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Phenotypic Profiles of Lymphocytes in Adult C57BL/6N Mice Infected With *Cryptosporidium parvum*

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PHENOTYPIC PROFILES OF LYMPHOCYTES IN ADULT C57BL/6N MICE INFECTED WITH *CRYPTOSPORIDIUM PARVUM*

by

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ABSTRACT

Phenotypic Profiles of Lymphocytes in Adult C57BL/6N Mice Infected with *Cryptosporidium parvum*

by

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The purpose of this study was to quantitate the populations of lymphocytes in the spleens and intestines of normal and immunosuppressed adult C57BL/6N mice that were noninfected or infected with *Cryptosporidium parvum*. This was accomplished by using the following methodologies: immunohistochemistry, ELISA-spot assay, and flow cytometry.

Mice in groups 1 and 2 were immunosuppressed, but only group 2 was infected. Mice in group 3 were only infected, whereas group 4 served as the normal control. Mice were immunosuppressed with dexamethasone (DEX) at a dosage of 125µg/mouse/day. Infected mice received 10^6 oocysts per os. The numbers of lymphocytes were monitored from day 0 to day 18 postinfection. Flow cytometry using antibodies directed against CD4+ and CD8+ T cells (helper and cytotoxic, respectively) and B cells (expressing IgG, IgM, and IgA receptors) revealed that *C. parvum* did not evoke an

alteration in the phenotypic profile of lymphocytes within spleens or Peyer's patches (PP) of mice in groups 2 and 3 that was statistically different from groups 1 and 4. Immunosuppressed mice (groups 1 and 2) had significantly fewer lymphocytes (bearing CD4+, IgG, IgM, and IgA receptors) within the spleen when compared with mice in groups 3 and 4 ($P<0.05$). Splenic leucocytes expressing macrophage (CD11b) and CD8+ receptors failed to respond to DEX. Moreover, the CD4+ to CD8+ cell ratio was decreased in the spleens of immunosuppressed mice. After 6 days of DEX administration, the percentage of T cells (CD8+) and macrophages within the PP was significantly higher than nonimmunosuppressed mice ($P<0.05$). Administration of DEX had no apparent effect on B (bearing IgG, IgM, and IgA receptors) and T lymphocytes (CD4+) present within the PP. However, DEX treatment was associated with a lower CD4+ to CD8+ lymphocyte ratio within the PP. Preliminary studies using immunohistochemistry demonstrated that lymphocytes bearing IgA receptors are significantly less in mice that received DEX for 20 days ($P<0.05$), whereas the number of IgG and IgM receptor-bearing lymphocytes was not signif-icantly affected.

(101 pages)

INTRODUCTION

Cryptosporidium parvum is a protozoan parasite with worldwide distribution that infects humans and other vertebrates. Depending on the species and isolate of the parasite and the age and immunologic status of the host, the severity of infection can range from subclinical to severe (41). Cryptosporidiosis in immunocompromised patients (particularly those with acquired immunodeficiency syndrome {AIDS}) presents with a prolonged, life threatening, debilitating diarrhea. Clinical disease frequently includes weight loss, fever, abdominal pain, and occasionally hematogenous spread to extraintestinal sites (49, 132). *C. parvum* is a frequent cause of illness in immunocompetent individuals. These infections often present with a self-limiting diarrhea and are usually accompanied by abdominal cramps. These observations suggest that the host's immune status does not appear to affect susceptibility of humans to *C. parvum* infections (34). However, the marked differences in the outcomes between immunocompromised and immunocompetent persons infected with *C. parvum* may be explained by the development of a sufficient immune response to clear the parasite from the intestinal mucosa.

No consistently effective chemotherapeutic agent against *C. parvum* is available. The literature indicates that an acquired immune response is necessary to overcome

cryptosporidial infections. Therefore, immunoprophylactic and immunomodulation therapies should be able to prevent and treat cryptosporidiosis. A prerequisite to successful immunological intervention is a thorough understanding of the immune mechanisms responsible for arresting the development of this coccidian parasite. Previous studies have shown that cryptosporidial infection results in the appearance of fecal and serum antibodies that are specific to *C. parvum*. Although marked seroconversion occurs during or soon after recovery from intestinal cryptosporidiosis, it is not known if these antibodies play a role in protective immunity. Furthermore, the coproantibodies and serum response may not reflect the concentration or isotype of antibody available at the site of infection (69). Very little is known about the cell-mediated immune response within the intestinal lamina propria or the Peyer's patches (PP) of individuals infected with *C. parvum*.

Before addressing the immunobiology of *C. parvum*, a treatise of this complex protozoan parasite is presented. Such an account affords a better understanding of the immunobiology of the host-parasite relationship.

Purpose of this research

The purpose of this research was to quantify the subpopulations of T and B lymphocytes and MØ (macrophages expressing CD11b receptors) in the spleens and intestines

of immunocompetent and immunosuppressed adult C57BL/6N mice with cryptosporidiosis.

Objectives

Three objectives were designed to characterize the kinetics and phenotypic profiles of lymphocytes in adult C57BL/6N mice infected with *C. parvum*. These objectives included:

1) Quantify the populations of lymphoid cells present in the spleens and small intestines of normal and immunosuppressed adult C57BL/6N mice that were either infected or uninfected with *C. parvum*. Subsequently, determine the expression levels of these cells from day 0 to day 18 postinfection (PI).

2) Correlate oocyst shedding intensities with the expression of these lymphoid populations.

3) Quantify the populations of B lymphocytes (expressing immunoglobulin {Ig} G, M, and A receptors) present in the terminal ilea and spleens of mice infected with *C. parvum* using immunofluorescence techniques on tissue sections.

This research effort was undertaken to provide an understanding of the splenic and intestinal immune mechanisms that develop in response to *C. parvum* infections and dexamethasone (DEX) immunosuppression. Moreover, this research will serve as a first step toward developing

immunomodulation therapies for use in patients with debilitating cryptosporidiosis.

LITERATURE REVIEW

History

Cryptosporidium parvum is a protozoan assigned to the phylum Apicomplexa, class Sporozoasida, order Eucoccidiorida, and family Cryptosporidiidae that was identified and named in 1912 by Ernest E. Tyzzer (133). For nearly 50 years after Tyzzer's work, *C. parvum* was regarded as a commensal of no economical or medical importance. Increased veterinary investigation of *Cryptosporidium* began in the early 1970's, when it was discovered to be associated with severe bovine diarrhea (104). In 1976, *Cryptosporidium* was first reported as a human pathogen in an immunocompetent 3-year-old girl with acute enteritis who recovered spontaneously (102). After that, relatively few cases were subsequently reported until 1982, when the Center for Disease Control received reports that 21 males had protracted diarrhea caused by *Cryptosporidium* in association with AIDS. Medical and veterinary interest in the epidemiology, diagnosis, treatment, and prevention increased substantially thereafter throughout the world (41).

Life cycle and epidemiology

The life cycle of *C. parvum* is illustrated in Fig. 1. Infection of a new host occurs when thick-walled oocysts in food, water, or the general environment are ingested or

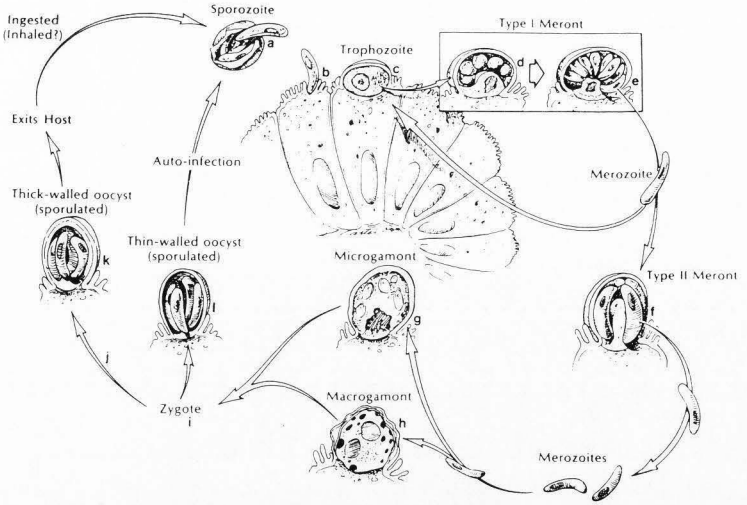

FIG. 1. Life Cycle of *Cryptosporidium parvum*. Infection of a new host occurs when oocysts are ingested or inhaled. Sporozoites excyst from the oocyst (a), and invaginate microvilli (b). The sporozoite then differentiates into a trophozoite (c), which undergoes schizogony (d). Each schizonts nucleus incorporates into a merozoite (e). These merozoites can develop into a Type I (d & e) or Type II schizont (f). Merozoites from Type II schizonts initiate gametogony. These merozoites differentiate into either microgamonts (g), or macrogamonts (h). The microgamete fertilizes the macrogamont, which results in a zygote (i). About 80% of the zygotes mature into thick-walled oocysts and leave the hosts body (k). The remaining zygotes mature into thin-walled oocysts (l) and serve as a source of auto-infection. Drawing by Kip Carter, University of Georgia. Reprinted with permission from CRC Press Inc., Boca Raton, Florida.

inhaled. Modes of transmission include aspiration of gastric contents, aspiration associated with esophageal or oropharyngeal infection, fecal-oral spread with aspiration, and hematogenous spread (49, 132). Electron microscopy indicates that the oocyst wall is composed of two layers; an outer, irregular 10-nm wall separated from a thicker inner wall. The inner layer of the oocyst contains a suture which dissolves during excystation (41, 111). Banana-shaped sporozoites excyst from the oocyst and invaginate microvilli. Subsequently, the host cell membrane evaginates and thin extensions of the microvillus membranes surround the parasite and form an intracellular but extracytoplasmic parasitiphorous vacuole which is located at the luminal surface of the host cell (89, 107, 111). A vacuolar membrane separates the sporozoite cytoplasm from the host cell cytoplasm and appears to be the precursor of the feeder organelle which may serve in the exchange of material between the host cell and the parasite (89, 111). Sporozoites reabsorb cytoplasmic organelles and then differentiate into a spherical trophozoite with a prominent nucleus. The nucleus of the matured trophozoite undergoes two to three asexual multiplications referred to as merogony or schizogony. Two morphologic types of schizonts have been observed. As the type I meront matures, each nucleus becomes incorporated into a merozoite (41). Set free from the ruptured schizont, the merozoite

becomes a motile, feeding trophozoite that can invade another host cell where it can develop into another type I or type II schizont (12). Mature type I and type II meronts contain six to eight and four merozoites, respectively (41, 66). It is believed that merozoites from type II schizonts initiate sexual multiplication (gametogony) when they leave the schizont and parasitize new host cells. These merozoites differentiate into either microgamonts or macrogamonts, the male and female stages, respectively. Early in development, microgamonts become multinucleate and, upon maturation, each nucleus becomes a microgamete (41). The microgamete fertilizes the macrogamont, resulting in a zygote. The zygote secretes a protective protein and lipid shell, thereby transforming into an oocyst (79), which sporulates *in situ*. About 80% of the zygotes mature into thick-walled oocysts and leave the host's body. Oocysts in the gastrointestinal tract are excreted with the feces and those in the respiratory tract are carried out with respiratory or nasal secretions. The thick-walled oocysts that are released into the environment serve as the infective stage for new hosts. Over 10^8 oocysts can be excreted daily in human feces, and infective calves can excrete up to 10^{10} oocysts each day for up to 14 days (17). The remaining zygotes (~20%) mature into thin-walled oocysts and serve as a source of autoinfection in that they

release their sporozoites, which then repeat cycles of schizogony, gametogony, and sporogony.

It is not known how cryptosporidia cause clinical disease. Based on histological findings of villous atrophy, crypt hyperplasia, intimate association of the parasite with absorptive cells, and inflammatory cell infiltration of the intestinal lamina propria, a number of mechanisms of diarrhea could be postulated. These include impaired digestion and absorption as a consequence of decreased villous surface area, the presence of immature, cryptlike cells on the villi, or injury to absorptive cells by the organism or products of the inflammatory response. Additionally, it is possible the toxic material produced by the parasite could result in secretory diarrhea (6).

