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Characterization of a Dexamethasone-Immunosuppressed C57BL/6N Mouse Model for Chronic Cryptosporidiosis

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CHARACTERIZATION OF A DEXAMETHASONE-IMMUNOSUPPRESSED C57BL/6N MOUSE MODEL FOR CHRONIC CRYPTOSPORIDIOSIS

by

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ABSTRACT

Characterization of a Dexamethasone-Immunosuppressed C57BL/6N Mouse Model for Chronic Cryptosporidiosis

by

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Cryptosporidium parvum is a coccidian protozoan that colonizes epithelial cells lining respiratory and digestive tracts of animals and humans. Cryptosporidiosis is a well-recognized zoonotic disease infecting primarily neonates and immunocompromised hosts, including human immunodeficiency virus-infected patients. Clinical disease is manifested as a chronic diarrheal illness that is self-limiting in immunocompetent hosts and prolonged and often life-threatening in hosts with compromised immune systems. The lack of a suitable small animal model for screening anti-cryptosporidial drugs and for examining the pathogenicity and immunobiology of chronic cryptosporidiosis was the impetus for this research effort.

The objectives of the present study were three-fold: to characterize chronic *Cryptosporidium parvum* infections in dexamethasone-immunosuppressed mice; evaluate the effects of *Cryptosporidium parvum* and dexamethasone on B and T lymphocyte proliferation; and determine the effects of the immunomodulator dehydroepiandrosterone on oocyst shedding intensities of mice infected with *Cryptosporidium parvum*.

Adult C57BL/6N mice were immunosuppressed with the synthetic glucocorticoid dexamethasone, then infected with *Cryptosporidium parvum* (10^6 oocysts/mouse) and

investigated for their ability to sustain a four-month chronic infection. Dexamethasone was administered intraperitoneally (125 μg/mouse/day) or orally (8 μg/ml) in the drinking water *ad libitum*. Infection chronicity was characterized by evaluating mouse mortality, oocyst excretion in the feces, tissue distribution of the parasite, and parasite-induced pathology.

A progressive infection with *Cryptosporidium parvum* occurred in mice immunosuppressed intraperitoneally and orally as long as dexamethsone was administered. Mice receiving dexamethasone given intraperitoneally had a shorter prepatent period and a more consistent, although cyclic, oocyst shedding pattern when compared with mice given dexamethasone orally. Mice given dexamethasone orally exhibited a delayed prepatent period, with a steady increase in oocyst shedding. All mice receiving dexamethasone orally died within three months following oocyst inoculation. Clinical signs included dehydration, icterus, and reduction in spleen and body weights. Clinical signs were more abrupt in mice receiving oral dexamethasone.

Parasite colonization involved the entire intestinal tract, including the pyloric ring and Peyer's patches, but was the heaviest in the terminal ileum. Parasites were present in the lungs, gallbladder, and pancreatic ducts. Pathologic abnormalities were isolated to the terminal small intestine and included blunting and fusion of intestinal villi and crypt hyperplasia.

Cryptosporidium parvum and dexamethasone administered *in vivo* reduced B and T lymphocyte responses to the mitogens lipopolysaccharide and concanavalin A.

Dehydroepiandrosterone and dehydroepiandrosterone-sulfate resulted in no significant reductions in cryptosporidial activity as determined by oocyst shedding in the feces. (100 pages)

INTRODUCTION

Background. *Cryptosporidium parvum* is not a "new" organism. Ernest E. Tyzzer first described *C. parvum* in 1912 (116) and at the time, the intestinal protozoan was regarded as a benign commensal. In the early 1970's, *C. parvum* was recognized as a significant pathogen for calves. Since that time, the parasite has been isolated from many species of wild and domestic animals (39). Consequently, cryptosporidiosis became more fully appreciated as a cause of great economic loss to agriculture almost half a century following its original description (117). During the last 18 years, *C. parvum* infections have been recognized as severe, life-threatening illnesses of people (44). Cryptosporidiosis has been reported worldwide in more than 20 countries on 6 continents (44). Today, *C. parvum* is thought to be one of the most significant enteropathogens in under-developed countries. The highest prevalence of the disease appears to be in malnourished children, patients receiving chemotherapy, and individuals with AIDS (39). Detection of *C. parvum* in patients with AIDS heightened physician awareness of the pathogenic potential of the parasite in immunocompetent and immunocompromised hosts. Between 1976 and 1982, fewer than 10 reports of human cryptosporidiosis had been documented (44). Since that time, an explosion of publications has attracted the attentions of scientists worldwide. Thus, tremendous interest has been generated regarding cryptosporidiosis and the thrust of experimental research has been in the development of *in vitro* and *in vivo* models for screening anti-cryptosporidial agents (26, 39, 44, 117).

More success has been experienced with the development of *in vivo* models as opposed to *in vitro* systems. A variety of laboratory animals have been examined for their use in studying *C. parvum* infection and valuable information has been gleaned from these experiments (5, 15, 19, 28, 41, 52, 65, 74, 75, 79, 94, 97, 98, 104, 122, 123). In order to establish a chronic *C. parvum* infection, the animal model must be

immunocompromised, either genetically or by exogenous immunosuppression. It is also important that the model possess an immune system comparable to hosts that readily contract and succumb to *C. parvum* infection. These requirements for a model improve both its reliability and utility in determining the immunobiology and pathophysiology involved in developing resistance to cryptosporidiosis.

Neonates were initially explored as potential animal models for cryptosporidiosis because of their susceptibility to *C. parvum* without the use of exogenous immunosuppression. To date, the most commonly used small animal model for cryptosporidiosis is the neonatal mouse (28, 119). The use of neonates has been limited because they do not provide sufficient time for screening drugs before they clear the infection.

A number of genetically immunodeficient animals have also been investigated as possible models for cryptosporidial infection (74, 75, 122). This type of animal is susceptible to *C. parvum* and can maintain a chronic infection. Information regarding parasite migration in host tissues during a chronic infection has been gleaned using immunodeficient models (75). Because of their genetic immunodeficiency, it is difficult to accurately determine the immunologic response associated with the development of resistance to an infection. The housing demands and high cost of a genetically immunodeficient animal decrease its practical use when compared with other models. Thus, the use of immunodeficient animals has been limited.

Finally, animals requiring exogenous immunosuppression have also been examined for studying cryptosporidiosis (15, 94, 97, 98, 123). This type of animal model is useful for studying acute and chronic phases of the infection and can be used to assess the immunologic response to infection. However, a readily available small animal model for examining the chronic nature of cryptosporidiosis as seen in AIDS and other immunocompromised patients is still lacking. Evidence of genetic preference of *C.*

parvum for its host and the presence of species susceptibility to the parasite are important factors to consider when selecting a reliable laboratory animal model.

Statement of the Problem. To date, there is no known effective chemotherapeutic agent for animals or humans infected with *C. parvum*. In fact, cryptosporidiosis is the only known opportunistic infection in AIDS patients for which there is no effective treatment. The two main factors limiting the development of effective anti-cryptosporidial drugs is the lack of a reliable *in vitro* cell cultivation system and the lack of an easily adaptable laboratory animal model for screening drug efficacy. It is the latter which is the impetus for the present research.

Purpose of this Research. The purpose of the present study was to develop and characterize a reliable mouse model for cryptosporidiosis and investigate the immune parameters involved in a chronic infection. Adult C57BL/6N female mice were used in these studies because of their susceptibility to *C. parvum* following exogenous immunosuppression (92). Dexamethasone (DEX), a synthetic glucocorticoid previously described as an effective drug capable of inducing susceptibility to *C. parvum* in other rodent models (92, 94, 98), was used to immunosuppress the mice. The DEX is known to induce a state of leukocytopenia as a result of immediate and profound lymphocytolysis in mice (20).

Objectives. Three objectives relating to the development and characterization of a laboratory mouse model for chronic cryptosporidiosis were identified:

1. Characterize *Cryptosporidium parvum* infections in dexamethasone-immunosuppressed mice by evaluating infection duration, tissue distribution, tissue pathology, and parasite load.

2. Determine the effects that *Cryptosporidium parvum* and dexamethasone have on the immune status of mice (including B and T lymphocytes and spleen weights) relative to oocyst shedding intensities.

3. Assess the therapeutic utility of the immunomodulator dehydroepiandrosterone as a means to enhance resistance to *Cryptosporidium parvum* in mice immunosuppressed with dexamethasone.

LITERATURE REVIEW

History. In 1912, the American parasitologist, Ernest Edward Tyzzer, isolated the coccidian parasite, *Cryptosporidium parvum,* from the intestinal epithelia of the common mouse (116). During that same year, Tyzzer named *C. parvum* as a new species and provided remarkable morphologic detail that has been confirmed by the use of electron microscopy (39). Taxonomists have found many detailed biological characteristics that classify *Cryptosporidium* as one of several genera of coccidian protozoans belonging to the phylum Apicomplexa (26). Although many species of *Cryptosporidium* have been named on the basis of the host in which they were originally described (39), recent reviews and cross transmission studies cast doubt on the validity of most of these species. Currently, *C. parvum*, *C. muris* (another mammalian species) and two avian species, *C. baileyi* and *C. meleagridis*, are accepted as valid and distinct species. Of these species, *C. parvum* is unique in that it appears to be infectious to all mammals, including humans.

Nearly half a century passed from the original description of *C. parvum* before cryptosporidiosis was recognized by more than taxonomists. Large economic losses in the early 1970's associated with severe bovine diarrhea captured the interests of veterinarians (39). In 1976, two independent findings linked cryptosporidiosis with morbidity in humans (77, 85). It was not until the early 1980's that the emergence of the acquired immunodeficiency syndrome (AIDS) epidemic heightened awareness that *C. parvum* was becoming an important human pathogen (26, 44, 58).

The parasite is found as an opportunistic invader of various mucosal surfaces, especially those of the digestive and respiratory tracts in hosts with a compromised immune system. It is also recognized as a probable extraintestinal pathogen (39). The acute recognition of *C. parvum* as a potentially fatal human enteric pathogen stimulated activity in the research community. Investigators began refining diagnostic techniques,

defining the epidemiology, and developing anticryptosporidial drugs via *in vitro* and *in vivo* systems in hopes of interrupting the parasite's life cycle (39, 44).

Life Cycle. The life cycle of *C. parvum* is representative of other coccidians in that it possesses both asexual and sexual stages of reproduction. Figure 1 identifies six major developmental phases which have been recognized (44, 58): excystation (release of infective sporozoites); merogony (asexual multiplication) resulting in the formation of type I and type II meronts; gametogony (gamete formation); fertilization yielding immature oocysts or zygotes; oocyst wall formation giving rise to thick-walled (80%) and thin-walled (20%) oocysts; and sporogony (sporozoite formation).

The prepatent period, from acquisition of the parasite to the demonstration of oocysts in the feces, ranges from 5 to 28 days with a mean of 7 days (39). The timing of appearance of specific developmental stages is not certain. Current et al. (31) has demonstrated that sporozoite-infected cell cultures developed into mature type I meronts, type II meronts, gamonts and oocysts at 12, 24, 48, and 72 hours, respectively. The patent period, which is defined as the duration of oocyst shedding, varies with the host. The persistance of this parasite, especially in immunocompromised patients, is due primarily to its ability to undergo unlimited cycles of asexual schizogony and produce thin-walled oocysts important for initiating an auto-infection.

When sporulated oocysts from contaminated food or water are ingested by a suitable host, they pass through the stomach and excyst in the small intestine, releasing infective sporozoites. The process of excystation is initiated by the presence of pancreatic enzymes, bile salts, and other reducing agents. The sporozoites invade and replicate within the microvillous border of epithelial cells that line the digestive and respiratory organs of the host (29, 32). As the sporozoites and merozoites penetrate epithelial cells,

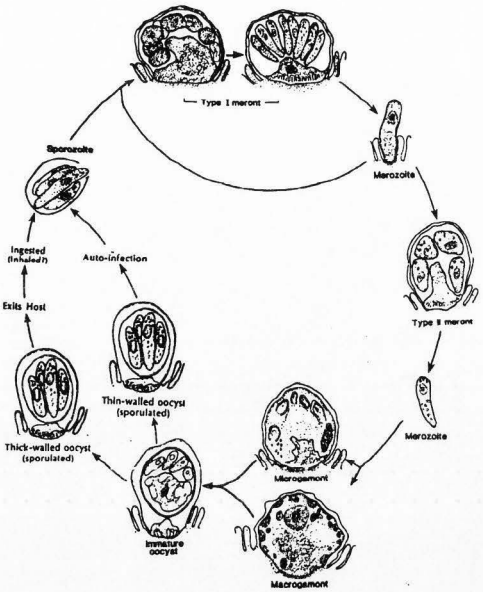

Figure 1. Diagrammatic representation of the proposed life cycle of *Cryptosporidium parvum* as it occurs in experimentally infected mice. Adapted from Current (29) and Dubey (39).

they become enveloped by host cells and eventually reside within individual parasitophorous vacuoles. However, the developmental stages do not reside within the cytoplasmic portion of the host cell as do most other coccidians. Thus, developmental stages of *C. parvum* are confined to an intracellular-extracytoplasmic location: intracellular because each stage is surrounded by a parasitophorous vacuole of host cell origin; extracytoplasmic because the parasite-containing vacuole remains at the microvillous surface of the host epithelial cell. It is at this location that *C. parvum* gives rise to the pathological lesions and clinical signs associated with this disease.

