinct regions of the intestine, including stomach and duodenum, jejunum, ileum, cecum, and colon. Individual biliary tract scores were assigned to the gallbladder and common bile duct, intrahepatic and extrahepatic ducts, and pancreatic ducts. The scores were determined by estimating the percentage of mucosa infected by *C. parvum* (0, absence of infection; 1, 1 to 33% of mucosa parasitized; 2, 34 to 66% of mucosa parasitized; 3, more than 67% of mucosa parasitized) (21). The sum of the individual scores for different regions of the intestine (maximum possible score = 15) and the biliary and pancreatic systems (maximum possible score = 9) were used to compute mean infection scores for each treatment group. The Wilcoxon rank sum test was used to examine differences in *C. parvum* infection among the MAb 17.41-treated, isotype control MAb-treated, and nontreated groups (18).

The number of *Cryptosporidium* endogenous stages infecting the biliary and pancreatic-duct epithelia was similar in MAb 17.41-treated and isotype control MAb 7.3.4-treated mice (Table 1). The histologic changes within the biliary and pancreatic systems were similar in all three groups (Fig. 2). Treatment with neutralizing MAb 17.41 did not significantly affect the levels of biliary and pancreatic cryptosporidial infection compared with levels in the isotype control MAb-treated group. It has been hypothesized that the biliary system serves as a reservoir for infection (5, 6). Therefore, it may be crucial to ensure that therapeutic agents directed at the autoinfective sporozoite and merozoite stages have access to the biliary and pancreatic ductal system in order to eliminate this potential reservoir of infection.

A significant reduction in intestinal *Cryptosporidium* infection was demonstrated when oral passive immunotherapy with MAb 17.41 was used rather than isotype control MAb 7.3.4 treatment or no treatment (Table 1 and Fig. 3). The number of *C. parvum* endogenous stages within the intestinal epithelium was similar in the isotype control MAb 7.3.4-treated and the nontreated groups, as reflected by the mean infection scores (Table 1). Intestinal infection was more severe in the anterior duodenum, cecum, and colon in