The broad host range, together with the high output of oocysts, ensures a high level of contamination of the environment, and favors waterborne transmission. Infected humans, domestic animals, and wildlife may all contribute to the pool of oocysts in a watershed through wastewater discharges (127). There is a lack of host specificity for *C. parvum*. Isolates from humans are infectious for a variety of mammals, and isolates from one mammalian species are infectious for others (41). Opportunities for zoonotic transmission exist when persons are closely associated with infected livestock (24, 118) and companion animals (42). Additional sources of contamination include urban and

agricultural slurry, septic tank leakage, recreational bathing, agricultural runoff, and erosion of soils exposed to infected feces (142). Waterborne outbreaks have demonstrated that *C. parvum* oocysts can pass through filtration systems currently in use and are insensitive to the standard chlorination regime used in the water industry (126). The fact that surface and groundwater may be contaminated by cryptosporidial oocysts and the lack of effective treatment stress the importance of techniques to remove *C. parvum* from drinking water.

C. parvum oocysts are resistant to many disinfectants. The survival of *C. parvum* oocysts after 18 hours incubation in several disinfectants was examined. Only formol saline and ammonia were effective in destroying the viability of the oocysts (21). Both ozone and chlorine dioxide constitute a means of removing most *C. parvum* oocysts from drinking water, although some oocysts may remain viable (105). Since oocysts are resistant to many disinfectants, prevention of waterborne spread of cryptosporidiosis relies on wastewater treatment and improved filtration methods. The efficiency of oocyst removal in sewage treatment facilities using activated sludge approaches 79 to 84% when one compares the number of organisms in raw versus treated sewage (86, 142). Chapman and Rush (1990) show that when purified oocysts are applied to the top of a sand filter, oocysts do not easily pass through the filter when eluted

with distilled water at a filtration rate of 15 m^3/m^2/hour (26). Treatment plants using sand filtration along with activated sludge have significantly lower levels of oocysts in their finished effluents than those using activated sludge treatment alone (86). These studies establish that water treatment processes are capable of removing a percentage of oocysts. However, it is important to realize that the proportion of oocysts that penetrate the system is dependent not only on the design and operation of the treatment process, but also upon the number of oocysts challenging the filters and water treatment system (126). Lastly, altering the temperature of the contaminated water may kill the oocysts. Oocysts are unable to survive freezing (126) or temperatures above 45°C (25). However, the fiscal and practical implications of altering water temperature are considerable.

Treatment

Attempts to treat cryptosporidiosis have met with limited success. At the time of this writing, no consistently effective parasiticidal agent or preventive vaccine is available (51). Treatment remains limited to oral or intravenous hydration and hyperalimentation (administration of greater than optimal amount of nutrients) (33).

Few chemotherapeutics have shown any efficacy. Some success has been reported for arprinocid (117), spiramycin

(100), octreotide (87), paromomycin (7, 36, 52, 56), eflornithine (121), and halofuginone lactate (101). The effectiveness of halofuginone remains questionable because Tzipori et al. (1982) show that halofuginone neither prevents nor modifies the course of infection in neonatal mice (135). Two synthetic lytic peptides (Hectate 1 and Shiva 10) exhibit significant activity against *C. parvum* in an *in vitro* assay with unproven activity *in vivo* (8). Greenberg et al. (1989) describe a patient with severe secretory diarrhea and malabsorption who had clinical, microbiologic, and histologic resolution of cryptosporidiosis after therapy with azidothymidine. This beneficial effect is believed to be secondary to improved cell-mediated immune functions mediated by suppression of human immunodeficiency virus by azidothymidine (53).

Other treatments, such as hyperimmune bovine colostrum (HBC) and anti-cryptosporidial monoclonal antibodies (Mabs), show varying degrees of success. Hyperimmune bovine colostral immunoglobulins have been successful in treating an AIDS patient (103, 141) and neonatal BALB/c mice with severe cryptosporidiosis (44). The efficacy of HBC as a immunotherapeutic agent may be explained by the fact that each immunoglobulin isotype in colostral whey recognizes meronts, merozoites, microgametocytes, microgametes, and macrogamonts (43). Based on these findings, it seems that antigens in all parasitic stages provide targets

of opportunity for the anti-parasitic activity of HBC. Neonatal BALB/c or nude mice treated with anti-cryptosporidial murine Mabs had lower parasite loads than did control mice (9, 14). Similarly, suckling BALB/c mice are protected when sporozoites or merozoites are incubated with Mabs or HBC before gastric inoculation (16, 108, 120). In contrast, dams that recovered from enteric cryptosporidiosis did not protect their infants from experimental cryptosporidiosis (9, 62, 97, 98).

Immunomodulation therapies appear to hold some promise for treating patients with cryptosporidial infections. Rasmussen et al. demonstrate that dehydroepiandrosterone (an immunomodulator) treatment has significant anti-cryptosporidial activity in experimentally infected immunosuppressed rats (112, 116) and Syrian golden hamsters (113). A treatment trial with oral bovine transfer factor results in clinical and parasitologic cure in one of eight patients with cryptosporidiosis and clinical improvement in four others (85). Studies with interferon gamma show promise in treating cryptosporidial infections (61, 91, 138). Adoptive transfer of murine lymphocytes to nude (136) and mice with severe combined immunodeficiency (SCID) mice with chronic cryptosporidiosis causes functional immunologic reconstitution, followed by complete eradication of cryptosporidial infection (94). Severe combined immunodeficient mice injected with unprimed BALB/c spleen

cells prior to inoculation with *C. parvum* oocysts are resistant to infection (91). Conversely, neither spleen nor mesenteric lymph node cells, nor cells or supernatant fractions harvested from *in vitro* cultures, transfer protection from resistant adult donors to susceptible infant recipients (60).

Immunobiology of *C. parvum*

Most investigators believe that humoral and cell-mediated immunities are essential in removing *C. parvum* from the infected host. The role of antibodies in the natural resolution of cryptosporidial infections is supported by the observation that many of the early case reports of human cryptosporidiosis were from patients with immunoglobulin deficiencies (79, 125, 144).

Although the nature of acquired immunity to *C. parvum* is not clear, it does result in the appearance of serum antibodies that are specific to *C. parvum*. An immunofluorescent antibody assay using tissues from experimentally infected animals (28, 35, 134) or oocysts as antigen (23, 70) demonstrates that recovery from intestinal cryptosporidiosis is usually accompanied by a marked parasite-specific seroconversion. The first description of antibodies to *Cryptosporidium* sp. was reported by Tzipori in 1981 in 10 animal species, including humans. Antibody was detected by fluorescent antibody techniques performed on

cryostat sections of gut tissue from an experimentally infected lamb. Antibody is present in a high proportion of serum samples tested (134). Campbell and Current (1983) show that five of five immunocompetent persons who were tested between 360 and 400 days after recovery from a single intestinal infection with *Cryptosporidium* had detectable serum antibodies that were specific for the parasite and which recognized all life cycle stages (22). Neither of these studies using histochemical methods defined precisely the nature of the immune response in terms of immunoglobulin isotypes. An immunofluorescent study using oocysts as antigen shows that *Cryptosporidium*-specific IgG, IgM, IgA, and IgE are present in the sera of persons who recover from intestinal cryptosporidiosis (23).

Procedures using sonicated oocysts as antigen have demonstrated that there are *Cryptosporidium*-specific IgG and IgM antibodies in the sera of persons who have recovered from intestinal cryptosporidiosis (80, 137, 139, 140). Ungar et al. (1986) demonstrate the presence of both classes of *Cryptosporidium*-specific antibodies in AIDS patients with prolonged cryptosporidiosis (139, 140). Moreover, specific anti-*Cryptosporidium* antibodies are detected by this method in the sera of cattle (84, 99, 106, 146), rats (47), and mice (128) that are infected with *Cryptosporidium*.

Coproantibodies have been detected in humans and animals after natural or experimental cryptosporidial infections. An enzyme-linked immunosorbent assay (ELISA) was used to measure the *C. parvum*-specific IgA, IgG, and IgM levels in stool and duodenal fluid of Filipino children (80). Antibody levels were measured on admission to the hospital, 1 week later, and at 6-week follow-up examinations. The duodenal fluid samples show higher levels of IgA at the 6-week collection. Stool samples have significantly higher levels of IgM at all three collections and for IgG at the acute collection. Immunoglobulin A is found in detectable levels, though there was no difference when compared with the controls (80). Fecal anti-*C. parvum* immunoglobulins were monitored by an ELISA after infection of calves with *C. parvum*. Experimental infection is followed by a rise in local anti-*C. parvum* IgM levels from day 5 PI. Immunoglobulin M peaks at day 14 PI and then disappears quickly. Anti-*C. parvum* IgA levels increase between days 7 and 14 PI and then slowly decrease. Fecal anti-*C. parvum* IgG levels increase slightly during oocyst output, and IgG disappears 3 weeks PI (106). Hill et al. (1990) demonstrate that *C. parvum*-specific IgA is the only isotype detected by immunofluorescent assay in fecal extracts from infected lambs. Specific IgA reach a mean peak titre in these lambs on day 16 and then decline (70).

Immunoblot analysis of sera and intestinal secretions of BALB/c mice orally infected with oocysts reveals that: 1) the intestinal antibody response to the parasite is characterized by a strong recognition of antigens by IgA and a similar but less important pattern of binding with local IgG; 2) no specific IgM is detected in intestinal secretions; 3) specific IgA, IgG, and IgM are found in the sera of infected mice; and 4) the antibody response appears in serum and intestinal secretions between day 8 and day 15 PI (119).

Although marked humoral immune responses occur, it is unlikely that antibodies alone play a major role in acquired immunity because the membrane of host cell origin that separates *C. parvum* sessile stages from the intestinal lumen may prevent antibodies from binding to the nonmotile parasitic stages. It is likely that a protective response against *C. parvum* involves T lymphocyte activation and induction of specific antibody responses (34). Responses mediated by B or T lymphocytes, or both, appear to play a role in eradicating *C. parvum* from the intestinal mucosa, since animals with SCID develop a severe and persistent infection (15, 57, 78, 91, 94, 95). Severe combined immunodeficient animals, in addition to possessing nonfunctional B and T lymphocytes, have normal natural killer (NK) cell counts. The fact that chronic infections develop in SCID mice suggests that either NK cells play a minor role

in host responses to *C. parvum* or are dependent on T lymphocytes or T lymphocyte products (e.g., cytokines) for part of their function (95).

The importance of T lymphocytes in the clearance of *C. parvum* from the mammalian intestine is supported by several studies. B cell deficient (anti-μ treated) neonatal mice do not differ from untreated controls in the onset, peak, or duration of cryptosporidiosis (128). Parasite exposure of nude mice (63, 95, 136, 138) and rats (47) with greatly reduced T lymphocyte function results in chronic symptomatic cryptosporidiosis. In contrast, control animals (euthymic) have self-resolving infections with no detectable signs of disease. An *in vitro* cell-mediated immune response against *C. parvum* is demonstrated in the lymphocyte blastogenesis assay. Peripheral blood lymphocytes from calves (146) and murine spleens (145) exhibit a significant antigen-specific blastogenic response.

Considerable effort has been spent in determining which subset of T lymphocytes is responsible for clearance of *C. parvum* from the infected mucosa and for rendering the host resistant to reinfection. Flanigan et al. (1992) show that self-limited cryptosporidiosis is associated with a higher CD4+ count (helper T cells), CD8+ count (cytotoxic T cells), and CD4+ to CD8+ cell ratio, although only the CD4+ count is an independent predictor of self-limited disease (45). Chronic *C. parvum* infection can be produced in

infected mice that are treated with anti-CD4 Mab with or without anti-CD8 Mab treatment (136, 138). In contrast, infected mice that receive anti-CD8 Mab alone cease shedding detectable oocysts. In a similar study MHC class II deficient mice (lacking functional CD4+ cells) dosed with oocysts remain infected 8 weeks postexposure (1). In contrast, MHC class I deficient mice (lacking functional CD8+ lymphocytes) clear *C. parvum* infections similar to age-matched controls. Unlike those studies performed in T lymphocyte deficient mice, others have shown the effect of *C. parvum* on lymphocyte subpopulations. Experimental cryptosporidial infections in adult female C57BL/6 mice show that T cells (thymic and splenic) are significantly higher than in the uninfected controls. Moreover, CD4+ lymphocytes in the spleen are increased in those mice with cryptosporidiosis (37).

Another study used neonatal mice to characterize the leucocyte subgroups present in PP from the ileum and jejunum of *C. parvum*-infected mice. This study shows that ileal and jejunal PP are functionally different in response to *C. parvum*. Moreover, Boher et al. (1994) suggest an involvement of jejunal PP in T lymphocyte dependent immunity against the parasite, whereas ileal patches may be associated with B lymphocyte expansion and maturation (18).