Pathogenesis and Clinical Signs. The invasive parasitic stages rapidly destroy the integrity of infected epithelial cells (118). The respiratory tree, gastrointestinal tract, and associated organs (pancreas, liver, and gall bladder) are particularily susceptible to infection by *C. parvum* in both human and non-human hosts (50, 75, 122) . Epithelial cells lining the terminal small intestine (jejunum and ileum) are usually most heavily colonized (77). The location of the parasite at the epithelial surface results in a loss of microvilli and a decrease in the levels of digestive enzymes. This, in turn, interferes with absorptive and other functions of the cells, leading to malabsorption and malnutrition. Thus, it is not surprising that one of the most prominent clinical features associated with this disease in humans is a watery, cholera-like diarrhea. In rodents, diarrhea is rare because of the efficient fluid and electrolyte absorption occuring in the cecum. Other clinical presentations include hepatitis, cholecystitis, pancreatitis, reactive arthritis, conjunctivitis, and a variety of respiratory problems (39). These are due undoubtedly to the parasite's proposed ability to migrate to distal sites during a chronic infection.

At the microscopic level, a number of pathologic findings are characteristic for *C. parvum* infection. Infected intestinal cells appear swollen and vacuolated, with more of a cubodial or squamous cellular morphology as opposed to the normal columnar appearance (39). Intestinal villi are blunt, shortened, and wider than normal and may be fused with adjacent villi if the infection is heavy. Crypts appear larger than normal due to parasite-induced hyperplasia. Inflammatory changes of the subepithelial lamina propria include cellular infiltrations of neutrophils, plasma cells, macrophages, and lymphocytes (29). Mesenteric lymph nodes may be swollen, and Peyer's patches appear reactive (39).

Cryptosporidiosis in Domestic Animals. Due to its broad host specificity, *C. parvum* has proven to be of significant agricultural importance as a cause of neonatal diarrhea in numerous domestic animals, in particular, calves, kids, and lambs (119).

Numerous studies suggest that cryptosporidia are common enteropathogens of neonatal ruminants (39, 44, 117). However, the role of *C. parvum* as a primary pathogen in these hosts is not clear. Field studies have demonstrated that the parasite can cause clinical diarrhea in the absence of other common agents normally associated with neonatal diarrhea (39). In the United States, the average annual livestock losses attributed to cryptosporidial infections are believed to exceed $6.2 million. Consequently, many outbreaks of intestinal *C. parvum* infections have engaged the attentions of veterinarians, particularly where farmed ruminants are involved. Despite more than a decade of experience involving *C. parvum* infections in livestock, virtually all that has been achieved is a capability for diagnosing the presence of the oocyst stage in feces, disinfecting premises, and interrupting the spiral of infection encountered in contaminated environments by modifying existing management procedures (3). Effective prophylactic and therapeutic measures for controlling *C. parvum* infections in animals are still lacking.

Cryptosporidiosis in Humans. The earliest documented cases of cryptosporidiosis in humans were in 1976. A 3-year-old child and a 39-year-old college administrator, both from farming communities, were diagnosed with *C. parvum* infections associated with abdominal pain, cramping, and severe watery diarrhea (77, 85). Both patients recovered within two weeks following symptomatic treatment. During the next several years, especially when the AIDS epidemic gave new importance to cryptosporidiosis, several worldwide reports of human cryptosporidiosis appeared in the literature. These findings gave researchers crucial information concerning transmission, immunobiology, and pathophysiology of this parasite.

Humans may acquire the infective *C. parvum* oocysts from other persons (32), contaminated drinking water (33, 54), and infected animals (64, 99). Young malnourished children are most commonly infected (44, 119), but previously unexposed adults with intact immune systems are also susceptible to *C. parvum* (54, 64). Studies

have shown that there is a link between the severity of illness and degree of immune competence in the host (29, 32).

Cryptosporidium parvum infection in immunocompetent individuals is characterized as a benign, self-limiting, flulike illness generally producing mild signs and symptoms of disease. Diarrhea is the most common clinical feature of this intestinal infection. Clinical illness in patients with intact immune systems usually resolves within 30 days, in some cases maintaining a persistence for transmission for up to 60 days (40, 59, 112). There have been several reports of asymptomatic human infection as well. In contrast, immunodeficient or immunocompromised hosts, especially human immunodeficiency virus (HIV)-infected persons, have a severe, fulminate, and frequently unremitting illness often contributing to mortality. Although a cholera-like diarrhea is the most noteworthy clinical sign, abdominal pain, nausea, fever, malaise, and respiratory manifestations of the infection are also common. Most immunocompromised patients harboring *C. parvum* in their respiratory tracts succumb to infection (39). Since the early 1980's, most of the reported cases of human cryptosporidiosis (respiratory and intestinal) have been associated with AIDS patients (58). Relatively few cases were diagnosed, however, until the disease was reported to be life-threatening (44). Ultimately, clinical disease in patients with immune deficiencies varies with their ability to reverse the immunosuppression.

Immunological Response of the Host. Information regarding the function of the immune response in hosts infected with *C. parvum* is scant. Nonetheless, the severity of infection can range from subclinical to severe, depending upon the host's age and immunological status. Age-related susceptibility as well as resistance to cryptosporidiosis has been documented (10, 29, 93). Generally, young infants and animals with immature immune systems are more susceptible to infection. They exhibit severe and sometimes fatal clinical disease, but with symptomatic treatment, it allows

their immune systems to mature and they rid themselves of the infection. Adults, on the other hand, may appear asymptomatic, demonstrate acute illness, or exhibit chronic illness lasting months to years. Thus, distinct differences exist between the clinical course of the disease in immunocompetent versus immunocompromised hosts. These differences have been attributed to the immune response to the parasite.

Clinical evidence has indicated that impairment of cellular and/or humoral immunity delays and often prevents recovery from cryptosporidiosis (29, 60, 118). There is still some controversy as to which branch of the immune system plays the more significant role in clearing the infection. Numerous scenarios predispose individuals to *C. parvum* infection, including exogenous immunosuppressive agents for cancer chemotherapy, hypo- or agammaglobulinemia, malnourishment, intercurrent viral infections, pregnancy, and AIDS (32, 39). Specific bovine immunoglobulins (IgM, IgA, and IgG) against *C. parvum* oocysts and sporozoites have been identified. Also, anti-*C. parvum* IgG, IgM, IgA, and IgE have been identified in the sera of previously infected humans. Though studies have demonstrated that sporozoites can be neutralized by antibodies, a measurable humoral response alone does not provide sufficient protection against the disease (60). For instance, AIDS patients produce both IgM and IgG and yet suffer from chronic cryptosporidiosis. In contrast, studies in patients with B lymphocyte dysfunctions (hypo- or agammaglobulinemia), but with T lymphocyte functions intact, still develop chronic cryptosporidiosis (60). Experimental studies have demonstrated the important role T lymphocytes play in the clearance of and resistance to cryptosporidiosis in mammals (29, 60, 122, 123), suggesting the likelihood that a successful response to infection involves a T lymphocyte-dependent induction of specific secretory antibody responses (29). Thus, a protective immune response may require the orchestration of specific humoral and cell-mediated immune mechanisms.

To appropriately mimic the chronic, life-threatening nature of *C. parvum* infections in AIDS and other immunocompromised patients, and to further elucidate the immune mechanisms involved in the host's immunological response to infection, suitable *in vitro* and *in vivo* models are essential.

***In Vitro* Cultivation.** The lack of a simplistic, standardized *in vitro* cultivation system has not only limited testing of possible therapeutics for cryptosporidiosis, but has also limited an understanding of the host's immunological response to this parasite. Although there is a species of *Cryptosporidium* (*C. baileyi*) that grows readily in numerous *in vitro* culture systems, the mammalian species (*C. parvum*) is much more selective. The latter species has been found to complete its entire life cycle, from sporozoite to oocyst, in only a select few chicken embryos and cultured cell systems (30, 31). Chorioallantoic endodermal cells (30), primary chicken kidney cells, human fetal lung cells, pig kidney cells (31), human endometrial cells (95), and human colon carcinoma cells (34), have been proposed as *in vitro* cultures for evaluation of antiprotozoan drugs. However, these systems limit the extent of multiplication, provide few infective oocysts, and are difficult to perform. This marked preference of *C. parvum* to some culture systems as opposed to others is not yet understood and deserves further exploration.

***In Vivo* Experimentation.** Experimental research involving numerous animal species has been employed in the study of *C. parvum* infection in hopes of characterizing a reliable model. To date, the most widely used and inexpensive animal models have been rodents. Wild as well as laboratory rodents have been experimentally infected with *C. parvum* (28, 41, 44, 52, 55, 65, 111, 116, 120) and examined for their use in characterizing the pathogenesis and immune mechanisms associated with cryptosporidiosis. Attempts to develop a reliable immunocompetent adult rodent model have been unsuccessful (52, 119).

In selecting animal models, considerable attention has been devoted to their immune status and subsequent susceptibility to *C. parvum*. As in human infections, persistent animal infections require that the host be immunocompromised. This fact has led to the examination of experimental infections in animals with immature immune systems (neonates) and animals that are immunocompromised as a result of either genetic immunodeficiency or administration of exogneous immunosuppressive drugs.

Neonatal animal models available to study cryptosporidiosis include suckling mice, calves, piglets, and lambs (28, 41, 119, 121). Experimental studies conducted in neonates indicate that, although they are initially susceptible to *C. parvum* infections, the rapid maturation of their immune systems and development of innate immunity quickly eliminates *C. parvum*. Initial studies showed that while neonatal (1-4 days old) mice could be asymptomatically infected, mice 21 days or older exhibited a transient infection (111). Other research involving neonatal mice demonstrated development of patent infections in approximately 4 days, oocyst shedding for up to 9 days, followed by spontaneous clearing of the infections within 3 weeks (28, 41, 55). However, when the host was immunosuppressed, the disease became chronic, with the host exhibiting severe signs of infection closely paralleling those seen in human infections. Hence, the use of conventional neonatal animal models is limited due to the short-term nature of the disease. This short duration of infection limits the period necessary to effectively screen prophylactic or therapeutic agents. In addition, drug administration to neonates usually requires labor-intensive procedures because neonates are neither metabolically nor biologically well developed. Thus, the neonatal model fails to lend itself as a reliable model for effective evaluation of anti-*C. parvum* drugs.

Attempts to infect immunodeficient adult animals have also received some attention. Ungar et al. (122) has described adult athymic and T-cell subset-depleted mice as possible models of chronic symptomatic cryptosporidiosis. More recently, severe

combined immune deficient (SCID) and NIH-III (bg/nu/xid) mice have been examined as possible animal models for *C. parvum* infections (75). These and similar studies have shown that genetically immunodeficient mice are susceptible to *C. parvum* infections as neonates and as adults (75). In addition, these models have utility for screening potential anti-cryptosporidial agents. However, their inbred genetic disorder does not allow one to determine the immunological status of the animal. Furthermore, immunomodulating agents can not be screened using a genetically immunodeficient model. This proves to be a disadvantage based on the general agreement that immunological as opposed to physiological responses of the host result in termination of the parasite's life cycle and full recovery from infection (29, 60).

Other laboratory animal models previously tested for cryptosporidiosis include guinea pigs, hamsters, primates, and a number of wild and domestic species (4, 19, 39, 79, 104). However, demands on expense, availability, and handling of these animals, as well as their resistance to glucocorticoid-induced immunosuppression (20), have limited their use. Controversy regarding the etiological agent of disease (a species other than *C. parvum*) has also caused some concerns (4, 19, 124, 125). Whether or not the disease in these animals is due to *C. parvum* or another species is still not clear, and thus merits further investigation.