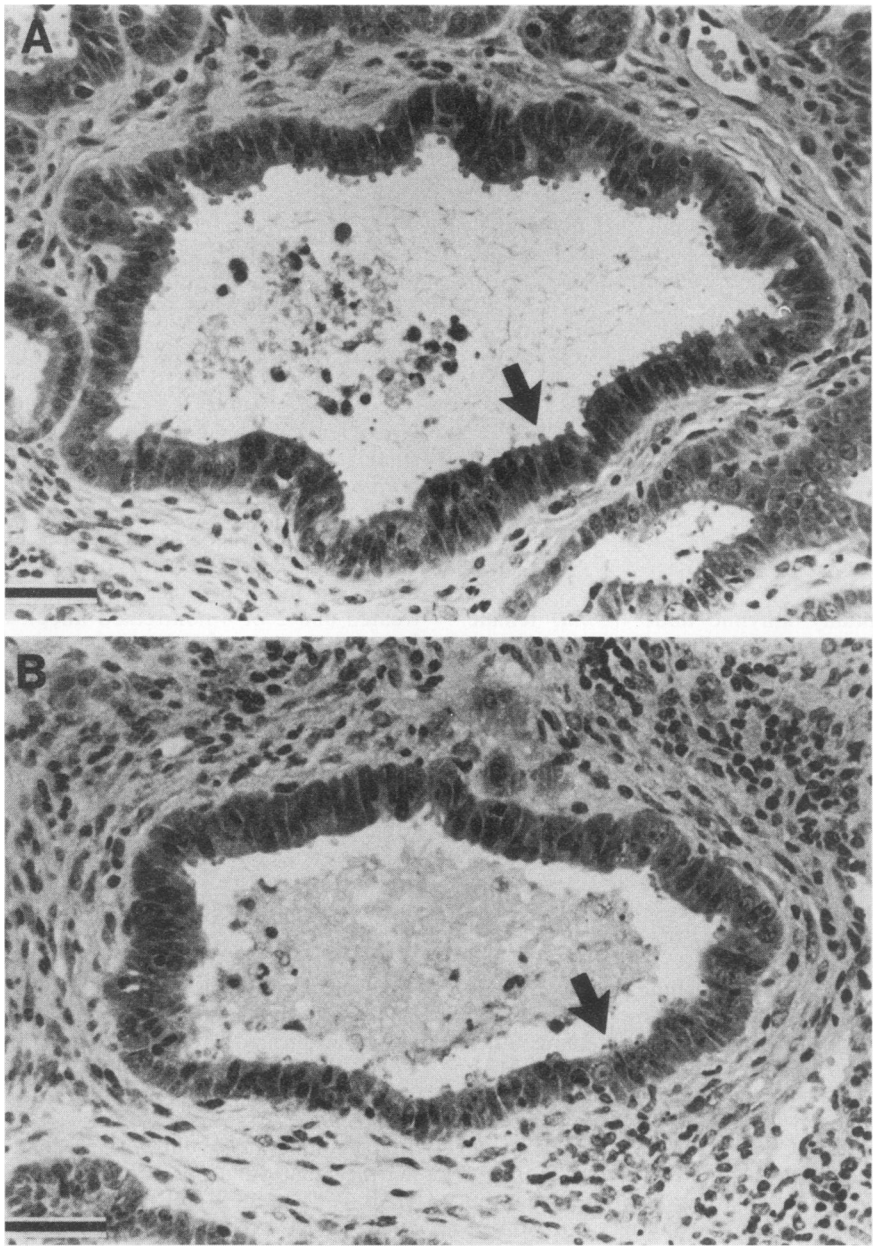


FIG. 2. Photomicrographs of common bile ducts from nude mice persistently infected with *C. parvum*. There was no significant difference in biliary tract infection between MAb 17.41-treated mice (A) and isotype control MAb 7.3.4-treated mice (B). Bar, 20 μm. Arrows indicate *C. parvum* organisms.

all groups. Even though our treatment protocol significantly decreased the number of cryptosporidial organisms observed histologically, it did not terminate infection. Continuous oral treatment with higher concentrations of neutralizing MAb may provide further reductions in intestinal *C. parvum* infection.

Previous studies have demonstrated partial protection against cryptosporidiosis in animals treated with bovine colostrum, MAb, and ascites fluid (1, 9–11, 19). Others have used hyperimmune bovine colostrum to effectively treat persistent cryptosporidiosis in immunodeficient humans (22, 23, 25). These studies provide supportive evidence that oral passive immunotherapy with neutralizing anti-*Cryptosporidium* antibodies may be a useful treatment approach. In this report we discuss three important facts concerning therapy of persistent cryptosporidiosis in an immunodeficient host. First, oral administration of MAb 17.41, which recognizes shared neutralization-sensitive epitopes on *C. parvum* sporozoites and merozoites (2, 20), can significantly decrease the number of cryptosporidial organisms in persistently infected immunodeficient mice. Second, MAb 17.41 defines epitopes that can be studied and isolated for induction of host immune responses necessary for prevention or treatment of cryptosporidial infections (2, 20). Finally, the data demonstrate that delivery of a specific amount of treatment reagent to intestinal, biliary, and pancreatic mucosal sur-