The systemic and localized immune responses have been determined in similar studies of intestinal and extrain-

testinal parasites, such as *Eimeria* spp. (74) *Ascaris suum* (88), *Giardia* spp. (65, 66), *Trichinella spiralis* (32), *Strongyloides stercoralis* (48), *Taenia hydatigena* (96), and the larval form of *Dermatobia hominis* (54). Although there are substantial differences in the biology of *Giardia* spp. and *C. parvum*, the immunological responses to these parasites are astoundingly similar (Table 1). As in the case of cryptosporidial infections, immunologic clearance of giardiasis depends on lymphocytes bearing CD4+ receptors. One difference that should be noted, however, is that the susceptibility of immunocompetent rodents to *C. parvum* is age related. Nevertheless, studies investigating the dynamics of the immune response to these parasites will be useful in developing parasiticidal agents and immunomodulation therapies.

Very little is known about the immunological response to cryptosporidiosis that does not involve the recognition of antigen by lymphocytes and the mounting of specific immune responses (e.g., nonspecific immunity, anatomical barriers, etc.). Experimental cryptosporidial infections in fetal lambs shows that hypercellularity of the intestinal lamina propria occurs and consists of a mixed infiltration of neutrophils, MØ, and eosinophils. Moreover, there appears to be an increase in phagocytic activity with time (77). The role that granulocytes play that *C. parvum* infections in mice with a deficit in ery

TABLE 1. List of cell phenotypes involved in *Giarida* spp. and *Cryptosporidium* infections

Cell Type	*Giardia* spp.	*Cryptosporidium*
CD4+	+(67)	+(1, 37)
CD8+	-(67)	-(1, 138)
IgG producing	+(13, 75)	+(80, 137)
IgM producing	+(13, 75)	+(80, 137)
IgA producing	+(13, 75)	+(70, 80)
IgE producing	Unknown?	+(23)
NK	-(68)	-(95)

+ plays a role in the clearance of parasitic infection (Ref.)
- plays no role in the clearance of parasitic infection (Ref.)
NK = natural killer

throcytes, granulocytes, and mast cells are similar to in the elimination of *C. parvum* is uncertain because Harp and Moon (1991) show infections in normal mice (58). The role of MØ is more complex than that of granulocytes. In addition to having important phagocytic functions, MØ can participate in the initiation of a specific immune response by processing the antigen and presenting it to lymphocytes (30, 82). Conceptually, MØ should exert an active defense against *C. parvum*, however the parasite appears to be resistant to lysosomal digestion. It is this resistance that permits *C. parvum* to multiply in the cytoplasm of MØ; therefore, parasites within MØ may gain access to other organs and tissues (90). Nevertheless, parasites within MØ are particularly vulnerable to the effects of MØ products if their defenses break down (31). Additional evidence

that supports the involvement of nonspecific mechanisms of resistance in cryptosporidiosis is supported by studies done with germ-free and flora-bearing mice (57, 60).

Intestinal immunity

The mucosal epithelium overlying the PP appears to be modified for immune function, specifically the uptake and transport of luminal antigens across the epithelium. In the small-intestinal PP, specialized epithelial cells, designated "M cells" (microfold cells), have been reported to be capable of transporting particles ranging in size from proteins to intact cryptosporidia (89) from the lumen to underlying lymphoid tissue. This path of entry presumably permits antigenic sampling by the intestinal immune system. Mechanisms other than invasion of the mucosa that might provoke an immune response includes apoptosis, which can take place in enterocytes. This "programmed" cell death may result in antigen being presented to the immune system by phagocytes that have eliminated these membrane-bound particles (23).

The majority of T and B lymphocytes continuously recirculate between the secondary organs (i.e., lymph nodes and PP) and the bloodstream. This continuous recirculation not only ensures that the appropriate lymphocytes will come into contact with antigen, but also ensures that appropriate lymphocytes will encounter each other. Lymphocyte

recirculation depends on specific interactions between the lymphocyte glycoproteins on the cell surface and the surface of specialized endothelial cells lining small veins (called postcapillary venules) in the secondary lymphoid organs. Some lymphocytes express specific glycoproteins that attracts them to the PP; these cells constitute a gut-specific subsystem of lymphocytes specialized for responding to antigens that enter the body from the intestine (2).

After the lymphocytes percolate through the PP, they accumulate in postcapillary venules that leave the PP and connect with other lymphatic venules. Passing into increasingly larger vessels, the lymphocytes eventually enter the systemic compartment (2). Within the systemic compartment (including the spleen), the lymphocytes differentiate (93, 131) and eventually enter the bloodstream. Lymphocytes then leave the blood stream and migrate to the lamina propria of the intestinal villi and other mucosal sites by squeezing between specialized endothelial cells (2).

Hypercellularity of the intestinal lamina propria is an essential component of intestinal immunity. Many lymphocytes and immunoglobulins make their way through the lining epithelium to enter the intestinal lumen (55). These antibodies presumably eliminate the parasite by mediating biological processes including motility, attachment to the host cell, modification of the host membrane, and entry into the host cell (111). Moreover, antibodies

may result in opsonization of the parasite for killing by complement or by the mechanism of antibody-dependent cellular cytotoxicity (38). Lastly, secretory antibodies (IgA) can be transported across epithelial cells of the intestine from the basal to the apical surface of these cells by transcytosis (2). Consequently, it is reasonable to speculate that *C. parvum*-specific IgA might reach the basal membrane of attached cryptosporidia directly via the epithelial cell cytoplasm.

Immunosuppressive agents

Dexamethasone is a potent synthetic glucocorticoid that has striking pharmacologic effects on lymphoid tissues and cells. These effects form part of the basis for the widespread use of corticosteroids in the treatment of a variety of diseases involving immunologic, inflammatory, or neoplastic processes. The efficacy of DEX in the treatment of various inflammatory and immunological processes can be accounted for by a combination of mechanisms such as inhibitory action on the production of important lymphokines and monokines. Additional mechanisms include: 1) effective change in the traffic of various leucocyte populations; and 2) ability to lyse lymphocytes through induction or enhancement of the action of a specific DNAase (4), resulting in digestion of cellular DNA. Animals have been divided into glucocorticoid-sensitive and gluco-

corticoid-resistant groups. Glucocorticoid-resistant species include most domestic animals as well as man, ferret, guinea pig, and monkey, whereas the rat, mouse, hamster, and rabbit are considered glucocorticoid-sensitive. The differentiation is usually based on the relative ease of producing lymphoid depletion after a given regimen of systemic glucocorticoids (29). The differences in susceptibility to glucocorticoids are often overlooked. However, they are of crucial importance in the interpretation of data. Therefore, the emphasis of this review concerns the effects of DEX in murine models. In sensitive animals, glucocorticoids profoundly affect lymphoid tissues. The animals exhibit lymphopenia (20) and shrinkage of the thymus, spleen, and lymph nodes.

Inhibition of circulating antibody production by DEX has repeatedly been shown in glucocorticoid-sensitive animals. For example, rats treated with DEX have lower IgG levels in serum (115, 147), saliva , and vaginal secretions (147). Rodents treated with DEX have elevated IgA levels in serum, while IgA levels decrease in saliva, vaginal secretions (147), and bile (3). Moreover, C57BL/6N mice that are immunosuppressed with DEX have fewer total B lymphocytes in the spleen (115).

In glucocorticosteroid-sensitive species, there has been relatively little study of classic manifestations of cell-mediated immunity. Immunosuppression of murine CD4+

cells (115, 124) and total T lymphocytes (115) is brought about by DEX. Helper T lymphocytes are believed to be more responsive to glucocorticoid-mediated immunoregulatory effects than CD8+ cells because they are more migratory as immature cells (27).

MATERIALS AND METHODS

Animal housing and husbandry

Female C57BL/6N strain mice weighing 14 to 16 grams (approximately 5 to 6 weeks old) were purchased from Simonson Laboratories (Gilroy, CA) and maintained in the Laboratory Animal Research Center at Utah State University. Mice were housed in transparent plastic cages with stainless steel wire lids at approximately 22°C and a 12-hour light-dark cycle. These animal cages were kept in high-efficiency particulate air-filtered laminar flow units. Five mice (within the same treatment group) were housed per cage, on corncob bedding with food and water provided *ad libitum*. The mice were randomly placed into one of the following groups (see Fig. 2 for a graphic illustration of the groups described below):

Group 1. Mice were immunosuppressed with daily DEX intraperitoneal injections for the duration of the experiment.

Group 2. Mice were immunosuppressed with DEX by daily intraperitoneal injections and oocysts of *C. parvum* were intragastrically administered (10^6 oocysts/mouse) 3 days after immunosuppression began. All mice within this group continued to receive DEX intraperitoneally after oocyst inoculation and throughout the duration of the experiment.

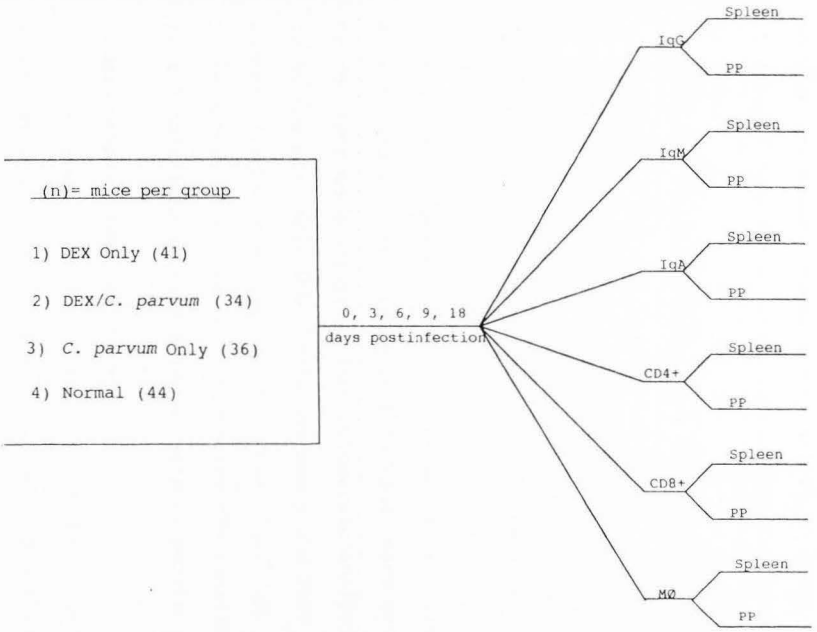

Figure 2. Experimental design for investigating the phenotypic profiles of cells in mice treated with dexamethasone (DEX) and infected with *Cryptosporidium parvum*. Monoclonal antibodies directed against CD4+ and CD8+ T cells (helper and cytotoxic, respectively), and B cells (expressing IgG, IgM, and IgA receptors), and macrophages (MØ) were used to quantify these leucocytes within the spleens and Peyer's patches (PP).

Since the prepatent period of *C. parvum* is 3 days, no mice were killed on day 0.

Group 3. Mice received intragastrically 10^6 *C. parvum* oocysts per mouse. No mice were killed on day 0, because it takes 3 days for a patent cryptosporidial infection to develop.

Group 4. This group of mice served as the nonimmunosuppressed/noninfected (normal) controls.

Spleen tissues were used to represent the systemic immune response and the PP from the small intestine were used to determine the localized immune response. In order to determine the best methodology for this study, avidin biotin complex staining, the ELISA-spot assay, flow cytometric, and immunofluorescence techniques were employed. Of the four methodologies, flow cytometric analysis proved to be the most suitable. Flow cytometry and Mabs directed against lymphocytes bearing CD4+, CD8+, IgG, IgM, IgA, and MØ receptors were used to characterize the kinetics of the cell populations in mice infected with *C. parvum*.

Immunosuppression of mice

The immunosuppressed adult mouse model established by Rasmussen and Healey (114) for chronic cryptosporidiosis was used in this experiment. Limitations within this experiment necessitated slight modifications of their originally proposed protocol. Briefly, DEX (Sigma Chemical

Co., St. Louis, MO) was diluted from a stock solution (6.25 mg/ml in absolute ethanol) to a working solution (0.625 mg/ml in sterile water) and administered in a daily regimen of 125 µg per mouse. All mice within groups 1 and 2 received DEX by intraperitoneal injection through a 26 1/2 gauge syringe needle throughout the duration of the experiment. Mice in group 2 received DEX 3 days before infection with *C. parvum*. Nonimmunosuppressed mice received similar injections with 0.2 ml of sterile water.