Based on findings which suggest that patent and pre-patent periods of *C. parvum* differ between immunocompetent and immunocompromised hosts, anticryptosporidial drugs intended for use in immunocompromised hosts should be evaluated in an immunocompromised model (98). Also, as stated by Brasseur et al. (15:1037), "the validity of a rodent model as a model for human cryptosporidiosis is supported by 1) the identification of the parasite in the different hosts, and 2) the similarity of infections in both immunosuppressed humans and rodents."

Immunocompromising Agents. In order for chronic *C. parvum* infection to persist, the host needs to maintain a state of immunosuppression. This is supported by research involving cryptosporidial infections in malnourished children, genetically immune deficient rodents, humans receiving immunosuppressive therapy, and AIDS patients (39). All of these hosts exhibit chronic and often fatal cases of the disease. Therefore, in order to mimic the signs and symptoms associated with chronic cryptosporidiosis, as would be seen in immunosuppressed or immunodeficient patients, the animal model must be immunosuppressed.

Some of the more common immunosuppressants that have been used in animals and humans include cyclosporin, hydrocortisone acetate, cyclophosphamide, and dexamethasone (15, 57, 73, 77, 80, 92, 94, 97, 98, 107). All of these agents have and are still being utilized in research as well as clinical work. Rodent models relying on the administration of the aforementioned immunosuppressants as a means of facilitating susceptibility to *C. parvum* infection have been described (15, 97, 98, 104).

Cyclosporin, a commonly used immunosuppressive agent in cancer chemotherapy and organ transplants has, for the most part, proven quite successful in this regard. Its mechanism of immunosuppression involves the inhibition of lymphokine secretion (63), and interference with enzymes essential for the tertiary folding of proteins (51). However, complications associated with the use of cyclosporin, including renal dysfunction, hypertension, tremors, and convulsions, have limited its use. In addition, excessive doses have proven to be highly hepatotoxic and nephrotoxic, with the later occurring in 25-75% of patients on cyclosporin therapy (107).

Hydrocortisone acetate has been used in conjunction with a low protein diet in rodents to induce susceptibility to *C. parvum* (15). However, this immunosuppressive regimen has not been attractive because it is expensive and may result in decreased antibody production with an apparent increase in cell-mediated immunity (CMI).

Because research has suggested an important role of both the humoral and CMI responses of the host in clearing cryptosporidiosis, this regimen has been avoided in most research protocols.

Cyclophosphamide is among the more common immunosuppressive agents associated with establishing susceptibility to cryptosporidiosis in humans (57, 73, 77, 80) and animals (5, 97). Studies demonstrated that cyclophosphamide therapy induced delayed toxicity in rodents (5, 97). In addition, cyclophosphamide administration was required in higher doses and for longer periods to provide sufficient immunosuppression in rats to allow for *C. parvum* colonization (97). Consequently, the anticryptosporidial activity of potential therapeutics could not be tested as soon with a cyclophosphamide-immunosuppressed animal model. Furthermore, cyclophosphamide has also been reported as being an anti-proliferative agent against a myriad of dividing cells, and therefore is not specific for cells of the immune system.

Dexamethasone (DEX) is a synthetic glucocorticoid (GC) known to have potent anti-inflammatory and immunosuppressive activity (20, 21, 27, 51, 70, 90). Natural glucocorticoids are hormones secreted from the adrenal cortex in response to various stimuli, and have been shown to have striking pharmacologic effects on tissues and cells of lymphoid origin (20). Natural steroid therapy generally requires large doses and at times localized administration, which has often limited the use of these compounds (81). The overall potency and effectiveness of synthetic steroid analogs can readily be controlled by slight conformational and structural alterations (70).

Dexamethasone is known to suppress the immune system by non-selective and selective mechanism(s). The mechanisms, though obscure, are considered non-selective for the following reasons: species differences in susceptibility to glucocorticoids, heterogeneity of lymphoid cells within the same species, and presence of specific intracellular glucocorticoid receptors (11) and biochemical events responsible for

subcellular effects (20). Nonetheless, the majority of DEX-mediated mechanisms are selective. The mechanism of action of this steroid involves controlling the rate of protein synthesis at the cellular level (22, 51). The most widely accepted mechanism suggests that DEX accomplishes this by reacting with specific protein receptors located in the cytoplasm of sensitive cells, resulting in the formation of a steroid-receptor complex (22, 51). Recent studies have demonstrated, however, that the initial binding of hormone and receptor may actually occur inside the nucleus. The hormone-receptor complex, after undergoing structural modification involving the dissociation of a 90 Kd protein from the receptor, migrates to the chromatin of the cell where it binds to DNA and regulates gene transcription. In most cases, transcription is enhanced, resulting in an increase of mRNA. However, some known examples have shown evidence of a decrease in gene transcription (51).

Animals have been divided into GC-sensitive and GC-resistant species based on the ease of producing lymphoid depletion following a regimen of systemic GC administration (20). 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 GC-sensitive. Most of the information concerning the effects of GC on immunologic processes and lymphoid cells have been derived from experiments involving GC-sensitive species. Therefore, extrapolation of these experimental results to GC-resistant species should be done with discretion. Opposing effects of DEX on lymphocyte populations in GC-resistant versus GC-sensitive species have been documented (22).

The biologic half-life of DEX varies depending upon the route of administration and the species receiving the immunosuppressive therapy. In humans, where DEX has been administered intravenously or orally, the half-life has been reported to range from 36 to 72 hours (14, 51), with blood lymphcyte counts reaching minimum values between 4-8

hours postdosing (14). In other studies, the half-life has ranged from 2 to 9 hours in humans (20). Unfortunately, there is very little information regarding kinetic modeling of DEX in rodents.

Data suggest that GC administration has suppressive effects on both humoral and cell-mediated immunity (94), with the latter requiring prolonged treatment or large doses before measurable effects are apparent (20). Humoral immune responses are indirectly influenced by GC and are believed to be differentially affected by GC immunosuppression depending on species sensitivity. In GC-sensitive species, DEX results in decreased production of circulating antibodies and inhibits lymphocyte response to mitogen stimulation (12, 22, 48, 72, 94). In GC-resistant species, DEX treatment produces enhanced responses of B and T lymphocytes to mitogens (22). Some of the immunosuppressive effects of DEX on lymphoid cells include *in vivo* thymic involution (20), *in vitro* thymocyte destruction (71), inhibition of cellular activation and metabolism (20), redistribution of lymphocytes (20), and profound effects on peripheral lymphoid tissues such as spleen and lymph node shrinkage (20).

Dexamethasone-immunosuppressed rodent models for studying cryptosporidiosis have been partially characterized (92, 94, 98). The dose and route of DEX administration appear to be critical when attempting to induce an infection (98). Genetic differences between rodent species also play a role in the effects DEX has on the immune system (92). In any event, the consistent, low-toxic immunosuppression produced by DEX, and the subsequent susceptibility of the host to *C. parvum*, supports the use of this drug in the development of a rodent model for studying chronic cryptosporidiosis.

Adrenal Steroids as Chemotherapeutics. The adrenal cortex synthesizes two classes of steroids, namely corticosteroids (mineralocorticoids and glucocorticoids) and androgens, which includes dehydroepiandrosterone (DHEA). Dehydroepiandrosterone, 3-beta-hydroxyandrost-5-en-17-one is one of the most

abundantly secreted adrenal cortical steroids in humans and other mammals (67, 88, 126). The level of DHEA increases during early adolescence and decreases to nearly 5% of its original level with old age (67, 88). There are substantially lower levels of DHEA in people exhibiting signs of chronic illness and stress (110), Alzheimer's disease (113), those with type A personalities (43) and HIV-infected patients (78). The physiological significance of the progressive decline in DHEA is not yet fully understood.

A number of *in vitro* and *in vivo* experimental findings provide evidence that DHEA administration to research animals has beneficial effects on a number of physiological and metabolic parameters. Some of these effects include treatment of diabetes in genetically obese (23) and diabetic mice (24), increased tissue sensitivity to insulin in aged normal mice (25), and decreased weight gain in young animals without altering food intake (127). In rodents, DHEA has been shown to increase lifespan (68), inhibit spontaneous breast cancer (109), increase resistance to carcinogen-induced cellular transformations (45, 86), restrain autoimmune diseases (68, 114), and up-regulate the immune system (13, 67, 103). Administration of DHEA has been shown to be associated with enlargement of splenic germinal centers, suggesting a direct stimulation of the B lymphocyte dependent areas (67). In contrast, as reported by Risdon et al. (101), normal mice given dietary DHEA showed an appreciable decrease (50%-60%) in lymphoid organ cellularity, suggesting that the route of DHEA administration may be an important factor influencing its potentiating effects on the immune system.

The molecular basis for the potentiating effect of DHEA on the immune system is not clear. As the concentration of DHEA declines, so does the competency of the immune system (13, 71, 103, 115). In humans and to a lesser degree in rodents, DHEA is converted by an adrenal sulfokinase to its sulfate ester metabolite, dehydroepiandrosterone sulphate (DHEAS). Observations based on the research of Loria et al. (67) suggest that protection with DHEA occurs through a pathway other than

sulfation. Their findings are supported by Browne et al. (16) who suggested that the prohormone DHEAS is biologically inactive and much less potent than DHEA. In contrast, Daynes et al. (36) administered DHEAS to aging mice and completely restored immune function. Risdon et al. (102) have shown that lymphopoiesis (lymphocyte production from bone marrow and thymus) is inhibited by DHEA, while having no effect on myelopoiesis (red blood cell production from the spleen). The DHEA may possess its own thymus cell-receptor, thus allowing it to exert effects directly on the thymus (71). However, May et al. (71) questioned the independent DHEA thymus receptor, observing that the protective effects of DHEA were present only when administered *in vivo*. This suggests that the effect may possibly be mediated at a site located elsewhere in the body, or the effect may be due to a necessary androgenic metabolite of DHEA. Experiments utilizing androstenedione, the most active metabolite of DHEA, failed to show any immune protective effect, ruling out the likelihood that androgens are involved in the mediating pathway (13, 71, 103). Experiments showing evidence of a direct effect of DHEA on central nervous tissues (46) support the possibility of a distant receptor site for this antiglucocorticoid. A DHEA-specific receptor has been found on murine T lymphocytes (76). Whether or not this receptor is present on lymphocytes of other animal species is speculative. Risdon et al. (102) concluded that DHEA acts selectively by suppressing lymphocyte progenitors as opposed to mature lymphocytes and that growth and maturation of B, T, and natural killer cells occurred by mechanisms other than interleukin 2 (IL-2) inhibition. Daynes et al. (35, 37, 38) have demonstrated that DHEA enhances IL-2 production by activated helper T lymphocytes. *In vitro* and *in vivo* administration of DHEA overcomes GC-induced depressions in IL-2 and gamma interferon (IFN) production (38). Studies in mice have demonstrated that protective immunity to opportunistic infections like cryptosporidiosis, requires helper T lymphocytes expressing IL-2 and gamma-IFN production (55, 75, 122). Individuals

infected with HIV exhibit elevated blood levels of GC (55), yet display depressed levels of DHEA (cb). This shift in steroid hormones in AIDS patients could possibly facilitate their increased susceptibility to infectious agents such as *C. parvum*. Furthermore, age-related changes in host defense mechanisms have been reversed by the administration of DHEA (36, 93).

Dehydroepiandrosterone has been used clinically in both topical and systemic regimens to treat such conditions as gout, psoriasis, hyperlipemia, and postcoronary illnesses. In animals and humans, DHEA has shown promise as an anti-obesity (84, 127) and anti-carcinogenic agent (56, 66, 105, 108). Some of the more prevalent findings involve the potentiating effects DHEA has on the immune system (13, 67, 103). Side effects of DHEA have been minimal as reported by Loria et al. (67) and others (84, 96). Despite the efficacy of DHEA in treating such a myriad of disorders, there is no undisputed mechanism(s) of chemoprevention. Potential mechanisms have been proposed by Risdon et al. (101), including induction of peroxisomal proliferation and/or modification resulting in alterations of liver metabolism, caloric restriction, conversion of oral DHEA to active metabolites such as estrogens and androgens, and inhibition of glucose-6-phosphate dehydrogenase.