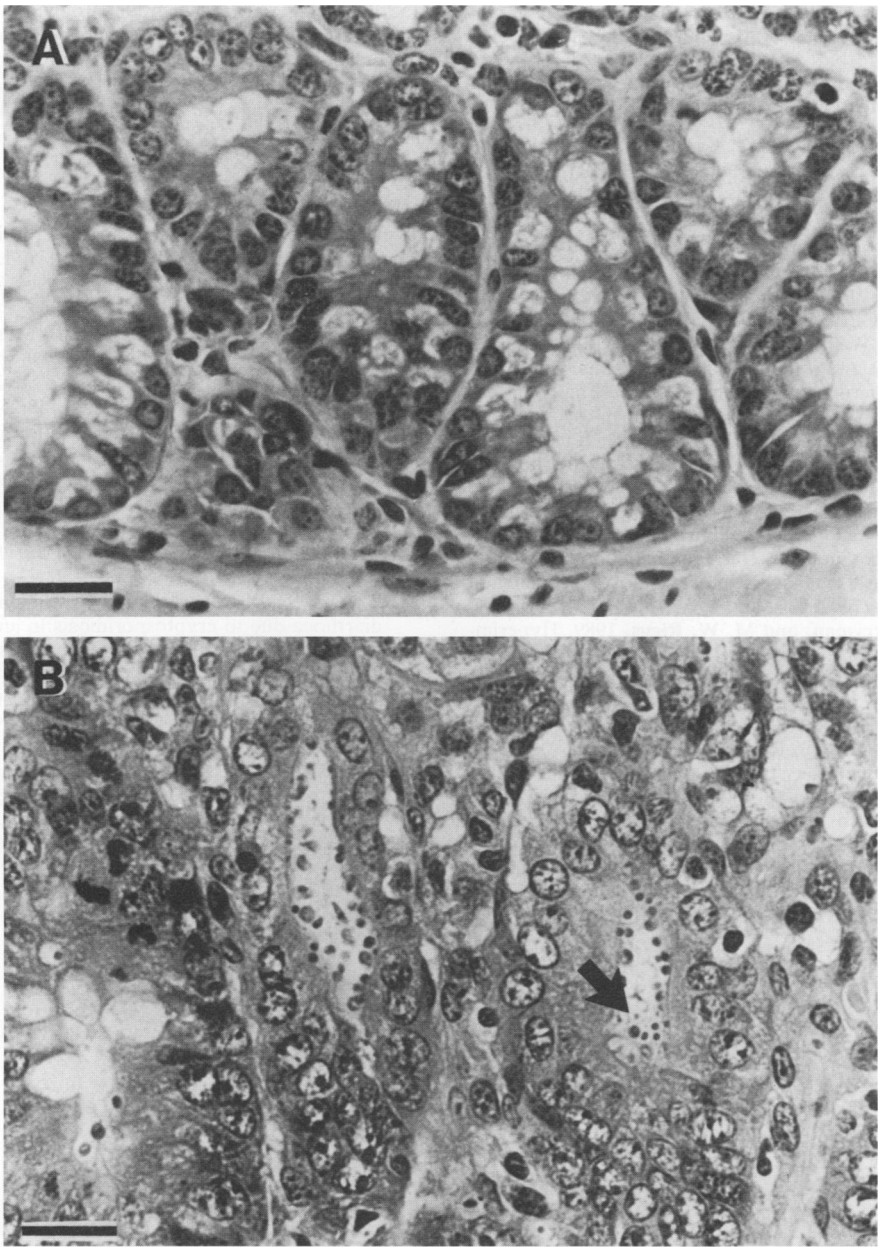


FIG. 3. Photomicrographs of ceca from nude mice persistently infected with *C. parvum*. MAb 17.41-treated mice (A) had significantly diminished cryptosporidial infection compared with isotype control MAb 7.3.4-treated mice (B). Bar, 20 μm. The arrow indicates *C. parvum* organisms.

faces may be necessary for a positive treatment effect. Since there is no chemotherapy currently available that can terminate persistent cryptosporidiosis in immunodeficient patients (3, 4, 6, 14), passive immunotherapy with neutralizing MAb may reduce the severity of this debilitating disease.

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## REFERENCES

1. **Arrowood, M. J., J. R. Mead, J. L. Mahrt, and C. R. Sterling.** 1989. Effects of immune colostrum and orally administered antisporozoite monoclonal antibodies on the outcome of *Cryptosporidium parvum* infections in neonatal mice. Infect. Immun. **57**:2283–2288.

1a. **Bjorneby, J. M.** 1990. Ph.D. thesis. Washington State University, Pullman.

1b. **Bjorneby, J. M., B. D. Hunsaker, M. W. Riggs, and L. E. Perryman.** 1990. Abstr. Annu. Meet. Conf. Res. Workers Anim. Dis. 1990, abstr. 167, p. 29.

2. **Bjorneby, J. M., M. W. Riggs, and L. E. Perryman.** 1990. *Cryptosporidium parvum* merozoites share neutralization-sensitive epitopes with sporozoites. J. Immunol. **145**:298–304.