Oocyst production and purification

C. parvum oocysts originally isolated from Holstein calves and obtained from Dr. Harley Moon (NADC, Ames, IA) were used to infect 1- to 2-day-old Holstein calves. At the peak of oocyst shedding, feces were collected daily, mixed with an equal volume of 5% potassium dichromate ($K_2Cr_2O_7$), and stored at 4°C. Feces were strained sequentially through stainless steel screens with a final mesh size of 230 (63 µm porosity). Oocysts were purified from feces using discontinuous sucrose gradient centrifugation techniques (10). In brief, the discontinuous sucrose gradients used in the oocyst purification process were prepared from Sheather's solution (320 ml H_2O, 500 g sucrose) diluted with 0.025M PBS and supplemented with 1% Tween 80. Eighty milliliters of 1:4 solution (specific gravity 1.064) were layered over 80 ml of the 1:2 solution

(specific gravity 1.103) in 250-ml polycarbonate centrifuge bottles. A 40-ml aliquot of the strained feces in potassium dichromate was layered over the 1:4 solution. Tubes were centrifuged at 1500 X *g* for 25 min and the oocysts were recovered from the interface of the sucrose layers and washed with 0.85% saline at 1500 X *g* (twice for 10 min). The pellets were resuspended to 40 ml with 2.5% potassium dichromate, dispensed in 5-ml aliquots over new gradient tubes (10 ml 1:2 Sheather's layered over 10 ml of 1:4 solution), and centrifuged as before. Pellets from the oocyst-containing layers of the second centrifugation were resuspended in potassium dichromate and stored at 4°C.

Oocyst preparation and inoculation of mice

Experimental murine cryptosporidial infections of groups 2 and 3 were established by using oocysts (less than 4 months old). Oocyst inoculations were prepared by washing oocysts three times with sterilized RPMI 1640 cell culture medium (Sigma Chemical Co.) to remove the potassium dichromate. Washed oocysts were then enumerated using a hemocytometer observed under bright field microscopy and diluted to 10^6 oocysts per 100 µl of RPMI 1640 base medium. Mice were inoculated intragastrically with 10^6 purified oocysts through a 22-gauge straight feeding needle.

Fecal collection and examination

Fecal pellets were collected from each mouse on every third day of the experiment to monitor oocyst shedding. Mice were removed from their cages and held briefly in a laminar flow hood to allow for collection of feces. Fecal samples were stored in microfuge tubes at 4°C in 300 µl of 2.5% potassium dichromate solution until analyzed. Fecal samples were prepared on microscope slides and assayed for the presence of oocysts using a fluorescent-labeled oocyst-specific Mab-based indirect immunofluorescent assay (11). Briefly, fecal samples were broken apart with a disposable applicator stick and then well vortexed. Four bacterial loops of feces were smeared on the slide to cover approximately the area equivalent to a quarter dollar. Slides were air-dried and fixed by heat. Fifty microliters of undiluted hybridoma supernatant containing anti-oocyst Mab 9D10 (isotype IgM) produced in this laboratory were applied over the fecal smears. Fecal smears were then incubated for 30 min at 41°C and 100% humidity. Unbound antibody was removed by washing slides in three changes of PBS for 3 min each. Care was taken to wipe off excess PBS from the slides before applying the second antibody. Fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgM (Hyclone Laboratories Inc., Logan, UT) diluted 1:60 in PBS was then applied to the fecal smear (90 µl) and incubated as before

for 30 min. Specimens were washed in PBS as before to remove unbound antibody. Slides were mounted with 1:1 glycerol/PBS and covered with glass coverslips (0.17-0.25 mm thick). Prepared slides were stored at -20°C until examined with epifluorescence microscopy. Slides were examined using an ultraviolet light microscope (Carl Zeiss, Oberkochen, Germany) and the number of oocysts observed in a single pass (40x objective) through the center of the fecal smear was counted.

Histological collection and examination

Terminal ilea were harvested and fixed in 10 ml of 10% buffered formalin (pH 7.5), embedded in paraffin, sectioned (4 µm), and stained with hematoxylin and eosin. Stained sections were examined for *C. parvum* colonization by using bright field microscopy in a blinded fashion. Parasites were quantified by counting the intestinal epithelial cells and the parasites from the apex of a villi to the apex of the adjacent villi. Five random villi were counted.

Avidin biotin complex (ABC) staining

Immunoperoxidase staining was done on paraffin-embedded tissue sections according to the method of Loose et al. (83). Briefly, the spleen, terminal ileum, and semitendinosus muscle were harvested and fixed in B-5

fixative (described in the following section) for either 3, 5, 7, or 7.5 hours. Tissues were then processed, embedded in paraffin, and sectioned in a routine manner. Sections were heated to 70°C for 5 min. The slides were then treated with the following reagents: 1) xylene for 5 min; 2) cold 95% ethanol for 5 min; 3) cold PBS wash for 10 min; 4) 0.3% hydrogen peroxidase-methanol mixture for 30 min to quench endogenous peroxidase activity; 5) PBS wash for 10 min; 6) normal goat blocking serum for 30 min; 7) biotinylated anti-mouse Ig (Hyclone Laboratories Inc.), IgA (Sigma Chemical Co.), or CD4 (PharMingen, San Diego, CA) Mabs for 30 min. Negative controls included biotinylated goat anti-swine IgG (Vector Laboratories., Burlingame, CA) and the omission of i) primary antibody ii) streptavidin horseradish peroxidase, and iii) diaminobenzidine tetrahydrochloride (DAB); 8) three PBS washes for 5 min each; 9) streptavidin horseradish peroxidase complex reagent (Hyclone Laboratories Inc.) for 45 min; 10) three PBS washes for 5 min each; 11) DAB (32 mg in 15 ml of Tris buffered saline with 12 µl of 30% hydrogen peroxide) for a 5 min incubation; and 12) a tap water rinse to stop the reaction.

To eliminate background staining, modifications of previously described procedures were used and adapted to this methodology (5, 19, 39, 64, 71-73, 81, 122, 143).

Immunofluorescence techniques

Tissues were harvested and prepared immediately after mice were killed. In order to determine the optimal fixation conditions of the tissues, trials using the following fixatives, at varying time lengths, were attempted (40, 149):

1) B-5 fixative: 90 ml aqua dest, 6 g mercuric chloride, 2.074 g sodium acetate and 10 ml of 37% formaldehyde solution, pH 5.7;
2) 10% buffered formalin: 10 ml of 37% formaldehyde solution and 90 ml of PBS, pH 7.5;
3) Bouin's solution (Sigma Chemical Co.); and
4) Histochoice tissue preservative (Ameresco, Solon, OH).

Specimens were processed, embedded in paraffin, and sectioned in a routine manner. After embedding, sections (4 μm) were cut and prepared for deparaffinization and direct immunofluorescent staining according to the method of Dorsett and Ioachim (40), with slight modifications. Slides were heated for 30 min at 80°C. Deparaffinization was accomplished by a 5-min wash in xylene and sequential passage through two changes each of absolute ethanol, 95% ethanol, 80% ethanol, and PBS (0.025M PBS, pH 7.3). Rehydrated sections were washed for 2 hours with constant agitation in PBS to remove the remaining fixative. Sections for direct immunofluorescence were then overlayed

with FITC-labeled antiserum diluted appropriately in PBS and incubated in a humid chamber at 41°C for 1 hour. Controls consisted of sections in which a Mab of irrelevant specificity was substituted for the FITC-labeled antibody. Unbound antibody was removed by washing in three changes of PBS for 10 min with constant agitation. Slides were counterstained with 3% methyl green (diluted in methanol) for 1 to 2 min, followed by a brief rinse in distilled water. Coverslips were added with 1:1 glycerol/PBS. Tissue sections were examined microscopically in a blinded fashion.

Because the antibodies may have been affected by buffers, incubation times, and relative conditions, this protocol was altered. The following buffers were tried for washing and to dilute the antibodies: 0.9% saline; 50mM Tris buffered saline; and 0.025M PBS. All buffers were tested with or without bovine serum albumin (BSA). This was done because BSA may effect antigen recognition by certain antibodies (130). Slides were overlaid with antibodies (undiluted to 1:1000) and incubated at either 4°C or 41°C and 100% relative humidity. Tissue sections were incubated under these conditions for 0.5, 1.0, 1.5, or 14.0 hours.

An indirect immunofluorescent assay was carried out as described in the previous paragraph, with the following modifications. Before the tissue sections were overlayed

with FITC-labeled antiserum, rat antibodies directed against mouse CD4+ and CD8+ lymphocytes (Gibco BRL, Gaithersburg, MD) were applied to the sections. Slides were incubated for 1 hour at 41°C and 100% relative humidity. Slides were washed in three changes of PBS for 10 min each.

Cryostat preparation

The cryostat chuck was stored at -20°C. A thin layer of OCT-embedding compound was applied to the chuck. A spleen was placed on the chuck and additional OCT-embedding compound was applied to cover the entire tissue. The spleen was cut on the microtome and applied to gelatin coated (subbed) slides. Slides were allowed to air-dry and then fixed by immersing in acetone for 5 min. Slides were stored at -20°C (<24 hours) until stained by immunofluorescent techniques as previously described in the section outlining tissue preparation and immunocyte detection.

ELISA-spot technique

A modification of the ELISA-spot techniques of Franci et al. (46) and Pestka et al. (109, 110) was attempted to quantify splenic lymphocytes in immunocompetent mice. In brief, a 96-well microtiter ELISA plate was uniformly coated with gelatin solution or poly-L-lysine. Each well was washed in PBS, emptied, and 0.3 ml of lymphocyte suspension (10^5 to 10^6 cells/ml) in RPMI 1640 containing

0.5% BSA was added to uniformly cover the bottom of the plate. The plates were centrifuged for 5 min at 450 *g*. Biotin conjugated goat anti-mouse Igs, IgA, and CD4 Mabs (diluted appropriately in 1% BSA-PBS) were added and plates were incubated for 60 min at 41°C. Plates were washed four times with PBS containing 0.5% Tween 20, and streptavidin horseradish peroxidase was added. Plates were covered and incubated at room temperature for 30 min. Following three PBS washes, DAB solution was applied. Incubation was continued for 5 min on ice and in the dark. The reaction was stopped with a PBS wash. Plates were examined at low power under a dissecting microscope for brownish spots which were indicative of lymphocytes.

Cell staining for flow cytometry

Immediately after the mice were killed, spleens and intestines were removed from the peritoneal cavities. The intestine was cut ~0.5 cm below the stomach and just above the cecum. Both spleen and small intestine were immediately placed in plastic petri dishes with RPMI 1640 containing 1 mM EDTA (ethylenediaminetetraacetic acid), to prevent drying. Fat and connective tissue were removed from the tissues. The spleen was weighed and set aside until the PP were harvested. Peyer's patches were removed by grasping them with a forceps and excising with a scalpel. Spleens and PP were put into separate stomach

bags with RPMI/EDTA solution and homogenized in a stomacher (Tekmar Co., Cincinnati, OH) for approximately 1 min. The resulting cell suspension was poured into 15-ml tubes. Visible intestinal pieces and spleen capsules were removed with disposable applicator sticks. Cell suspensions were centrifuged for 5 min at 300 *g*, followed by decantation of the supernatant. Nine ml of double distilled water was added to the spleen cell suspension to lyse erythrocytes. One to 2 seconds after hemolysis, 1 ml of 10X PBS (80 g NaCl, 2 g KCl, 11.5 g Na_2HPO_4, 2 g KH_2PO_4, and 1000 ml water) was added to prevent from lysing. After centrifugation at 300 X *g* for 5 min and decantation of the supernatant, the cells were washed two times in RPMI solution with 2% fetal bovine serum (FBS). Before staining, the cells were washed with RPMI solution without FBS. The pellet was resuspended in 0.5 ml of RPMI solution. The remaining leucocytes were counted with a Coulter Counter (Coulter Corp., Hialeah, FL) and aliquoted into polypropylene sample tubes (10^6 cells/100 µl). This 100 µl aliquot was stained with a mixture of Mabs: R-phycoerythrin (R-PE)-labeled anti-mouse CD8 and FITC-labeled anti-mouse IgM Mabs. A second tube with a 100 µl aliquot of cells was stained under identical conditions with a mixture of R-PE-labeled anti-mouse CD4 and FITC-labeled anti-mouse IgG Mabs. Similarly, a third tube with a 100 µl aliquot of cells was stained with R-PE-labeled anti-mouse MØ and FITC-

labeled anti-mouse IgA. All antibodies were diluted in 0.025 M PBS as recommended by the supplier. Staining was done at room temperature in the dark for 20 min. The cells were then fixed with 1 ml of 0.5% paraformaldehyde (Sigma Chemical Co.). Samples were immediately centrifuged as before. Supernatant was decanted and 0.5 ml of PBS added. Samples were kept <24 hours in the dark at 4°C until analyzed by flow cytometry.