The implications of using this native steroid to treat a variety of disorders are numerous, and through continued experimental research, the biological mechanism(s) of DHEA will be elucidated. Because clinical studies using DHEA have shown evidence of minimal side effects, the utility of this immunomodulator as a possible prophylactic and/or therapeutic agent against opportunistic infections like cryptosporidiosis merits intensive study.

MATERIALS and METHODS

Animals. Five- to six-week-old, female C57BL/6N mice (Simonsen Laboratories, Gilroy, CA), weighing 14 to 16 g, were used in this study. Rasmussen et al. (92) showed that the DEX-immunosuppressed C57BL/6N mouse could be successfully infected with *C. parvum*, resulting in a chronic infection. Female mice were used to decrease variability associated with steroid regimens and because their behavior in caged groups is less aggressive.

All mice were maintained in the American Association for the Accreditation of Laboratory Animal Care (AAALAC)-accredited Laboratory Animal Research Center at Utah State University. The animals were housed five per cage (unless otherwise indicated) in plastic shoe-box cages on corn-cob bedding and received commercial lab chow (Wayne Lab Blox, Allied Mills, Chicago, IL) and water *ad libitum*. Animal cages, bedding, and drinking water were changed once per week. Room conditions were maintained with a 12 hour light-dark cycle at 20-22°C with 40-60% relative humidity and 14-18 air changes per hour using 100% fresh air exchange. The room static pressure remained negative relative to the adjoining hallway. The animal cages were housed in a ventilated animal rack (Lab Products). Exhaust air from the rack was HEPA-filtered and recirculated back into the room. All live animal handling was done in a Bio-hazard hood. To prevent accidental infection between animals and personnel, BSL-2 safety standards were maintained (91).

Immunosuppression of Mice. The synthetic glucocorticoid, dexamethasone (DEX; Sigma Chemical Co., St. Louis, MO) was used to immunosuppress the mice. The mice were immunosuppressed for 12 days (unless otherwise specified) prior to *C. parvum* oocyst inoculation, and were continued on DEX throughout the remainder of the experiments. The immunosuppressive regimen consisted of daily intraperitoneal (i.p.)

injections of DEX at a concentration of 125 µg/mouse/day unless otherwise indicated. In short, DEX was dissolved in 100% ethyl alcohol and stored as a stock solution at 4°C. Just prior to i.p. injection, DEX was diluted in sterile deionized water to the desired concentration and a total volume of 200 µl per mouse was injected using a sterile 27-gage needle. To reduce physical trauma caused by daily i.p. injections, needles were replaced often (e.g., a maximum of five injections per needle). A group of mice were also immunosuppressed with DEX administered orally in the drinking water. A stock solution of DEX at a concentration of 8 µg/ml was prepared weekly as described. The DEX was added to individual water bottles for each cage and the mice had free access to the water.

Parasites. The *C. parvum* oocysts used to infect the mice were obtained originally from Harley Moon (U.S. Department of Agriculture, Ames, IA) (8). This Iowa isolate was used to orally infect newborn Holstein calves, which provided a source of fresh oocysts. The oocysts were purified from the calf feces using discontinuous sucrose gradients as described by Arrowood et al. (6), and stored in 2.5% potassium dichromate at 4°C for a maximum of four months before being used to infect the mice.

Prior to orogastric administration, the oocysts were washed to remove the potassium dichromate. Briefly, a calculated volume of oocyst suspension was placed into a polypropylene centrifuge tube (Corning Glass Works, Corning, NY) that had been precoated with RPMI-1640 (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). This protein coating prevented the oocysts from adhering to the wall of the centrifuge tube. An equal volume of RPMI-1640 base medium was added to the oocyst suspension and mixed gently. The oocyst-RPMI suspension was centrifuged for 10 min. at 2000 x g, the supernatant discarded, and the pellet resuspended in 20 ml of RPMI-1640 base medium. The suspension was centrifuged, the supernatant discarded, and the pellet resuspended two

more times as described. The final total volume of resuspension was in 2-5 ml of RPMI-1640 base medium. Immediately before administration, the oocysts were counted using a hemocytometer. Each mouse was intragastrically inoculated with approximately 10^6 clean oocysts in a volume of 200 µl of RPMI-1640 base medium. The oocysts were administered using a 22-gage straight feeding needle (Thomas Scientific, Swedesboro, NJ). The inoculum was repeatedly mixed to insure each mouse received equal numbers of oocysts.

Fecal Collection and Examination. Infection intensity was determined by the number of *C. parvum* oocysts excreted in the feces. To monitor the oocyst shedding in infected mice, fecal pellets were collected from each mouse per rectum throughout the experiment. The fecal pellets were suspended in a volume of 2.5% potassium dichromate approximately equal to that of the feces and stored at 4°C for a minimum of 24 hrs. Fecal material was mixed and smeared onto glass microscope slides and observed for the presence of *C. parvum* oocysts using an oocyst-specific monoclonal antibody-based indirect immunofluorescence assay (7). Generally, 40 microscopic fields (400x) were examined for the presence of oocysts. Fecal smears were examined in a blind fashion and different treatment groups were compared according to mean oocyst numbers per fecal smear. Raw fecal counts for each treatment group were subjected to statistical analysis.

Histological Collection and Examination. Periodically, mice from each treatment group were euthanized and necropsied, and tissues were collected and examined for parasite colonization. Prior dates were established for sacrificing mice. However, at times it was necessary to sacrifice mice displaying abrupt clinical deterioration (loss of skin tone, lethargy, emaciation). Mice were euthanized by overexposure to carbon dioxide. Following sacrifice, a complete gross examination of all organs was performed and any abnormalities were noted. Spleens were collected

aseptically and weighed as a representation of the immune status of the animal. The spleens intended for use in immunological assays were temporarily stored in RPMI-1640 base medium supplemented with 10% FBS while other spleens were fixed in 10% neutral buffered formalin for histological analysis.

Tissue samples harvested from the heart, lungs, liver, gall bladder, kidney, uterus, urinary bladder, stomach, pancreas, duodenum, jejunum, ileum, cecum, and colon were fixed in 10% neutral buffered formalin. Tissues were stored at least one week in formalin prior to sectioning. Histological sections were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H & E). Stained tissues were examined using brightfield microscopy in a blind fashion to determine parasite colonization and presence of histopathologic lesions. Generally, intestinal tissue was scored numerically by viewing 10 random fields (400x) examining a total of 0.5 cm of intestinal tissue with raw parasite numbers recorded. Nonintestinal tissues were examined and scored on a percentage basis as follows: 0 = parasites not observed; 1+ = < 10% of epithelial surface parasitized; 2+ = 10-50% of epithelial surface parasitized; 3+ = 50-90% of epithelial surface parasitized; and 4+ = >90% of epithelial surface parasitized. For each treatment group, the average number of cryptosporidia at the epithelial surface of infected tissues was determined and parasite counts were subjected to statistical analysis (9).

Immunologic Paramaters. The immunologic parameters examined were the functionality of splenic B and T lymphocytes in response to the mitogens lipopolysaccharide (LPS) and concanavalin A (ConA). The mitogens served as agents (antigens) to stimulate mitosis in mouse B and T lymphocytes.

Splenocyte preparation. Spleens were collected aseptically, weighed, and temporarily maintained in 15 ml polypropylene centrifuge tubes (Corning) containing 5 ml of RPMI-1640 base medium (GIBCO). The base medium consisted of 99% RPMI-1640 (GIBCO) and 1% penicillin-streptomycin (1000 U penicillin-G/ml plus 1000 µg

streptomycin/ml) (GIBCO). The penicillin-streptomycin served to inhibit bacterial growth from possible contamination. Individual spleens and media were poured into plastic seward medical stomacher bags (Tekmar Company, Cincinnati, OH). Three bags were simultaneously homogenized using a stomacher (Tekmar) to create single cell suspensions. Spleen cell suspensions were poured back into their original 15 ml tubes, centrifuged at 200 x g for 10 minutes, and the supernatant discarded. Red blood cells were removed by hemolysis using 9 ml of sterile, deionized water. The remaining lymphoid cells were quickly (within 5 seconds after adding water) brought back to physiological pH by adding 1 ml of 10 X phosphate buffered saline (PBS) adjusted to a pH of 7.2-7.6. Any connective tissue debris was removed using sterile wooden applicator sticks. Remaining splenocytes were centrifuged a second time (10 min. at 200 x g), the supernatant discarded, and the pellet resuspended in 10 ml of RPMI-1640 base medium. Cell suspensions were centrifuged a third time (10 min. at 200 x g), the supernatant discarded, and the pellet resuspended in 5-10 ml of RPMI-1640 complete medium containing 20% FBS (Hyclone). The complete medium consisted of RPMI-1640 with 1% penicillin-streptomycin, 1% 5 mM 2-mercaptoethanol (2-ME; Sigma), and 1% sodium pyruvate (GIBCO). The FBS provides necessary growth factors for cell division. The 2-ME stimulates murine macrophages which, in turn, are necessary for T lymphocyte stimulation. And, the sodium pyruvate provides essential energy requirements of the cells. The individual cell suspensions were then counted using a Coulter Counter (Coulter Electronics, Hialeah, FL) and adjusted to 5 x 10^5 cells/ml. Splenocytes were used in B and T lymphocyte mitogen-induced proliferation assays to determine the immune paramaters affected by DEX immunosuppression and *C. parvum* infection.

B and T Lymphocyte Proliferation Assays. The blastogenic response of spleen cells to the B lymphocyte mitogen *Escherichia coli* lipopolysaccharide

(10, 5 and 2.5 μg/ml; Sigma) and the T lymphocyte mitogen concanavalin A (5, 2.5 and 1.25 μg/ml; Sigma) were determined to assess general B and T lymphocyte function. The mitogen doses chosen were based on previous findings representing maximal response in our cell culture system (unpublished data). A total of 5 x 10^5 spleen cells in 0.1 ml volumes of RPMI, 10% FBS, and 2-ME was added to triplicate wells of 96-well flat-bottom microplates (Corning). A volume of 0.1 ml of each mitogen dilution in a solution containing 10% FBS in RPMI-1640 was added to each well in triplicate. Controls consisted of wells containing only RPMI-1640 base medium and 0.1 ml spleen cells (5 x 10^5). The plates were incubated for 72 hrs at 37°C in a humidified atmosphere of 5% CO_2. Synthesis of DNA was measured by the addition of 0.5 μCi/well [^{3}H]thymidine (4 μCi/ml; New England Nuclear Corp., Boston, MA) for 4 hrs. Thymidine incorporation was determined by harvesting cells on glass fiber filter paper, followed by counting on a Packard 1500 scintillation counter. Lymphocyte proliferative response was determined using counts per minute (CPM) and represented as mean CPM. Mean CPM were calculated by dividing the average of the sample CPM by the average of the control CPM for that same mouse.

Statistical Analysis. For each day that fecal specimens were collected, the number of *C. parvum* oocysts per mouse per 40 microscopic fields (400x) was averaged for each experimental group to obtain a group mean. Tissue colonization by the parasite was determined for each infected mouse by counting total parasite numbers associated with 0.5 cm of tissue (intestinal sections) or percent colonized (nonintestinal sections) and group means were calculated. Group means ± standard error (SE) were reported for each experimental group. One-way and two-way analyses of variance (ANOVA) were performed to compare differences within and between groups when appropriate. For ANOVA, Fisher Protected LSD or Scheffe tests (Macintosh version) were used to do

mean comparisons when differences existed among treatment groups. A significance level of 95% was set for each test to denote significant differences between groups.

EXPERIMENTAL DESIGN

Characterization of Chronic Cryptosporidiosis in Immunosuppressed Mice. This study was designed to examine the chronicity of *C. parvum* infections in DEX-immunosuppressed C57BL/6N mice. Two experiments were performed, the first of which was a "pilot" study used to define the parameters associated with a chronic infection in this mouse model. The second experiment was a "follow-up" to the pilot study in which modifications to optimize the model were incorporated.

In the pilot study, four groups of mice were studied (Table 1). Group-1 (Normal) consisted of eight mice housed four per cage, none of which were immunosuppressed or challenged with the parasite. Group-2 (Cp only) consisted of 12 mice housed five per cage with the exception of one cage which contained only two mice. All mice in group-2 were challenged with the parasite but were never immunosuppressed. Group-3 (DEX only) consisted of 20 mice housed five per cage, none of which were challenged with parasite, but all were immunosuppressed. Group-4 (DEX + Cp) consisted of 22 mice housed five per cage with the exception of one cage containing two mice. All mice in group-4 were immunosuppressed and challenged with the parasite.