3. **Centers for Disease Control.** 1984. Update: treatment of cryptosporidiosis in patients with acquired immunodeficiency syndrome (AIDS). Morbid. Mortal. Weekly Rep. **33**:117–119.
4. **Connolly, G. M., M. S. Dryden, D. C. Shanson, and B. G. Gazzard.** 1988. Cryptosporidial diarrhoea in AIDS and its treatment. Gut **29**:593–597.
5. **Crawford, F. G., and S. H. Vermund.** 1988. Human cryptosporidiosis. Crit. Rev. Microbiol. **16**:113–159.
6. **Current, W. L.** 1989. *Cryptosporidium* spp., p. 281–341. *In* P. D. Walzer and R. M. Genta (ed.), Parasitic infections in the compromised host, 1st ed. Marcel Dekker Inc., New York.
7. **Current, W. L., and P. H. Bick.** 1989. Immunobiology of *Cryptosporidium* spp. Pathol. Immunopathol. Res. **8**:141–160.
8. **Current, W. L., N. C. Reese, J. V. Ernst, W. S. Bailey, M. B. Heyman, and W. M. Weinstein.** 1983. Human cryptosporidiosis in immunocompetent and immunodeficient persons. Studies of an outbreak and experimental transmission. N. Engl. J. Med. **308**:1252–1257.
9. **Fayer, R., C. Andrews, B. L. P. Ungar, and B. L. Blagburn.** 1989. Efficacy of hyperimmune bovine colostrum for prophylaxis of cryptosporidiosis in neonatal calves. J. Parasitol. **75**: 393–397.
10. **Fayer, R., A. Guidry, and B. L. Blagburn.** 1990. Immunotherapeutic efficacy of bovine colostral immunoglobulins from a hyperimmunized cow against cryptosporidiosis in neonatal mice. Infect. Immun. **58**:2962–2965.
11. **Fayer, R., L. E. Perryman, and M. W. Riggs.** 1989. Hyperimmune bovine colostrum neutralizes *Cryptosporidium* sporozoites and protects mice against oocyst challenge. J. Parasitol. **75**:151–153.
12. **Fayer, R., and B. L. P. Ungar.** 1986. *Cryptosporidium* spp. and cryptosporidiosis. Microbiol. Rev. **50**:458–483.
13. **Garcia, L. S., D. A. Bruckner, T. C. Brewer, and R. Y. Shimizu.** 1983. Techniques for the recovery and identification of *Cryptosporidium* oocysts from stool specimens. J. Clin. Microbiol. **18**:185–190.
14. **Glatt, A. E., K. Chirgwin, and S. H. Landesman.** 1988. Treatment of infections associated with human immunodeficiency virus. N. Engl. J. Med. **318**:1439–1448.
15. **Harlow, E., and D. Lane.** 1988. Immunoblotting, p. 471–510. *In* E. Harlow and D. Lane (ed.), Antibodies: a laboratory manual, 1st ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Heine, J., H. W. Moon, and D. B. Woodmansee.** 1984. Persistent *Cryptosporidium* infection in congenitally athymic (nude) mice. Infect. Immun. **43**:856–859.
17. **Gross, T. L., J. Wheat, M. Bartlett, and K. W. O'Conner.** 1986. AIDS and multiple system involvement with *Cryptosporidium*. Am. J. Gastroenterol. **81**:456–458.
18. **Ott, L.** 1988. A nonparametric alternative: the Wilcoxon rank sum test, p. 183–188. *In* L. Ott (ed.), An introduction to statistical methods and data analysis, 3rd ed. P. W. S. Kent Publishing Co., Boston.
19. **Perryman, L. E., M. W. Riggs, P. H. Mason, and R. Fayer.** 1990. Kinetics of *Cryptosporidium parvum* sporozoite neutralization by monoclonal antibodies, immune bovine serum, and immune bovine colostrum. Infect. Immun. **58**:257–259.
20. **Riggs, M. W., T. C. McGuire, P. H. Mason, and L. E. Perryman.** 1989. Neutralization-sensitive epitopes are exposed on the surface of infectious *Cryptosporidium parvum* sporozoites. J. Immunol. **143**:1340–1345.
21. **Riggs, M. W., and L. E. Perryman.** 1987. Infectivity and neutralization of *Cryptosporidium parvum* sporozoites. Infect. Immun. **55**:2081–2087.
22. **Tzipori, S., D. Roberton, and C. Chapman.** 1986. Remission of diarrhoea due to cryptosporidiosis in an immunodeficient child treated with hyperimmune bovine colostrum. Br. Med. J. **293**: 1276–1277.
23. **Tzipori, S., D. Roberton, D. A. Cooper, and L. White.** 1987. Chronic cryptosporidial diarrhoea and hyperimmune cow colostrum. Lancet **i**:344–345.
24. **Ungar, B. L. P., J. A. Burris, C. A. Quinn, and F. D. Finkelman.** 1990. New mouse models for chronic *Cryptosporidium* infection in immunodeficient hosts. Infect. Immun. **58**:961–969.
25. **Ungar, B. L. P., D. J. Ward, R. Fayer, and C. A. Quinn.** 1990. Cessation of *Cryptosporidium*-associated diarrhea in an acquired immunodeficiency syndrome patient after treatment with hyperimmune bovine colostrum. Gastroenterology **98**:486–489.