Antibody specificities and controls for flow cytometry

Goat antibodies directed against mouse IgG, IgM, and IgA were obtained from Sigma Chemical Co. Rat antibodies directed against mouse CD4+ and CD8+ cells were purchased from PharMingen. Fluorescent-labeled rat anti-mouse MØ antibody was obtained from Caltag Laboratories (San Francisco, CA). These antibodies were affinity purified by the supplier and tested for specificity against the relevant class of mouse antibodies by immunoelectrophoresis. The affinity-purified antibodies were conjugated to FITC or R-PE by the supplier. Negative antibody controls for testing nonspecific binding were prepared as previously described in the paragraph on flow cytometric cell staining. However, the antiserum for the B lymphocyte populations was FITC-labeled goat IgG and R-PE-conjugated rat IgG2a Kappa isotype for the CD4+ and CD8+ populations. The isotype

control used for the MØ populations was R-PE-conjugated rat IgG2b.

Flow cytometric analyses

Dual-color fluorochrome analysis was performed with an EPICS-C model flow cytometer (Coulter Corp.) equipped with an argon laser set at 488 nm. Cells were carried in double distilled water as sheath fluid through a 76-micron flow tip. Twenty-five hundred cells were analyzed for each sample. Fluorescence data for FITC and R-PE were obtained using a bit-map format gated on the forward light scatter versus ninety-degree light scatter. Fluorescence intensity was standardized using latex beads of 10 microns (Coulter Corp.) and by adjusting the laser power to place the log-green histogram in channel 119 (148).

Statistical analyses

For each of the four treatment groups, eight mice were killed on each scheduled date, with the exception of groups 2 and 3 on day 0. Of the eight mice from each treatment group, the respective tissues (spleens and PP) from four mice were pooled, resulting in two repetitions. Spleen cell percentages reported by the flow cytometer were multiplied by the mean of their spleen weight to estimate absolute numbers. A square root transformation was required to stabilize variances prior to statistical analysis. Because data were not collected from groups 2 and 3

on day 0, multiple analyses of variance were performed to compare differences between animal groups on different dates. Least significant differences (LSD) were done to compare different groups within a date and different dates within a group.

RESULTS

Immunohistochemistry

Identification of lymphocytes in paraffin-embedded sections. Direct immunofluorescence techniques applied to paraffin-embedded sections stained B lymphocytes in the spleen and intestinal lamina propria. Three to 7 hours of fixation in B-5 fixative resulted in poor morphological preservation, although immunohistochemical reactivity with B lymphocyte receptors could be detected. Plasma cells stained weakly in the tissues that were fixed in Histochoice tissue preservative. Fixation in 10% buffered formalin for 4 to 7 hours showed reactivity for plasma cells, although it was relatively unsuitable for quantitating lymphocytes in the intestinal lamina propria. Fixation in Bouin's solution for 6 hours resulted in excellent preservation of morphology and proved to be a good fixative for lymphocytes bearing Ig receptors, which showed complete preservation of their capacity to react with FITC-labeled antibodies (Figs. 3 and 4).

A pilot study using direct immunofluorescence on paraffin-embedded tissues demonstrated that lymphocytes bearing IgA receptors are significantly decreased in 9-week-old mice that were immunosuppressed. Administration of DEX for 20 days significantly reduced the numbers

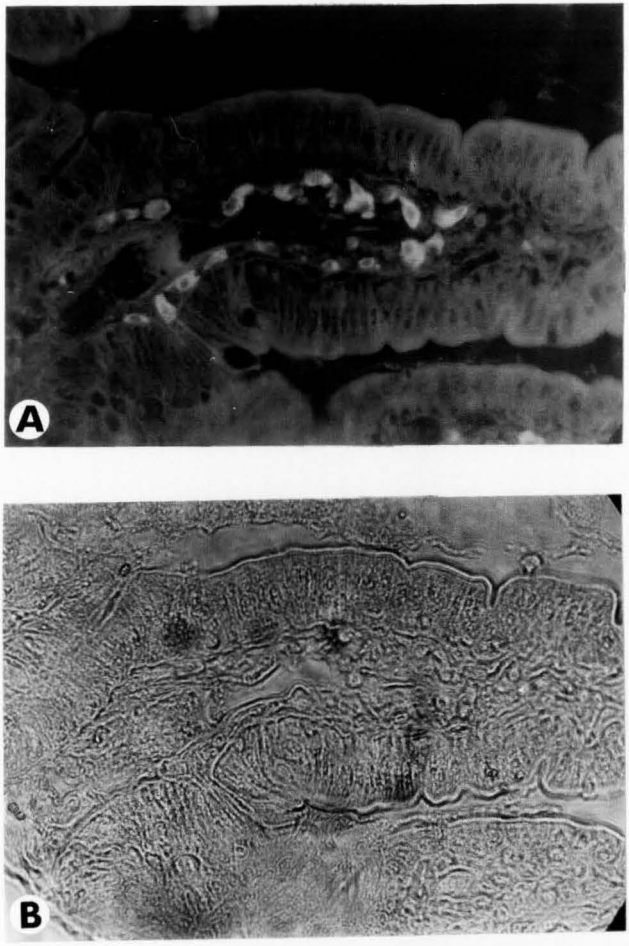

FIG. 3. Immunofluorescent (A) and methyl green (B) staining of the terminal ileum of a control mouse. Note the fluorescing B lymphocytes (expressing IgA receptors) present in the lamina propria (400x).

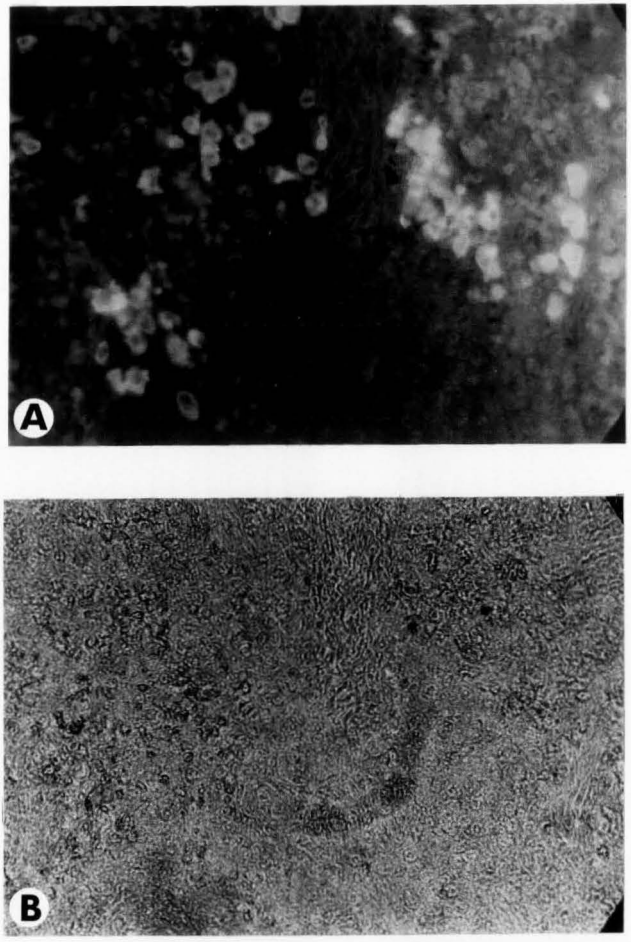

FIG. 4. Immunofluorescent (A) and methyl green (B) staining of the spleen of a control mouse. Note the fluorescing B lymphocytes expressing IgM receptors (400x).

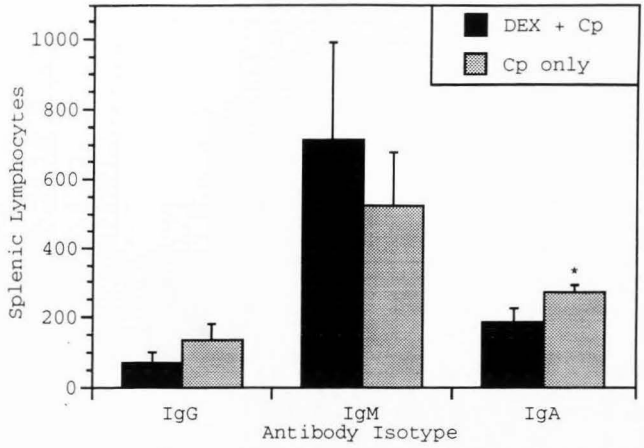

FIG. 5. Immunohistochemical evaluations comparing splenic lymphocytes in *Cryptosporidium parvum* (Cp)-infected mice to dexamethasone (DEX)-treated mice with chronic infections. Bar height represents the mean number of lymphocytes counted in 15 random fields (400x). Error bars indicate standard deviations of the mean. * indicates significance ($P<0.05$) when compared to DEX + Cp.

of B lymphocytes expressing IgA receptors in the spleen and intestinal lamina propria ($P<0.05$ and $P<0.02$, respectively). The number of IgG and IgM receptor bearing B cells in these mice were not significantly different from those of the nonimmunsuppressed controls (Figs. 5 and 6).

Direct and indirect immunofluorescent stainings were unsuitable for immunohistochemical examination of MØ and T lymphocytes. Altering the buffer conditions, incubation times, and temperatures failed to stain MØ, CD4+, or CD8+

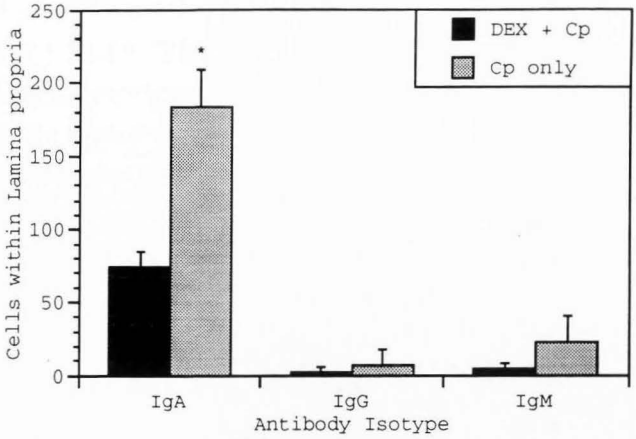

FIG. 6. Immunohistochemical evaluations comparing lymphocytes within the ileal lamina propria of *Cryptosporidium parvum* (Cp)-infected mice to dexamethasone (DEX)-treated mice with chronic infections. Bar height represent the mean number of lymphocytes counted in the lamina propria of 15 villi. Error bars indicate standard deviations of the mean. * indicates significance ($P<0.02$) when compared to DEX + Cp.

cells within the spleen or intestinal lamina propria. However, the plasma cells stained with each alteration.

Immunofluorescent staining of frozen sections. Immunofluorescent techniques applied to frozen sections had several shortcomings that limited their application to this study. The antiquated cryostat microtome cut the tissues in varying thicknesses that were thicker than desirable.

Although immunohistochemical reactivity of B lymphocytes was evident in the spleen, this technique resulted in poor morphological preservation, insufficient resolution, and nonspecific fluorescence.

Immunoperoxidase staining. Avidin biotin complex staining on paraffin-embedded tissues was unsuccessful. Modifications of the protocol (i.e., antibody and enzyme dilutions, filtration of substrate, incubation time and temperature, blocking nonspecific binding, and blocking endogenous peroxidase activity) failed to eliminate background staining.

ELISA-spot technique. Attempts to stain lymphocytes with the ELISA-spot technique were met with limited success. Some immunochemical reactivity was noticeable. However, the modifications made to this technique resulted in clumps of lymphocytes aggregating at the edge of the wells. In addition, there was considerable nonspecific binding.