The DEX was administered i.p. daily (125 µg/mouse/day) for 12 days prior to *C. parvum* oocyst inoculation and continued throughout the duration of the experiment. Each mouse receiving the parasite was intragastrically inoculated with 0.2 ml of a suspension containing 10^6 oocysts. Fecal samples were collected per rectum each day from individual mice for the first 2 weeks and weekly thereafter, until the end of the experiment. Oocyst shedding was monitored by immunoflourescent assay of the fecal pellets (9, 47). Individual mouse weights were recorded weekly and the overall appearance and health status of the mice was noted throughout the experiment. The study was carried out for 4 months in order to examine the chronic phase of the disease.

Mice from each treatment group were necropsied bimonthly or when moribund, beginning on day one following inoculation with the oocysts. Due to mortality, the number of mice sacrificed in each treatment group varied. Mice were euthanized by overexposure to carbon dioxide. Following euthanasia, spleens were collected aseptically and weighed as a general representation of the immune statue of the mice. The entire gastrointestinal tract of necropsied mice was dissected and sections of the heart, lungs, liver, gall bladder, kidney, uterus, stomach, pancreas, duodenum, jejunum, ileum, cecum, colon, and urinary bladder were fixed in 10% formalin. Histological sections were stained with hematoxylin and eosin (H & E) and examined using brightfield microscopy to determine parasite colonization. The average number of cryptosporidia at infected epithelial surfaces was determined and parasite counts were subjected to statistical analysis (9).

Table 1. Experimental design for the pilot study of chronic *Cryptosporidium parvum* infections in mice.

Group	n	DEX (125 µg/mouse/day)	*C. parvum* (10^6 oocysts/mouse)
1 (Normal)	8	-	-
2 (Cp only)	12	-	+
3 (DEX only)	20	+	-
4 (DEX + Cp)	22	+	+

Cp = *C. parvum*; DEX = dexamethasone

The chronic experiment was repeated with the following modifications and is recognized as a "follow-up" study. Five groups of mice were studied (Table 2). Group-1 (Normal) consisted of 10 mice housed five per cage and received neither DEX-immunosuppression nor were challenged with parasites. Group-1 mice were given i.p. injections of a 10% alcohol solution as a placebo on the same schedule as those mice receiving DEX i.p. Group-2 (Cp only) consisted of 20 mice housed five per cage, each

receiving the parasite without being immunosuppressed. Group-2 mice also received i.p. injections of a 10% alcohol placebo in accordance with the i.p. DEX schedule. Group-3 (DEX only) consisted of 50 mice housed five per cage. Each mouse in group-3 received DEX-immunosuppression but was never challenged with the parasite. Group-4 (DEX + Cp) consisted of 50 mice housed five per cage. Each mouse in group-4 received DEX-immunosuppression and parasite challenge. Group-5 (DEX + Cp) consisted of 47 mice housed five per cage with the exception of two cages which contained six mice. Each mouse in group-5 received parasite challenge; however, DEX was administered in the drinking water (8 µg/ml) *ad libitum* rather than being administered i.p.

In groups 1-4, DEX (125 µg/mouse/day) was administered i.p. daily for 12 days prior to *C. parvum* oocyst inoculation (10^6 oocysts) and continued until the 8th week post-infection. At this time, the DEX regimen was administered only 6 days per week and continued throughout the duration of the experiment. Parasite inoculation, fecal examination, euthanasia, and histological collection and examination followed the same protocol as described previously. The study was carried out for 4 months in order to examine the chronic phase of the disease.

Table 2. Experimental design for the follow-up study of chronic *Cryptosporidium parvum* infections in mice.

Group	n	Intraperitoneal Injection	*C. parvum* (10^6 oocysts/mouse)
1 (Normal)	10	10% alcohol	-
2 (Cp only)	20	10% alcohol	+
3 (DEX only)	50	DEX	-
4 (DEX + Cp)	50	DEX	+
5 (DEX + Cp)[a]	47	None	+

[a] mice in group-5 were immunosuppressed orally with DEX (8 µg/ml).
DEX = dexamethasone (125 µg/mouse/day); Cp = *C. parvum*

Effects of *Cryptosporidium parvum* and Dexamethasone on B and T Lymphocyte Proliferation. These studies were designed to determine if susceptibility to *C. parvum* infections in immunosuppressed mice correlated with decreased *in vitro* B and T lymphocyte responses to the mitogens LPS and ConA, respectively. Furthermore, these studies were designed to determine if *C. parvum* effected immunosuppression on the hosts.

Thirty-two mice were randomly assigned to four groups as shown in Table 3. Group-1 consisted of six mice receiving neither DEX nor parasite challenge (Normal); group-2 consisted of six mice receiving no DEX but were challenged with the parasite (Cp only); group-3 consisted of 10 mice receiving DEX without parasite challenge (DEX only); and group-4 consisted of 10 mice receiving DEX and parasite challenge (DEX + Cp). All mice were immunosuppressed daily for 14 days with i.p. injections of DEX (125 μg/mouse/day) after which each mouse (those assigned to receive parasite challenge) was intragastrically inoculated with 10^6 *C. parvum* oocyst. All mice on immunosuppression continued to receive DEX i.p. throughout the duration of the experiment. Mice and facilities were maintained as described.

Table 3. Experimental design for examining the effects of *Cryptosporidium parvum* and dexamethasone on B and T lymphocyte proliferation in mice.

Group	n	Mice sacrificed 1 week p.i.	Mice sacrificed 2 weeks p.i.
1 (Normal)	6	3	3
2 (Cp only)	6	3	3
3 (DEX only)	10	5	5
4 (DEX + Cp)	10	5	5

Cp = *C. parvum*; DEX = dexamethasone; p.i. = postinfection

At 1 week postinfection (p.i.), 16 mice (half from each group) were sacrificed and at 2 weeks p.i. the remaining 16 mice were sacrificed. At the time of sacrifice, spleens

were collected and prepared for use in a B and T lymphocyte proliferation assay as described in the Materials and Methods. The assay was used to determine the effects DEX and/or *C. parvum* had on lymphocyte functionality.

Effects of Dehydroepiandrosterone on Oocyst Shedding Intensities of Mice Infected with *Cryptosporidium parvum*. This experiment was designed to determine if the immunomodulator dehydroepiandrosterone (DHEA) and its sulfated conjugate, DHEA-sulphate (DHEAS), increased resistance to *C. parvum* in immunosuppressed mice.

All mice in this experiment were immunosuppressed intraperitoneally (i.p.) with DEX (125 µg/mouse/day) for 12 days and then challenged with 10^6 *C. parvum* oocysts as described previously. The mice continued to receive DEX i.p. throughout the duration of the experiment. Thearpeutic treatment with DHEA or DHEAS began 35 days p.i. and continued daily through day 63 p.i., resulting in 28 days of treatment for a chronic infection.

Thirty-seven mice were randomly assigned to four groups as shown in Table 4. Group-1 consisted of four mice and served as a control group receiving DEX and parasite challenge, but no DHEA or DHEAS. Group-2 consisted of 12 mice equally divided into three subgroups receiving DEX, parasite challenge, and DHEA per os (p.o.) at a dose of 50, 150, and 300 µg/mouse/day. The DHEA was administered in a total volume of 100 µl of a peanut-oil vehicle. Group-3 consisted of 12 mice equally divided into three subgroups receiving DEX, parasite challenge, and DHEA subcutaneously (s.c.) at a dose of 3, 6, and 12 mg/mouse/day dissolved in 250 µl of a 100% propylene glycol solution (36). Group-4 consisted of 9 mice equally divided into three subgroups receiving DEX, parasite challenge and DHEAS s.c. at a dose of 3, 6, and 12 mg/mouse/day dissolved in 250 µl of sterile, deionized water (36). The different DHEA and DHEAS solutions were prepared fresh every week and stored at 4°C as stock solutions. Each DHEA or DHEAS

suspension was warmed to room temperature before daily administration. Mice and facilities were maintained as described in the Materials and Methods.

Fecals were collected per rectum daily, and examined for *C. parvum* oocysts. The oocyst shedding patterns for the individual groups were used to determine infection intensity. The overall health status and activity of the mice were noted throughout the study. Mice were sacrificed when moribound up until day 28 posttreatment with DHEA or DHEAS, at which time all remaining animals were sacrificed. At the time of sacrifice, any gross abnormalities were noted and spleens were collected and weighed as a general representation of the immune status of the mice.

Table 4. Experimental design for examining the effects of dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulfate (DHEAS) on the oocyst shedding intensities of mice infected with *Cryptosporidium parvum*.

Group	n	DHEA(S) dosage	DHEA(S) route	Vehicle
1	4	none	none	none
2a	4	50 µg	p.o.	peanut oil
2b	4	150 µg	p.o.	peanut oil
2c	4	300 µg	p.o.	peanut oil
3a	4	3 mg	s.c.	propylene glycol
3b	4	6 mg	s.c.	propylene glycol
3c	4	12 mg	s.c.	propylene glycol
4a*	3	3 mg	s.c.	sterile water
4b*	3	6 mg	s.c.	sterile water
4c*	3	12 mg	s.c.	sterile water

* mice in group-4 received DHEA-sulfate (DHEAS)
p.o. = per os; s.c. = subcutaneous

RESULTS

Characterization of Chronic Cryptosporidiosis in Immunosuppressed Mice. This pilot study was designed to examine the chronic nature of cryptosporidiosis in the DEX-immunosuppressed mouse model (92). Initially, a predetermined number of mice from each treatment group (random selection) were assigned to be sacrificed on a bimonthly schedule. As the immunosuppression continued and the infection intensified, many of the mice began to show clinical signs of deterioration that resulted in a number of unexpected deaths. As a result of this unanticipated loss of mice and in an attempt to maintain sufficient animal numbers to last the 4-month duration, the DEX-immunosuppression was reduced. In short, between weeks 8 and 10 of the experiment DEX administration was reduced from daily dosing to dosing every other day. This decrease in immunosuppression resulted in a rapid decline in oocyst shedding between weeks 8 and 12 postinfection (Figure 2). As the condition of the mice improved, DEX dosing was again administered daily, which explains the rapid increase in oocyst shedding toward the end of the experiment.

The prepatent period for mice infected with *C. parvum* was 3 days. Group-1 (Normal) and group-3 (DEX only) were never challenged with the parasite and, therefore, did not shed oocysts. Group-2 (Cp only) and group-4 (DEX + Cp) were all shedding by day 3 p.i. However, by day 7 p.i. all mice in group-2 had ceased oocyst shedding and remained negative for parasites throughout the experiment. All mice in group-4 continued to shed oocysts until the experiment was terminated. Figure 2 shows the oocyst shedding intensity of group-4 observed throughout the 4-month experiment. There was a steady increase in oocyst shedding until the DEX regimen was altered at 8 weeks p.i. As a result of the decrease in immunosuppression, the shedding intensity also

decreased rapidly. As the DEX-immunosuppression regimen was again instituted, there was another steady increase in oocyst shedding until the end of the experiment.

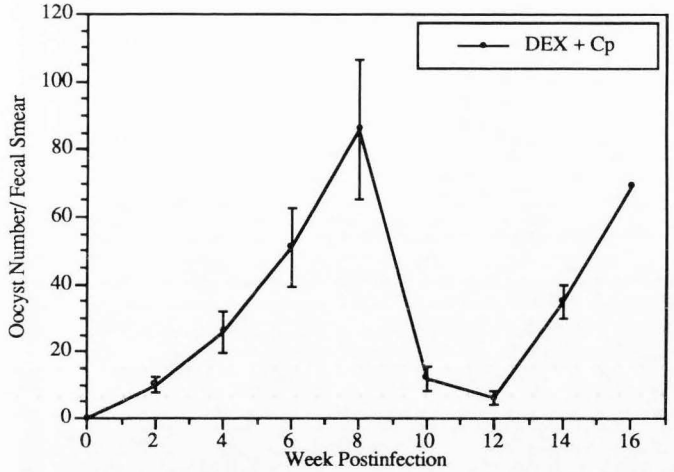

Figure 2. Pilot study of oocyst shedding intensity in dexamethasone-immunosuppressed mice with *Cryptosporidium parvum*.

On the day of sacrifice, individual mouse spleens were weighed and recorded as a representation of the overall immune status of the animal (Table 5). There was a significant decrease between the spleen weights of group-3 and group-4 when compared to both group-1 and group-2. Mice receiving DEX had depleted lymphoid follicles in the spleen compared to mice not receiving DEX. Histological observations of spleens from DEX-treated mice exhibited atrophy of both immature and mature lymphocytes. The spleen weights of mice receiving DEX-immunosuppression (group-3 and group-4) declined steadily from a mean of approximately 70 mg when the experiment began, to 20 mg at 4 weeks postimmunosuppression. The spleen weights from these two groups

remained at approxiamtely 20 mg until the DEX was reduced to dosing every other day. During the period of time when the DEX was given every other day, mice from group-3 had a peak mean spleen weight of 30 mg and mice from group-4 peaked at 50 mg. When the DEX was again administered daily, spleen weights from both DEX-treated groups dropped to a mean of approximately 20 mg.

Throughout the experiment, the body weights of the mice were also monitored; these data are recorded in Table 5. With respect to body weight, group-3 had a significantly ($P \leq 0.01$) lower body weight compared to group-2, and group-4 was significantly ($P \leq 0.01$) lower when compared to groups 1, 2, and 3. Altering the DEX-immunosuppression from daily to every other day injections did not have a significant effect on the body weights of the mice.

Table 5. Pilot study of spleen and body weights of mice infected with *Cryptosporidium parvum* represented as mean ± SE.

Group	n	Spleen Weight (mg)[a]	Body Weight (g)[a]
1 (Normal)	8	68.8 ± 4.0	20.5 ± 1.2
2 (Cp only)	12	81.7 ± 2.7	22.3 ± 0.4
3 (DEX only)	17	31.2 ± 5.8[bc]	19.4 ± 0.7[c]
4 (DEX + Cp)	14	32.1 ± 4.1[bc]	15.7 ± 0.8[bcd]

[a] values represent those data obtained on day of sacrifice.
[b] difference compared to group-1 is significant ($P \leq 0.01$).
[c] difference compared to group-2 is significant ($P \leq 0.01$).
[d] difference compared to group-3 is significant ($P \leq 0.01$).

The results of the tissue localization by cryptosporidia are presented in Table 6. Parasites were detected in the gallbladder as early as 2 weeks p.i. and the infection was intermittent throughout the experiment. Three mice had parasite colonization in the gallbladder; two with a score of 1+ and one with a score of 4+ (refer to the Materials and

Table 6. Pilot study of histologic location of *Cryptosporidium parvum* in dexamethasone-immunosuppressed mice with a chronic infection.

	Week Postinfection at Necropsy[a]						
TISSUE	2(n=3)	4(n=2)	6(n=2)	8(n=4)	12(n=1)	14(n=1)	16(n=1)
Heart	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-
Gallbladder	+[c]	+[c]	-	-	+	-	-
Kidney	-	-	-	-	-	-	-
Uterus	-	-	-	-	-	-	-
Lungs	+[c]	-	-	-	-	-	-
Stomach	-	-	-	-	-	-	-
Pancreatic ducts	NE	-	NE	+[b]	-	NE	NE
Duodenum	+[c]	+[c]	-	+[c]	-	-	+
Jejunum	+	+[c]	+[c]	+[d]	+	+	+
Ileum	+	+	+	+	+	+	+
Cecum	+[d]	+[c]	-	+[e]	-	-	-
Colon	+[d]	+[c]	-	+[d]	-	-	-

[a] no mice were sacrificed on week 10 p.i.
[b] one of the mice sacrificed on week 8 p.i. was not examined and one was (-) for *C. parvum*.
[c] only one of the total mice examined for the week indicated was (+) for *C. parvum*.
[d] two of the total mice examined for the week indicated were (+) for *C. parvum*.
[e] three of the total mice examined for the week indicated were (+) for *C. parvum*.
-, *C. parvum* absent; +, *C. parvum* present; NE, not examined.

Methods for a description of the scoring scale). There was no correlation between length of infection and infection intensity in the gallbladder. One mouse had parasite colonization in the lung (score = 1+) at 2 weeks p.i., while none of the other mice exhibited respiratory infections throughout the remainder of the experiment. The pancreatic duct of two mice at 8 weeks p.i. had detectable parasites, both with a score of 1+.

Microscopic examination of the intestinal tract, including duodenum, jejunum, ileum, cecum and colon, indicated that the ileum, especially the terminal ileum, was the most heavily infected section. The terminal ileum (T. ileum) is defined here as a 2-cm tissue section taken proximal to the ileal-cecal junction. Parasite intensity decreased proximal and distal with respect to the ileum and terminal ileum as demonstrated in Figure 3. Figure 3 is a pooled representation of all mice in group-4 (n = 14) sacrificed during

the 4-month experiment. Parasite colonization in the ileum and terminal ileum, as determined by the mean number of organisms in each intestinal section, was significantly ($P \leq 0.01$) higher when compared to the duodenum, jejunum, cecum, and colon.

There appeared to be a correlation between the infection intensity in the ileum and the oocyst shedding intensity in the feces. Figure 4 illustrates the parasite colonization detected in the ileum throughout the 4-month experiment. Altering the DEX regimen at 8 weeks p.i. resulted in similar tissue and fecal intensity patterns as is apparent when comparing the parasite colonization in the ileum (Figure 4) to the oocyst shedding pattern in the feces (Figure 2).

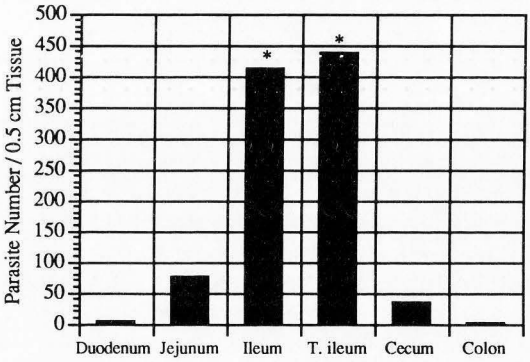

Figure 3. Pilot study of a pooled representation of *Cryptosporidium parvum* colonization in the intestinal tract of mice (group-4) with a chronic infection. * indicates significance ($P \leq 0.01$) when compared to duodenum, jejunum, cecum, and colon.

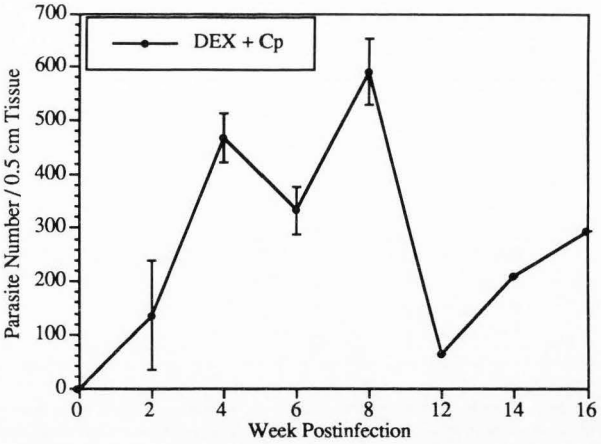

Figure 4. Pilot study of *Cryptosporidium parvum* colonization in the ileum of mice (group-4) with a chronic infection.

Pathologic findings associated with infection were isolated to the intestinal tract. Parasite colonization at extraintestinal sites, including the gallbladder and pancreatic ducts, did not result in any significant lesions. Heavy *C. parvum* infections in the ileum and terminal ileum produced villous atrophy, crypt hyperplasia, and light to modest inflammatory infiltration in the lamina propria. Figure 5 shows the parasites at the microvillar surface in a mouse terminal ileum at 2 weeks p.i. Figures 6 and 7 depict some of the salient features associated with a chronic infection, namely villar fusion and stunting, respectively. As the infection progressed, the parasite was found deep within the intestinal crypts resulting in hyperplasia (Figure 8).

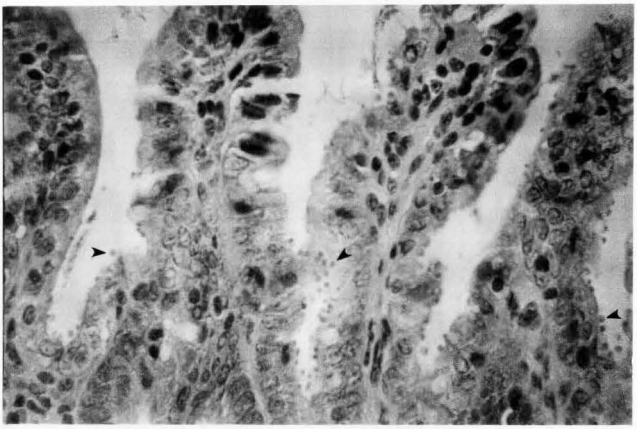

Figure 5. Photomicrograph of *Cryptosporidium parvum* (arrows) colonizing the terminal ileum of a mouse at 2 weeks postinfection (400x).

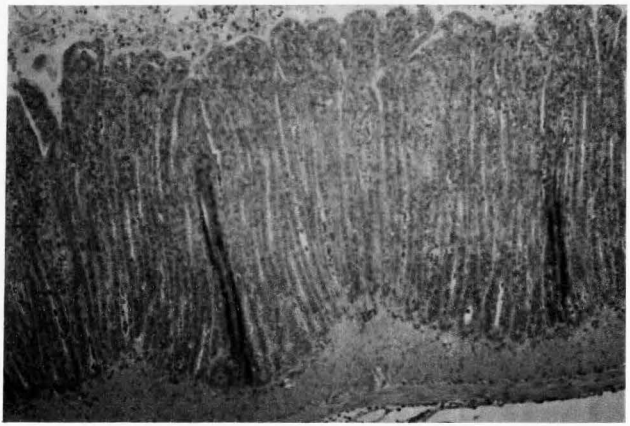

Figure 6. Photomicrograph of a mouse terminal ileum at 4 weeks postinfection showing *Cryptosporidium parvum*-induced villar fusion (100x).

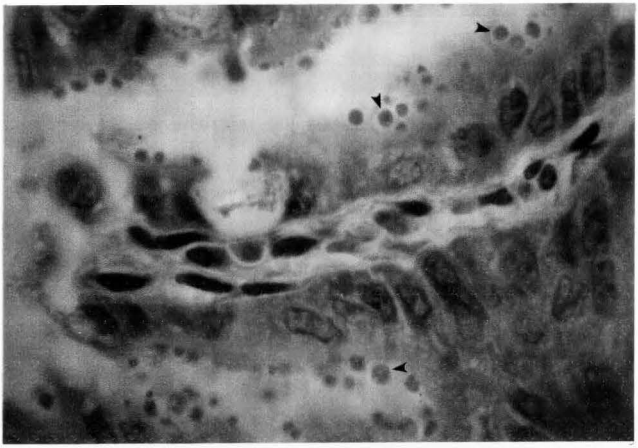

Figure 7. Photomicrograph of a single intestinal villus from a mouse ileum cononized by *Cryptosporidium parvum* (arrows). The villus has been shortened (stunted) due to colonization by the parasite (1000x).

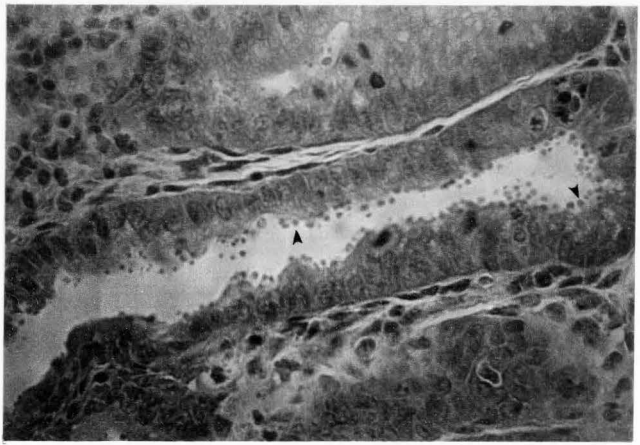

Figure 8. Photomicrograph of numerous *Cryptosporidium parvum* (arrows) colonizing a hyperplastic crypt of a mouse terminal ileum at 14 weeks postinfection (400x).