Infection dynamics

Oocyst production and intestinal colonization following *C. parvum* infection. All mice were confirmed to be uninfected with *C. parvum* when the experiment began. Both control groups (normal and immunosuppressed mice) remained uninfected with *C. parvum* throughout the experiment. All mice in groups 2 and 3, respectively, were

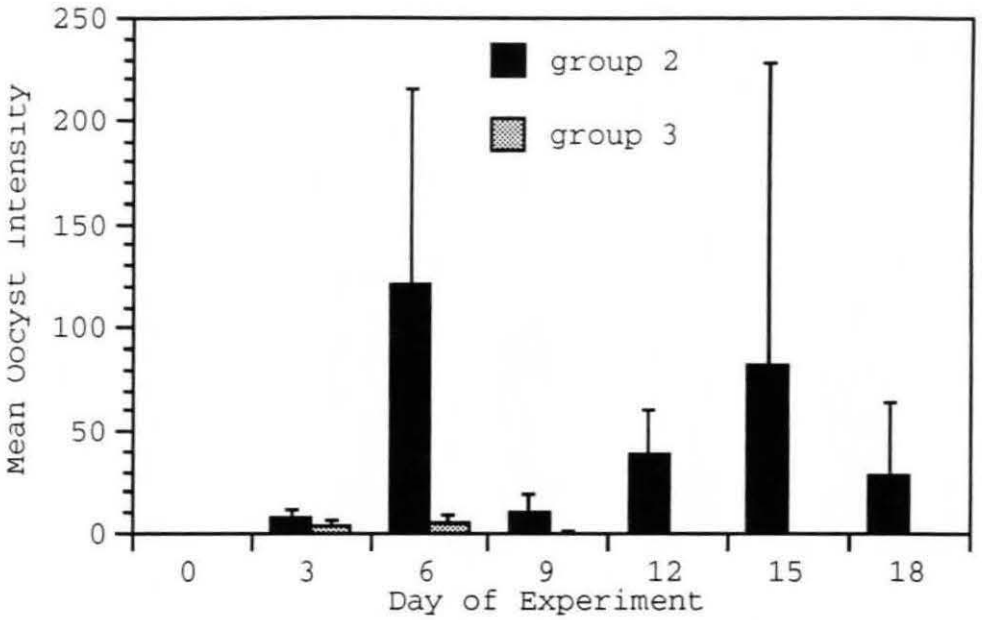

FIG. 7. Fecal oocyst shedding intensities comparing *Cryptosporidium parvum*-infected mice immunosuppressed with dexamethasone (group 2) and nonimmunosuppressed mice (group 3). Data are illustrated as mean oocyst intensity. Error bars indicate standard deviations of the mean.

shedding oocysts by day 3 PI. However, after day 9 PI, all mice in group 3 had ceased shedding and remained negative for fecal oocysts throughout the experiment. All mice in group 2 continued to shed oocysts until the experiment was terminated (Fig. 7).

Similar results were observed when ileal sections were examined for parasite colonization. However, there was a notable difference on day 3. Mice in groups 2 and 3 shed approximately an equal number of oocysts 3 days postinoculation, whereas histological examination of the terminal

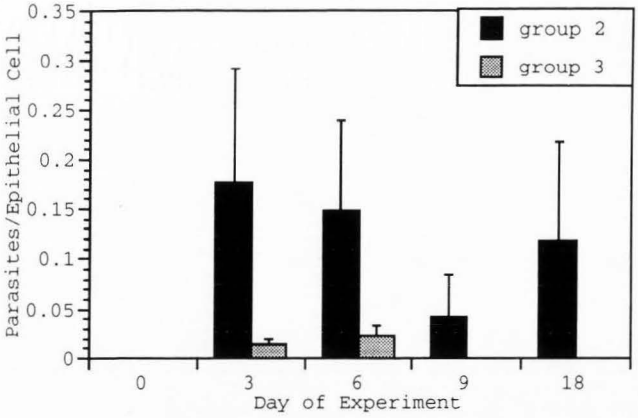

FIG. 8. Ileal colonization by *Cryptosporidium parvum* comparing mice treated with dexamethasone (group 2) and nonimmunosuppressed (group 3) mice. Data are shown as the mean parasite to epithelial cell ratio. Error bars indicate standard deviations of the mean.

ilea showed notably more parasite per epithelial cell in infected immunosuppressed mice (Fig. 8).

Effects of DEX and *C.parvum* on spleen weights. When DEX was administered for 3 consecutive days, the mean spleen weight of mice in groups 1 and 2 was about 30 mg less than the average spleen weight of nonimmunosuppressed mice in groups 3 and 4. After an initial decrease, spleen weights of immunosuppressed mice remained fairly constant. *C. parvum* did not significantly affect splenic weights.

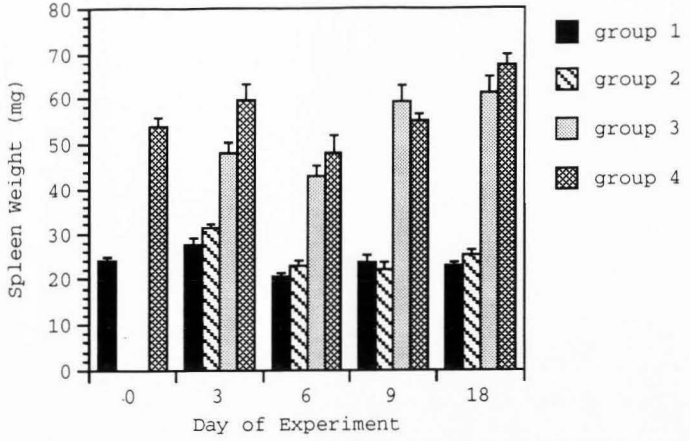

FIG. 9. Effects of *Cryptosporidium parvum* and dexamethasone on spleen weights. Data are presented as the mean of spleen weights. Error bars indicate standard deviations of the mean.

Effects of DEX and *C. parvum* on body weights. Mice that were not immunosuppressed gained weight steadily as the experiment progressed. Mice from groups 1 and 2 lost weight during the first 12 days of immunosuppression. On day 18 (21 days after immunosuppression began), the mice in groups 1 and 2 gained weight (Fig. 10).

Flow cytometric analysis

Comparative effect of *C. parvum* on splenic lymphocytes within treatment groups. With the exception of an isolated incident on day 3, no statistical

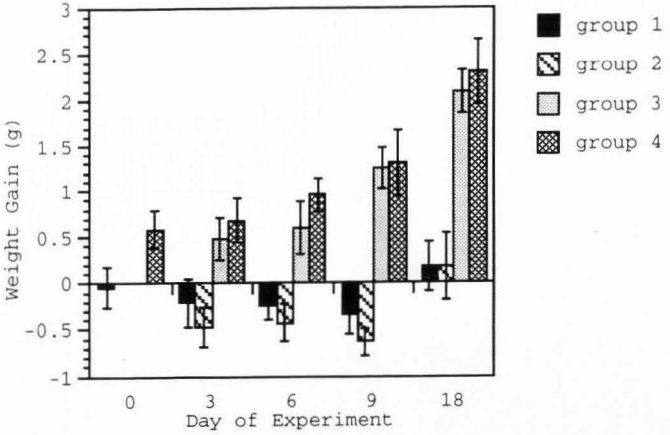

FIG. 10. Effect of *Cryptosporidium parvum* and dexamethasone on body weight. Data are expressed as the mean weight gain. Error bars indicate standard deviations of the mean.

difference was present when the absolute number of splenic lymphocytes of infected and normal mice were compared. On day 3 PI, *C. parvum*-infected mice had significantly lower levels ($P<0.05$) of cells bearing IgA receptors than the normal controls. Furthermore, there were no significant differences in the absolute numbers of splenic cells (expressing CD4+, CD8+, IgG, IgM, IgA and MØ receptors) when mice from groups 1 and 2 were compared (Table 2).

Comparative effect of *C. parvum* exposure on splenic lymphocytes over time. The absolute numbers of MØ and T lymphocytes bearing CD4+ and CD8+ receptors were

TABLE 2. Phenotypic profiles of lymphocytes* in the spleens of *Cryptosporidium parvum*-infected and control mice

Cell Type	Day	group 1	group 2	group 3	group 4
CD8+	0	2.11±0.10	ND[§]	ND	2.76±0.91
	3	2.23±0.08[d]	2.50±0.10[c]	2.30±0.09	2.38±0.02
	6	2.19±0.22[d]	2.05±0.13	2.08±0.25	2.25±0.22
	9	2.10±0.09[d]	1.62±0.08[d]	2.03±0.17	2.09±0.17
	18	2.77±0.05	2.48±0.08	2.35±0.01	2.32±0.26
CD4+	0	1.49±0.24[abcdB]	ND	ND	3.43±0.08[d]
	3	2.65±0.21[B]	3.25±0.90	3.40±0.24	3.62±0.07
	6	2.70±0.01[AB]	2.82±0.23[B]	3.72±0.51	3.96±0.29
	9	2.92±0.32[AB]	2.29±0.11[AB]	4.11±0.01	4.03±0.01
	18	2.41±0.18[AB]	2.56±0.33[AB]	3.87±0.05	4.11±0.44
IgG producing	0	2.94±0.31[dB]	ND	ND	5.07±0.13[d]
	3	3.52±0.25[dAB]	3.80±0.51[cdAB]	4.66±0.26[d]	4.99±0.11
	6	2.97±0.17[dAB]	3.07±0.33[dAB]	4.23±0.38	4.39±0.35
	9	2.94±0.52[dAB]	2.58±0.02[dAB]	4.42±0.26	4.63±0.30
	18	1.35±0.22[AB]	1.67±0.18[AB]	3.67±0.13	4.23±0.08
IgM producing	0	3.16±0.22[cd]	ND	ND	4.65±1.12
	3	3.30±0.21[cdAB]	2.98±0.95[dAB]	5.15±0.07[bcd]	5.89±0.55[bcd]
	6	2.26±0.27[dAB]	2.08±0.33[dAB]	3.76±0.15	4.01±0.21
	9	1.32±0.93[AB]	1.97±0.13[dAB]	3.65±0.35	3.67±0.19
	18	0.00±0.00[AB]	0.00±0.00[AB]	2.53±0.09	3.12±0.32

TABLE 2 (continued)

IgA producing	0	1.92±0.37^{d}	ND	ND	3.39±0.81^{b}
	3	2.40±0.07^{bcdAB}	2.09±0.10^{bcdAB}	3.15±0.18^{bcB}	3.98±0.23^{bcd}
	6	1.28±0.01^{dAB}	1.22±0.17^{AB}	2.23±0.18	2.20±0.29
	9	0.95±0.42^{dAB}	0.84±0.50^{AB}	2.24±0.06	2.37±0.60
	18	0.00±0.00^{AB}	0.51±0.02^{AB}	2.71±0.01	2.84±0.11
Macrophage	0	1.66±0.00^{b}	ND	ND	1.69±0.24^{b}
	3	1.74±0.05^{b}	2.01±0.61^{bAB}	1.09±0.11	1.09±0.05
	6	0.83±0.26^{d}	1.03±0.22^{d}	0.93±0.04	0.84±0.26^{d}
	9	1.29±0.09^{d}	1.23±0.06	1.19±0.13	1.17±0.16
	18	2.15±0.04	1.84±0.04	1.57±0.01	1.50±0.45

*Responses expressed as the square root of the absolute number ± standard deviation.
a significant when compared with day 3 in the same group (P<0.05).
b significant when compared with day 6 in the same group (P<0.05).
c significant when compared with day 9 in the same group (P<0.05).
d significant when compared with day 18 in the same group (P<0.05).
A significant when compared on the same day to group 3 (P<0.05).
B significant when compared on the same day to group 4 (P<0.05).
§ not done.

very similar throughout the experiment. Conversely, in infected mice (group 3), the absolute number of B cells (expressing IgG, IgM, and IgA receptors) varied significantly ($P<0.05$) when kill dates were compared. Lymphocytes with IgG receptors were significantly higher ($P<0.05$) on day 3 than on day 18. The levels of lymphocytes bearing IgM receptors were significantly higher ($P<0.05$) on day 3 than on days 6, 9, and 18. Similarly, IgA levels were significantly higher ($P<0.05$) on day 3 than on days 6 and 9. These trends seen in lymphocytes (expressing IgG, IgM, and IgA receptors) within the spleens of *C. parvum*-infected mice were also seen in the normal mice (Table 2).