A follow-up to the pilot study was performed with modifications as described in the Experimental Design section. This experiment was designed to further characterize chronic cryptosporidiosis in a murine model. To prepare for the unexpected mortality that was experienced in the pilot study, animal numbers were increased as described in the experimental design. Table 7 shows the mortality (mice that died as opposed to being sacrificed) of the mice during the 4-month period. None of the mice from group-1 died. Only one mouse from group-2 died during the second week of the experiment and had a very low original body weight compared to the other mice. Therefore, group-1 and group-2 are not represented in Table 7. The majority (95%) of the mice receiving DEX-immunosuppression without being challenged with *C. parvum* (group-3) remained active throughout the entire experiment. Aggressive activity began to decline in this group at approximately six weeks post-immunosuppression. At this time, hair coats of some of the mice appeared dull. Loss of skin turgor and clinical signs of emaciation were observed. Four mice from this group died during the fourth month of the experiment.

Group-4 (mice receiving DEX i.p. + *C. parvum*) and group-5 (mice receiving DEX orally + *C. parvum*) were compared on the basis of mortality, oocyst shedding intensities and tissue colonization. Six mice from group-4 were still alive by the end of the 4-month experiment whereas all mice from group-5 died by the end of the third month p.i. Twenty-four (48%) of the mice in group-4 died prematurely (before being sacrificed) compared to 29 (61.7%) of the mice that died prematurely from group-5. Mice in both of these groups exhibited clinical signs of dehydration and anorexia as early as 2 weeks p.i. Other signs included icterus, wasting, and abdominal distention. Mice in group-5 exhibited much more acute deterioration compared to the more chronic deterioration observed in group-4.

Table 7. Mortality of mice in groups 3, 4, and 5 during the 4-month follow-up study of chronic cryptosporidiosis represented as deaths per week and percent death.

	Group-3 (DEX only)		Group-4 (DEX + Cp)		Group-5 (DEX + Cp)[a]	
Week p.i.	Deaths/ Week	Percent Death	Deaths/ Week	Percent Death	Deaths/ Week	Percent Death
1	0	0.0	0	0.0	1	2.1
2	0		0		0	
3	0		0		0	
4	0		0		0	
5	0		0		0	
6	0		0		1	4.3
7	0		6	12.0	0	
8	0		5	22.0	2	8.5
9	0		4	30.0	3	13.9
10	0		4	38.0	9	34.0
11	0		0		9	53.2
12	0		1	40.0	4	61.7
13	0		0		-	-
14	1	1.8	4	48.0	-	-
15	3	7.3	0		-	-
16	0		0		-	-
Total	4	7.3	24	48.0	29	61.7

[a] mice in group-5 received DEX in drinking water (8 µg/ml).
p.i., postinfection with *C. parvum* oocysts.
- indicates all mice had succumbed to infection/immunosuppression.

The oocyst shedding intensities in mouse feces were monitored on a weekly basis. The normal mice (group-1) as well as the DEX treated mice (group-3) remained negative for oocyst shedding throughout the experiment, ruling out cross-contamination by the parasite. Approximately 80% of the mice infected with *C. parvum* (group-2, group-4, and group-5) began shedding oocysts by day three p.i. By day 4 p.i., 100% of the mice in groups-2 and 4 were shedding oocysts. All mice in group-5 were shedding by day 6 p.i., indicating a delayed prepatent period in this group. The mice in group-2 (Cp only) ceased shedding by the end of the first week p.i. and remained negative for detectable

parasites throughout the remainder of the experiment. Figure 9 shows the oocyst shedding pattern for group-4 and group-5.

At the time of sacrifice, spleens were weighed as a general representation of the immune status of the mice. Spleen weights, along with the mouse weights, are shown in Table 8 as means ± SE. The number of mice (n) for groups 3, 4, and 5 represented in Table 8 shows a discrepancy from the (n) shown in Table 2 of the experimental design because some mice died before spleens and final body weights could be collected.

Table 8. Follow-up study of spleen and body weights of mice chronically infected with *Cryptosporidium parvum* represented as mean ± SE.

Group	n	Spleen Weight (mg)[a]	Body Weight (g)[a]
1 (Normal)	10	66.0 ± 3.4	20.5 ± 1.0
2 (Cp only)	20	69.5 ± 3.2	20.4 ± 0.6
3 (DEX only)	46	26.3 ± 1.5[bc]	20.6 ± 0.5
4 (DEX + Cp)	46	25.9 ± 1.6[bc]	18.7 ± 0.4[cd]
5 (DEX + Cp)[f]	43	29.8 ± 4.0[bc]	17.3 ± 0.4[bcde]

[a] values represent those data obtained on day of sacrifice.
[b] difference compared to group-1 is significant ($P \leq 0.01$).
[c] difference compared to group-2 is significant ($P \leq 0.01$).
[d] difference compared to group-3 is significant ($P \leq 0.01$).
[e] difference compared to group-4 is significant ($P \leq 0.05$).
[f] mice received DEX in drinking water (8 µg/ml) as opposed to intraperitoneally.

Microscopic examination of infected tissues showed similar findings as those described in the pilot study. Histological sections of the gastrointestinal tract had significantly higher parasite colonization in the ileum and terminal ileum when compared with the duodenum, jejunum, cecum, and colon. Intestinal parasite colonization on a bimonthly basis is shown in Figures 10A and 10B for group-4 and group-5, respectively. Group-4 had fewer detectable parasites in the duodenum, jejunum, cecum, and colon compared with the colonization of the same sections in group-5.

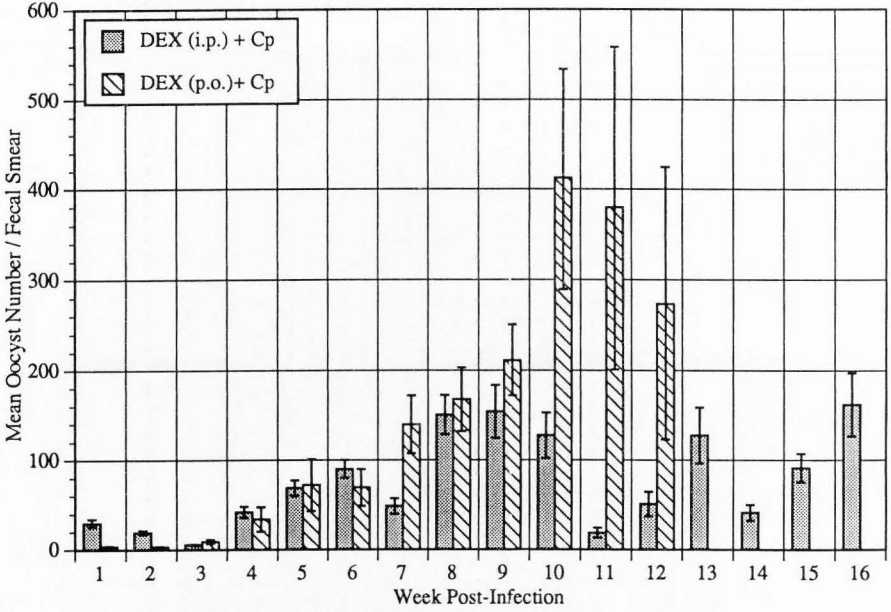

Figure 9. Oocyst shedding intensities comparing mice immunosuppressed with dexamethasone (DEX) administered intraperitoneally (i.p.) and orally (p.o). All mice receiving DEX orally died by week 12 postinfection with *C. parvum*.

Fig. 10A

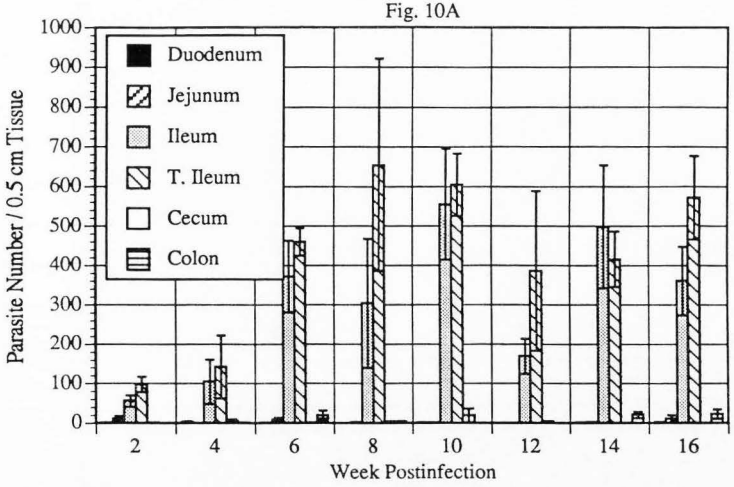

Fig. 10B

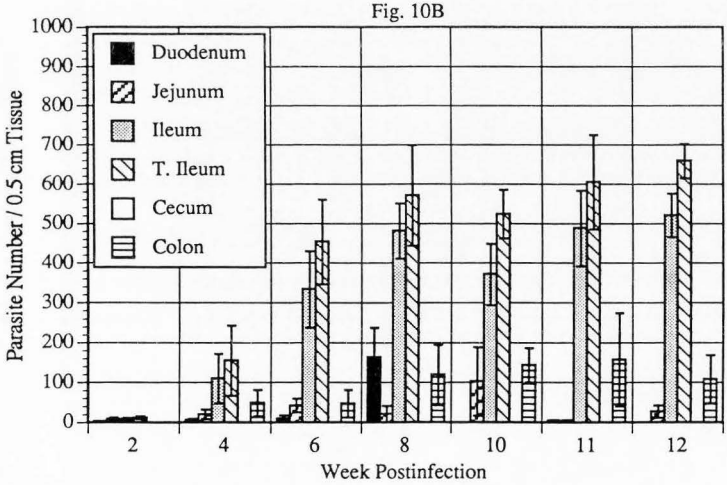

Figure 10. Intestinal colonization by *Cryptosporidium parvum* comparing mice immunosuppressed with dexamethasone administered intraperitoneally (Fig. 10A) and orally (Fig. 10B).

Figure 11 is a pooled comparison for groups 4 and 5, of *C. parvum* organisms detected in the intestinal sections throughout the entire experiment. Again, the ileum and terminal ileum were the most heavily parasitized with the intensity of infection decreasing significantly ($P \leq 0.01$) both proximal and distal. Group-5 had a significantly ($P \leq 0.05$) higher colonization in the duodenum, jejunum, and colon when compared to group-4. The infection in group-5 was more heavily disseminated throughout the intestinal tract compared to group-4. No parasites were observed in the cecum of group-5 and very few were detected in the cecum of group-4. All cecal sections from group-4 were disected from the ileal-cecal junction, whereas cecal sections from group-5 were from the tip of the cecum.

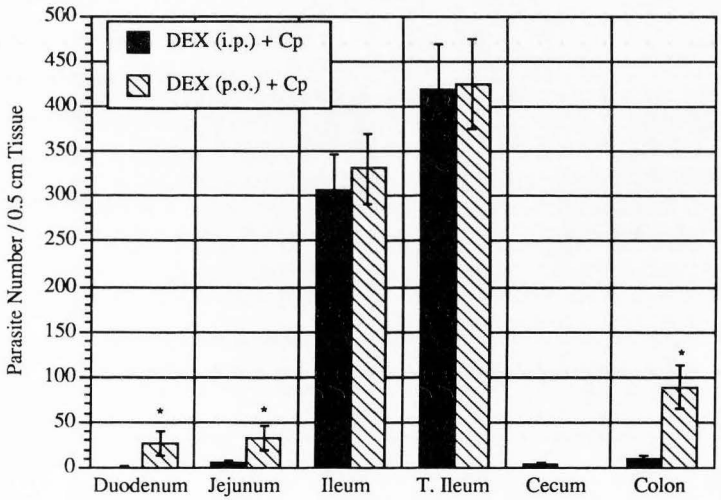

Figure 11. Pooled intestinal colonization by *Cryptosporidium parvum* comparing mice immunosuppressed with dexamethasone (DEX) administered intraperitoneally (i.p.) and orally (p.o.). * indicates a significant difference when compared to group-4 (DEX i.p. + Cp) for each respective tissue section ($P \leq 0.05$).

Throughout the experiment, parasites were detected in the gallbladder (Figure 12) and pancreatic ducts (Figure 13). Most of the mice examined demonstrated normal (noninfected) pancreatic and hepatobiliary systems. In group-4, five mice had *C. parvum* in the gallbladder with a score of 1+ and one mouse had *C. parvum* in the pancreatic duct with a score of 2+. In group-5, two mice had *C. parvum* in the gallbladder, one with a score of 1+ and the other with a score of 2+. A total of six mice in group-5 had parasites in the pancreatic duct with two scoring 1+, three scoring 2+ and one scoring 3+. For a description of the scoring scale, see Materials and Methods.