Comparative effect of DEX on splenic lymphocytes within treatment groups. Mice treated with DEX (group 1) for 3 consecutive days showed a significant decrease ($P<0.05$) in the absolute number of cells bearing IgG markers when compared to nonimmunosuppressed mice in group 4. After 6 days of daily DEX treatment, splenic IgG lymphocytes were consistently less than the nonimmunosuppressed mice. Both IgM ($P<0.05$) and IgA ($P<0.05$) levels were significantly decreased in the spleens of mice that received DEX for 6 consecutive days. The IgM, IgG, and IgA decreases seen in the spleens of DEX-treated animals were consistently lower than in nonimmunosuppressed mice for the duration of the experiment. When comparing groups within a

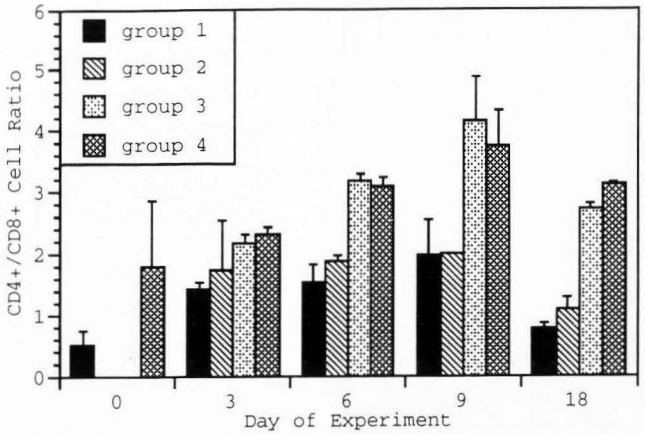

FIG. 11. Effect of dexamethasone and *Cryptosporidium parvum* on the splenic CD4+ to CD8+ lymphocyte ratio. Data are presented as the mean CD4+ to CD8+ cell ratio. Error bars indicate standard deviations of the mean.

given date, DEX did not significantly affect CD8+ T cells within the spleen. However, after 3 days of treatment, the number of CD4+ lymphocytes were significantly less ($P<0.05$) than in normal mice. After the initial decrease, the splenic CD4+ cells of immunosuppressed mice remained fairly constant throughout the experiment (Table 2). Furthermore, the CD4+ to CD8+ lymphocyte ratio was decreased in the spleens of mice treated with DEX when compared to the nonimmunosuppressed mice (Fig. 11). An isolated incidence on day 3 was seen when the splenic MØ of mice in group 2

were compared to the nonimmunosuppressed mice in groups 3 and 4. This significant increase in the absolute number of MØ may be due to an artifact associated with the sample, because no significance was seen between mice in group 1 and groups 3 and 4 (Table 2).

Comparative effect of DEX exposure on splenic lymphocytes over time. When the effects of DEX were observed over time, it was evident that the absolute number of B lymphocytes decreased. After 21 days of immunosuppression (day 18 of the experiment), the mice treated with DEX were nearly depleted of splenic IgG, IgM, and IgA-bearing cells. Although no trends were evident, there were significant differences in the absolute numbers of splenic leucocytes expressing CD4+, CD8+, and MØ receptors (Table 2).

Comparative effect of *C. parvum* on intestinal lymphocytes within treatment groups. When compared to mice in group 4, *C. parvum* had no significant effect on the percentage of lymphocytes in the PP of infected mice that were killed 3, 6, 9, and 18 days after the experiment began. Similarly, no significant effect of *C. parvum* was evident between mice in group 1 and mice in group 2 that were killed on days 3, 6, and 9 of the experiment (Table 3).

Comparative effect of *C. parvum* exposure on intestinal lymphocytes over time. Cryptosporidial

TABLE 3. Phenotypic profiles of lymphocytes* in the Peyer's patches of *Cryptosporidium parvum*-infected and control mice

Cell Type	Day	group 1	group 2	group 3	group 4
CD8+	0	3.39±0.10	ND[§]	ND	2.12±0.17
	3	4.86±0.87	3.52±0.50	3.38±0.31[bc]	3.52±0.50[abc]
	6	4.52±1.10[AB]	3.64±0.68[AB]	1.98±0.37	1.87±0.19
	9	4.41±0.24[AB]	3.93±0.27[AB]	2.24±0.00	2.34±0.15
	18	ND	ND	2.55±0.14	2.44±0.29
CD4+	0	3.39±0.10	ND	ND	3.94±0.09
	3	4.58±0.15	4.04±0.61	5.24±1.42	5.36±1.58
	6	5.43±1.04	4.63±0.38	4.80±0.96	4.51±0.55
	9	5.51±0.45	5.26±0.81	5.19±0.41	4.99±0.43
	18	ND	ND	4.69±0.15	4.85±0.07
IgG producing	0	7.28±0.19[a]	ND	ND	7.55±0.09
	3	7.40±1.19[a]	6.13±1.33	7.94±0.27[b]	7.33±0.77
	6	5.43±0.07	5.98±0.71	6.62±0.64	6.70±0.53
	9	6.18±0.86	5.82±0.61	6.36±0.06	6.16±0.12
	18	ND	ND	6.40±0.11	6.20±0.29
IgM producing	0	7.17±0.25	ND	ND	7.18±0.15
	3	4.41±1.76	5.24±0.34	5.61±1.77	3.87±2.65
	6	1.80±2.55	5.71±0.87	6.97±0.91	6.28±0.21
	9	3.67±1.45	3.76±1.32	5.37±0.66	5.25±0.94
	18	ND	ND	5.25±0.94	6.07±0.47

TABLE 3 (continued)

IgA producing	0	6.56±0.00[ab]	ND	ND	6.32±0.11[a]
	3	5.19±2.05[ab]	3.04±4.30	6.50±1.85	4.58±1.00
	6	1.83±0.58	1.87±0.19	2.00±0.00	2.55±0.14
	9	2.52±1.12	0.71±1.00	4.18±0.25	4.11±0.52
	18	ND	ND	4.39±0.73	4.90±0.00
Macrophage	0	1.00±0.00	ND	ND	1.00±0.00[ab]
	3	1.00±0.00	2.03±0.87	1.21±0.29	1.00±0.00[ab]
	6	1.21±0.29[B]	1.00±0.00	0.50±0.71	0.00±0.00
	9	1.21±0.29[B]	1.00±0.00	0.50±0.71	0.00±0.00
	18	ND	ND	0.00±0.00	0.00±0.00

*Responses are expressed as the square root of the mean cell percentage ± standard deviation.
a significant when compared with day 6 in the same group ($P<0.05$).
b significant when compared with day 9 in the same group ($P<0.05$).
c significant when compared with day 18 in the same group ($P<0.05$).
A significant when compared on the same day to group 3 ($P<0.05$).
B significant when compared on the same day to group 4 ($P<0.05$).
§ not done.

infections in immunosuppressed mice (group 2) did not affect the lymphocytes within the PP when days 3, 6, and 9 were compared. Mice within group 3 showed a significant difference ($P<0.05$) in IgG and CD8+ lymphocytes. However, these statistical differences between days 3, 6, and 9 were also seen in the normal mice. This observation suggests experimental variation rather than an immunological response to the parasite. Moreover, CD4+, MØ, IgM, and IgA-receptor bearing leucocytes within the PP showed no detectable response to cryptosporidial infections when days 3, 6, 9, and 18 were compared (Table 3).

Comparative effect of DEX on intestinal lymphocytes within treatment groups. When compared to nonimmunosuppressed mice, mice treated with DEX for 9 or 12 days had significantly higher levels ($P<0.05$) of and nearly a two-fold increase of CD8+ lymphocytes in the PP. Peyer's patch lymphocytes expressing IgG, IgM, IgA, and CD4+ receptors showed no detectable response to DEX 3, 6, 9, and 12 days of treatment (Table 3). Moreover, DEX treatment was associated with a lower CD4+ to CD8+ cell ratio within the PP when compared to the nonimmunosuppressed mice (Fig. 12).

Comparative effect of DEX exposure on intestinal lymphocytes over time. Immunoglobulin G receptor-bearing B cells within the PP of mice in group 1 decreased significantly ($P<0.05$) during the first 6 days following

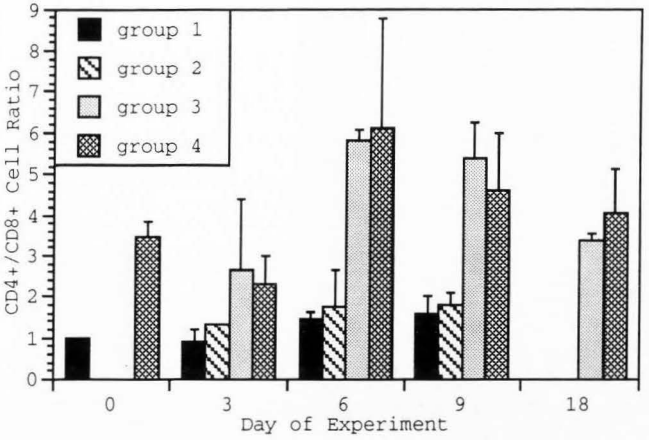

FIG. 12. Effect of dexamethasone and *Cryptosporidium parvum* on the CD4+ to CD8+ lymphocyte ratio in murine Peyer's patches. Data are shown as the mean CD4+ to CD8+ cell ratio. Error bars indicate standard deviations of the mean.

immunosuppression. After this initial decrease, cells expressing IgG receptors remained contant for the duration of the experiment. Similarly, cells bearing IgA receptors decreased significantly in these mice during the 12 days of DEX treatment ($P<0.05$). When comparing dates within a group, DEX failed to affect cells (bearing CD8+, CD4+, IgM, and MØ receptors) within the PP (Table 3).

Correlation between lymphocytes and oocyst shedding intensities. No strong linear correlation could be drawn between the number of leucocytes (attained

from flow cytometric analysis) and the intensity or duration of fecal oocyst shedding. A weak positive correlation was seen between oocyst shedding and the percentage of IgM bearing lymphocytes in the PP of immunosuppressed and nonimmunosuppressed mice (0.60 and 0.58, respectively). Similarly, a weak correlation could be drawn between the oocyst shedding and the MØ within the spleen (Table 4).

TABLE 4. Correlation coefficient r drawn between lymphocytes and oocyst shedding intensities

Tissue	Cell Type	Group 2	Group 3
Spleen	CD8+	-0.12	-0.07
	CD4+	-0.01	-0.32
	MØ	-0.51	-0.45
	IgG producing	-0.05	0.36
	IgM producing	-0.19	0.40
	IgA producing	-0.25	-0.04
PP	CD8+	-0.12	0.06
	CD4+	-0.01	0.02
	MØ	-0.40	0.45
	IgG producing	0.02	0.43
	IgM producing	0.60	0.58
	IgA producing	-0.24	-0.08

PP = Peyer's patches;
MØ = macrophages

DISCUSSION

Immunohistochemistry

To accomplish the goals of this study, considerable time and effort was spent developing a workable immunohistochemical technique. Initially, the ABC method was attempted on paraffin-embedded sections because of its superior staining sensitivity, which results from amplification of the antigen-antibody reaction (72). To enhance staining intensity and/or eliminate nonspecific binding, several alterations of the protocol were tried. Inasmuch as none of the modifications gave a satisfactory result, immunofluorescence techniques were attempted.

Direct and indirect fluorescent antibody techniques were applied to paraffin-embedded tissue sections. Due to great variation in sensitivities of antigens to different physical and chemical conditions, various fixation methods were tried prior to paraffinization. Regardless of the fixation method or modification to the protocol, MØ and T lymphocytes (CD4+, CD8+, and T_{Total}) showed no immunoreactivity. Apparently the fixatives (formalin, B-5, Histochoice, and Bouin's) failed to preserve the antigenic determinants of the MØ and T lymphocytes.

The capacity of B lymphocytes to react with FITC-labeled antibody was preserved by Bouin's solution. This allowed for the determination of the effects of DEX on the

humoral response in the spleen and intestinal lamina propria. The results and discussion generated from immunohistochemical techniques are of academic interest because the objective of this research was accomplished by flow cytometry that elucidated both cell-mediated and humoral immune responses. Nonetheless, preliminary data and observations from immunohistochemical techniques are presented. Immunohistochemical evaluation of paraffin-embedded sections showed that DEX reduced the number of lymphocytes expressing IgA receptors within the spleen and intestinal lamina propria. This suggests that these lymphocytes may play a role in immunity to cryptosporidiosis. Presumably this could occur because secretory antibodies present in the intestines can help eliminate *C. parvum* by mediating biological processes (111). Moreover, IgA can be transported across epithelial cells of the intestine from the basal to the apical surface of these cells by transcytosis (2). Accordingly, it is plausible that dimeric IgA might reach the basal membrane of attached cryptosporidia and hinder parasite feeding and development. Results produced from this preliminary study varied somewhat from those obtained by flow cytometric analyses. Immunohistochemical evaluations comparing lymphocytes in *C. parvum*-infected mice to DEX-treated mice with cryptosporidiosis showed that within the spleen and intestinal lamina propria, IgA was the only isotype affected by DEX. Because

these results were obtained from a preliminary study with a few animals, it is difficult to infer why lymphocytes expressing IgG and IgM markers were not significantly reduced in DEX-treated mice. To offer clarity to these findings, I am performing immunohistochemical techniques on terminal ilea of normal and immunosuppressed mice with cryptosporidiosis. Analysis by flow cytometry showed that B lymphocytes (expressing IgG, IgM, and IgA receptors) within the spleen, but not the PP, were affected by DEX. Any one of the following may contribute to the difference in the results of immunohistochemical evaluations and flow cytometric analyses: 1) The age of the mice used in the preliminary study (immunohistochemistry) were 4 weeks older than those mice used for flow cytometry; 2) The animal numbers in the study utilizing immunohistochemistry had three mice per treatment group, whereas the results generated from flow cytometry had eight mice per group; and 3) The duration of immunosuppression of mice evaluated by the immunohistochemical technique were treated with DEX for 20 days, whereas those used for flow cytometry were treated with DEX for 12 days.

Infection dynamics

Oocyst shedding patterns seen in this experiment were not unlike those previously observed in normal and DEX-treated adult C57BL/6N mice that were infected with *C.*

parvum (114). Infected mice that were not immunosuppressed quickly cleared the infection, whereas DEX-treated mice shed oocysts in fluctuant amounts until the experiment was terminated. The fluctuation of shedding intensities observed in this experiment and by others is possibly due to the inherent variation in sample preparation or to the course of autoinfection by thin-walled oocysts and merozoites. The trends of parasite colonization in the ileum and oocyst shedding were very similar with the exception of day 3. On day 3, oocyst shedding was markedly lower than ileal colonization by *C. parvum*. These observations are reasonable because the majority of the parasites may have had insufficient time to complete gametogony and formation of oocysts. Taghi-Kilani et al. (1990) report that no correlation could be drawn between the intensity of *Cryptosporidium*-specific responses and the severity or duration of cryptosporidiosis in neonatal BALB/c mice, although these mice exhibited a good IgM and IgG serum antibody response (128). Similar results were produced in this study in that no strong linear correlation was evident between the presence of lymphocytes in the tissues (spleen and PP) and the intensity of oocyst shedding. I believe that these observations reflect the insignificance between the cell numbers of infected and uninfected mice rather than suggesting that *in vivo* immune responses play a minor role in thwarting the development of *C. parvum*.

Even though cryptosporidial infections did not significantly affect splenic weights, mice in group 3 had an increase in splenic weights as the experiment progressed. This suggests that immunocompetent mice are responding to *C. parvum* while infected immunosuppressed mice do not. Dexamethasone rapidly reduced the weight and size of the spleen and PP. After 3 consecutive days of treatment with DEX (day 0 of the experiment), the mean spleen weight was approximately one half that of the nonimmunosuppressed mice. This reduction is profound not only because of how rapidly it occurred, but also because the spleen weights remained constant after the initial decrease despite continuous administration of DEX. The differences in spleen weight were taken into consideration by using the absolute number (cell percentage multiplied by the spleen weight) in the statistical analysis. Additionally, DEX reduced the size of the PP. One to 4 PP could be identified and harvested from the small intestine of an immunosuppressed mouse with the aid of a dissecting microscope. However, the unaided eye could see 6 to 8 PP on the small intestine of a nonimmunosuppressed mouse. Because DEX dramatically reduced the size of the lymphoid tissues, it was necessary to pool the tissues of four mice. Twenty-one days after immunosuppression began, it was impossible to collect the PP for flow cytometric analysis because they

were inconspicuous or absent (even with the aid of a dissecting microscope).

In addition to inducing a lymphopenia and decreasing the splenic weight, glucocorticoids affect carbohydrate metabolism by promoting gluconeogenesis and liver glycogen deposit and elevating blood glucose levels (129). Additionally, DEX inhibits insulin release by the ß-cells of the pancreatic islets (76), thus inhibiting the entry of glucose into cells, glycolysis, and production of ATP. These physiological events may explain the weight loss observed in mice treated with DEX (groups 1 and 2), because when the production and hydrolysis of ATP are affected, metabolic reactions, including synthesis of nucleic acids and proteins, are hampered.

Flow cytometric analysis

All strains of laboratory mice (inbred, outbred, immunodeficient, and germ-free) tested to date are difficult to infect with *C. parvum* once they are more than 3 weeks of age (34, 63, 123). These observations suggest that genetically based as well as age-related factors (immune status, gut physiology, or microflora) may be responsible for determining susceptibility or resistance to *C. parvum* in mice (34). Therefore, an immunocompromised animal model for chronic cryptosporidiosis was necessary to accomplish the goals of this study. The small animal model

that was used in the present study was developed by Rasmussen and Healey (114). These authors show that C57BL/6N mice immunosuppressed by intraperitoneal injections of DEX at a dosage of 125 µg per day are susceptible to *C. parvum* and developed chronic infections which persists at least 10 weeks. This immunocompromised laboratory animal model did not substantiate the immune response to *C. parvum*, but rather discerned the immunological defects which allow for the development of chronic infections. Therefore, the absence of statistical significance (in the number of cells expressing CD4+, CD8+, IgG, IgM, IgA, and MØ receptors) between mice in group 1 and group 2 was anticipated.

Nonspecific immunity and the role of MØ were discussed earlier in this thesis. In the present study there was no statistical difference in the numbers of MØ when groups 3 and 4 were compared. Moreover, flow cytometry yielded inconclusive data on the effects of DEX on MØ. These ambiguities are likely due to the paucity of MØ in the spleen and PP. Future research investigating the effects of DEX on MØ should consider *in vitro* techniques with peritoneal MØ to overcome this limitation.

The present study demonstrated that mice treated with DEX had significantly fewer CD4+, IgG, IgM, and IgA receptor-bearing lymphocytes within their spleens compared with nonimmunosuppressed mice. This observation implicates the

lymphocyte subpopulations that are responsible for arresting the development of *C. parvum*. Initially, it is tempting to dismiss the importance of Ig-bearing cells because the parasite is intracellular and others have shown that antibodies alone cannot eliminate *C. parvum* from the infected mucosa (62, 98, 128). Before the role of B lymphocytes is dismissed, the complex interaction between B and T lymphocytes needs to be considered. A B cell can activate a CD4+ cell by taking up antigen, converting it to a form that is recognizable to the T lymphocyte, and then presenting it to the T cell. After antigenic activation, the T lymphocyte is stimulated to release lymphokines, which in turn promote stimulation of other lymphocytes and MØ. Moreover, T lymphocytes (CD4+) are required for most B lymphocytes to respond to antigen. Without any further description of the immunological response, one can see that if a lymphocyte population is affected by DEX, it has the potential of affecting all lymphocytes associated with the network. In other words, the humoral immune response may not directly affect *C. parvum*, but the paucity of B cells may affect the ability of the cell-mediated immune response to control the infection. These findings are consistent with the hypothesis that a T lymphocyte is involved in recovery from cryptosporidial infection and that recovery is associated with both cellular and humoral immune responses to cryptosporidial antigens (34). The importance

of lymphocytes (bearing CD4+ receptors) in immunity to *C. parvum* is demonstrated in this study by the decrease of splenic helper T lymphocytes and by the decreased CD4+ to CD8+ cell ratios. Unfortunately, the fundamental physical or chemical processes (mediated by T lymphocytes) that are responsible for parasite killing remain unclear (92). Nonetheless, it is reasonable to speculate that the elimination of *C. parvum* by lymphocytes bearing CD4+ receptors may be due to the secretion of lymphokines and thus activation of MØ, B and T lymphocytes.

The increase of CD8+ lymphocytes in the PP of DEX-treated mice suggests that these cytotoxic cells play a role in immunity to cryptosporidiosis. Harp et al. (1988) (59) show that in mild cryptosporidial infections, *C. parvum* colonizes on or near the dome epithelium of the PP. This parasitic colonization may be facilitated by the CD8+ lymphocytes suppressing the intestinal immunological response. Conceptually, this could occur if the CD8+ cells kill other T lymphocytes (or B cells) bearing specific idiotypic determinants related to antigen recognition. Another way in which CD8+ lymphocytes could suppress immune responses to *C. parvum* would be by killing the active antigen presenting cells, thus removing the stimulus before an immune response could be generated (50). Alternatively, the presence of parasites juxapposed to the PP may be explained by the fact that T lymphocytes expressing CD8+

receptors secrete substances that facilitates parasite colonization.

Other studies have addressed the role of CD8+ cells in immunity to cryptosporidiosis. Adult BALB/c mice depleted of CD8+ lymphocytes through *in vivo* treatment with Mab did not develop persistent infections (138). Aguirre et al. (1994) show that major histocompatibility complex (MHC) class I-deficient mice (lacking functional CD8+ cells) infected at 5 to 6 weeks of age were no more susceptible to *C. parvum* infection than age-matched controls (1). The results of these studies do not support the hypothesis that CD8+ cells play a role in protective immunity to cryptosporidiosis. However, the present study and Rasmussen et al. (115) show that CD8+ lymphocytes are increased in immunosuppressed mice that are infected with *C. parvum*. Boher et al. (1994) show that after infection of neonatal mice with *C. parvum*, ileal PP show a predominant CD8+ T cell response (18). Additionally, McDonald et al. (1994) show that CD8+ cells appear to be involved in resistance to primary and secondary cryptosporidial infections (92). Future studies performing functional assays on CD8+ cells harvested from animals infected with *C. parvum* may offer clarity to these equivocal results.

One of the most striking results of the present study was the lack of any statistical significance in the number of lymphocytes (expressing CD4+, CD8+, IgG, IgM, and IgA

receptors) between nonimmunosuppressed mice infected with *C. parvum* and the mice in group 4. The results appear to contradict those studies that show B and T lymphocytes to be responsible for eliminating *C. parvum* from the intestinal mucosa (1, 14, 15, 18, 22, 23, 34, 37, 44, 45, 52, 56, 57, 62, 70, 78-80, 84, 85, 91, 94, 95, 99, 103, 106, 128, 134, 137, 139, 140, 144, 146). The apparent discrepency between the results of this study and previously published work may be explained by antibody specificities. Previous studies detecting serum, duodenal, and fecal antibodies used *C. parvum* antigens to probe for parasite-specific antibodies, whereas the antibodies against mouse immunocytes used in this study were not *C. parvum*-specific. Therefore, the evoked *C. parvum*-specific immune response is not significant above the indigenous B and T lymphocytes. This explanation is supported by other studies that assayed for total lymphocyte levels in humans and mice with cryptosporidiosis. Splenic CD4+ (115), CD8+, T_{Total}, and B_{Total} lymphocytes (37, 115) from infected mice were comparable to controls. Likewise, total IgG and IgM antibody levels in serum, stool, and duodenal fluid from infected children are not statistically different from control subjects (80). Alternatively, the discrepancy between the results of the present study and previously published work may be explained by animal numbers. Perhaps significance would be present between infected and normal mice if this experiment

was repeated with more animals per group. Increasing animal numbers would result in more repetitions, increased degrees of freedom, and decreased variance. Consequently, a greater possibility of statistical validity may become apparent. Alternatively, severity and length of infection may be a factor. Many of the researchers that demonstrated coproantibodies and seroconversion used specimens from human or large domestic animals that had severe infections and cryptosporidial diarrhea (22, 23, 70, 80, 99, 137, 139, 140, 146). Because tissues were harvested, this study was limited to small laboratory animals. The immunocompetent adult female C57BL/6N mice used in this study developed a mild infection without diarrhea for 6 days, after which the infection was cleared. Apparently, the length and intensity of the cryptosporidial infections were not sufficient to elicit a detectable immunological response within the spleen or PP.

Conclusions

This study was undertaken to quantify subpopulations of lymphoid cells present in the spleen and small intestine (lamina propria and PP) of normal and immunosuppressed adult C57BL/6N mice that were either noninfected or infected with *C. parvum*. The following conclusions may be drawn from this research: 1) The evoked *C. parvum*-specific immune response is not significant above the indigenous B and T

lymphocytes present in the spleen and PP; 2) DEX, a synthetic corticosteroid, significantly reduced splenic lymphocytes expressing CD4+, IgG, IgM, and IgA receptors. This differential effect explains why only immunosuppressed mice were capable of maintaining infections with *C. parvum*; 3) CD8+ cells within the PP may play a role in immunity to *C. parvum* by suppression or cytotoxic killing of other lymphocytes and antigen presenting cells bearing specific idiotypic determinants; and 4) Immunohistochemical evaluation of paraffin-embedded tissues suggests that IgA (within the spleen and intestinal lamina propria) may play a role in the protective immune response to cryptospordial infection.

Lastly, I believe that these preliminary findings will serve as a first step toward developing immunomodulation therapies for use in patients with debilitating cryptosporidial infections. Moreover, valuable data should emerge from future investigations that address specific immune responses within lymphoid cells associated with the intestine and systemic compartment.

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APPENDIX

Permission Letter

November 27, 1993
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