Histopathologic findings were similar to those observed in the pilot study. Gross observations at necropsy included hyperemia and distension of the terminal small intestine and colon. Microscopically, the upper small intestine, cecum, and colon were normal. Villi of the ileum and terminal ileum were blunted and thickened, especially as the infection progressed. Heavy parasite colonization was associated with villar fusion and infiltration of the lamia propria with large numbers of neutrophils. Crypts were hyperplastic, deepened, and lacked paneth cells, a finding that has been described previously (123).

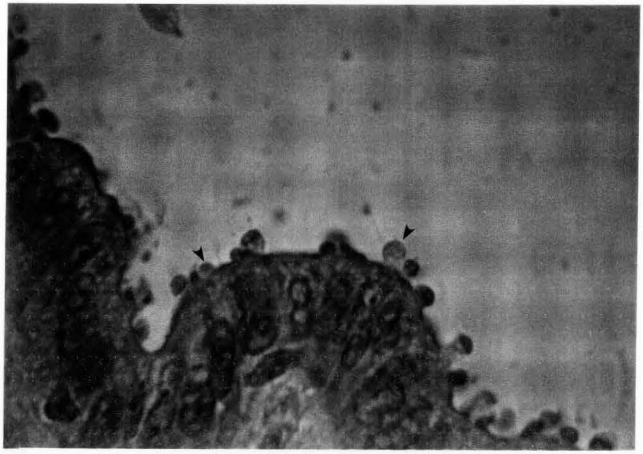

Figure 12. Photomicrograph of *Cryptosporidium parvum* (arrows) colonizing the gallbladder of a mouse with a chronic infection (1000x).

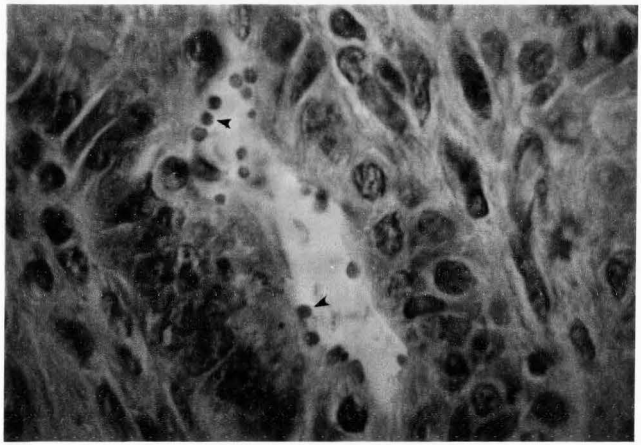

Figure 13. Photomicrograph of *Cryptosporidium parvum* (arrows) colonizing the pancreatic duct of a mouse with a chronic infection (1000x).

Effects of *Cryptosporidium parvum* and Dexamethasone on B and T Lymphocyte Proliferation. Lymphocyte functionality following DEX-immunosuppression and *C. parvum* inoculation was determined by examining the blastogenic response of B lymphocytes and T lymphocytes to the mitogens LPS and ConA, respectively. The optimal concentration of both mitogens, as determined by the stimulation induced in normal spleen cells, was 2.5 μg/ml. However, the difference between the concentrations was not significant and all three concentrations for both mitogens are reported for the purpose of emphasizing trends. Proliferative response is represented as mean counts per minute (CPM) as described in the Materials and Methods.

Figure 14 illustrates the blastogenic response of B lymphocytes to the mitogen LPS at 1 week (Fig. 14A) and 2 weeks (Fig. 14B) p.i. There was no statistical significance between groups 2-4 (Cp only, DEX only, and DEX + Cp, respectively) when compared to group-1 (Normal) for the first week p.i. with the exception of group-3 at 2.5 μg/ml LPS ($P \leq 0.05$). For week 2 p.i., there was a significant ($P \leq 0.05$) difference when comparing group-3 to group-1 at the lowest LPS concentration. Also, at 2 weeks p.i. there was a significant ($P \leq 0.05$) difference between group-4 and group-1 at 5 μg/ml LPS and a highly significant ($P \leq 0.01$) difference between group-4 and group-1 at 2.5 μg/ml LPS. Group-3 had a significantly ($P \leq 0.05$) lower blastogenic response at 2 weeks p.i. compared to 1 week p.i. at 10 μg/ml LPS.

The blastogenic response of T lymphocytes to ConA at 1 week (Fig. 15A) and 2 weeks (Fig. 15B) p.i. is shown in Figure 15. Again, there was no statistical significance between any of the groups at 1 week p.i. However, at 2 weeks p.i. there was a significant ($P \leq 0.05$) difference between groups 3 and 4 when compared to both groups 1 and 2 at 2.5 μg/ml ConA. Group-2 had a significantly ($P \leq 0.05$) higher blastogenic response at 2 weeks p.i. at 2.5 μg/ml ConA compared to the response at 1 week p.i.

Fig. 14A

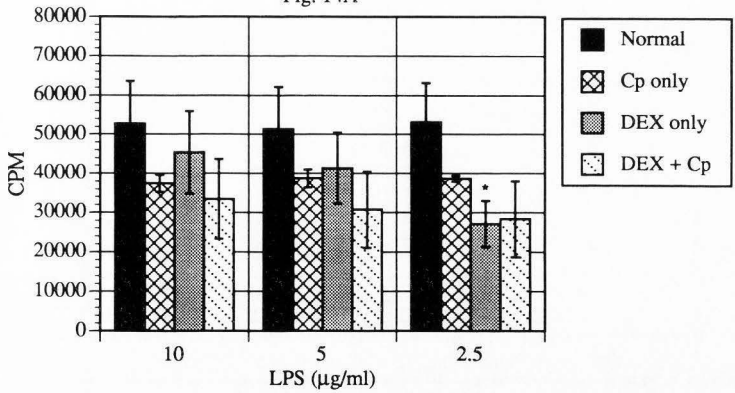

Fig. 14B

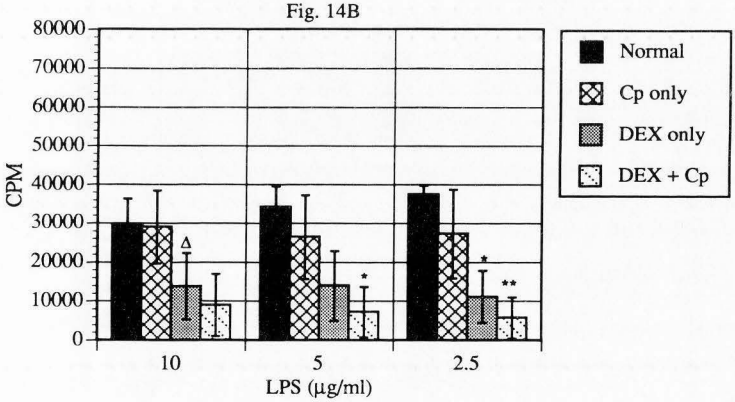

Figure 14. Lipopolysaccharide-induced incorporation of tritiated thymidine by mouse splenocytes expressed as counts per minute (CPM) and standard error of the mean (error bars). Fig. 14A represents results at 1 week postinfection and Fig. 14B represents results at 2 weeks postinfection. * indicates difference compared to normal controls is significant at $P \leq 0.05$ whereas ** indicates difference compared to normal controls is significant at $P \leq 0.01$. Δ indicates difference compared to same treatment group at 1 week postinfection is significant ($P \leq 0.05$).

Fig. 15A

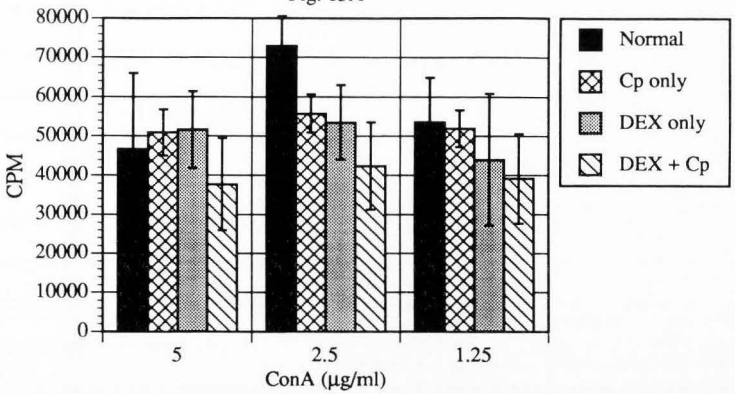

Fig. 15B

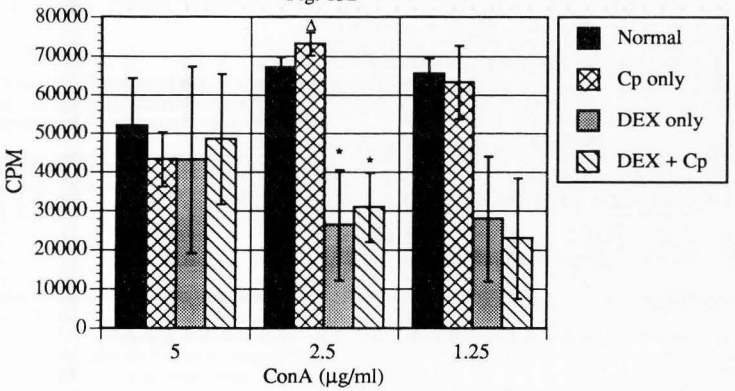

Figure 15. Concanavalin A-induced incorporation of tritiated thymidine by mouse splenocytes expressed as counts per minute (CPM) and standard error of the mean (error bars). Fig. 15A represents results at 1 week postinfection and Fig. 15B represents results at 2 weeks postinfection. * indicates difference compared to normal controls and group-2 (Cp only) is significant ($P \leq 0.05$). Δ indicates difference compared to same treatment group at 1 week postinfection is significant ($P \leq 0.05$).

Effects of Dehydroepiandrosterone on Oocyst Shedding Intensities of Mice Infected with *Cryptosporidium parvum*. Originally, 51 mice were DEX-immunosuppressed, infected with 10^6 oocysts/mouse, and continued on DEX-immunosuppression. However, one mouse died 5 days postimmunosuppression and thirteen more died following *C. parvum* inoculation before DHEA or DHEAS treatment began. Nine of the 13 mice died within the first week following parasite challenge as a result of a punctured esophagus (confirmed by necropsy) which occurred during *C. parvum* intragastric inoculation. Consequently, only 37 mice were used in the final study.

Table 9 shows the number of mice that died from each treatment group during either weeks 1, 2, 3, or 4 posttreatment with DHEA or DHEAS. The remaining mice were sacrificed at the end of the 4-week treatment period. Some of the routes and dosages of DHEA and DHEAS appeared to be toxic, as determined by mortality.

Table 9. Number of mice in each treatment group that died per week during four weeks of dehydroepiandrosterone (DHEA) or dehydroepiandrosterone-sulfate (DHEAS) treatment for chronic cryptosporidiosis.

Vehicle (Route)		Peanut Oil (p.o.)			Propylene Glycol (s.c.)			Sterile Water (s.c.)		
Week P.T.[a]	Control	50 µg DHEA	150 µg DHEA	300 µg DHEA	3 mg DHEA	6 mg DHEA	12 mg DHEA	3 mg DHEAS	6 mg DHEAS	12 mg DHEAS
1	0	0	0	1	4	2	1	0	0	2
2	0	1	1	0	0	0	3	0	1	1
3	0	0	0	0	-	0	-	0	0	-
4	0	0	3	0	-	2	-	3	2	-
Total	0	1	4	1	4	4	4	3	3	3

[a] P.T., posttreatment with DHEA or DHEAS; p.o = per os; s.c. = subcutaneous.
- indicates all mice in the respective group succumbed to treatment.
DHEA = dehydroepiandrosterone; DHEAS = DHEA-sulfate.

Following parasite challenge, mice from all groups were randomly sampled on a daily basis to ensure that all mice had *C. parvum* infection before DHEA or DHEAS treatment began. The number of detectable oocysts in all treatment groups (including controls) varied considerably throughout the experiment, a common finding with *C. parvum* oocyst shedding.

The shedding intensities in the mice treated with DHEA (50, 150, or 300 μg) orally in a peanut-oil vehicle (Figure 16) showed no significant decrease when compared to controls. Ironically, the mice treated with 300 μg of DHEA had a significantly ($P \leq 0.01$) higher shedding intensity on day 14 of treatment when compared to controls. Moreover, there was a significant ($P \leq 0.05$) increase in oocyst numbers detected on day 24 of treatment when mice receiving 50 μg DHEA were compared to controls.

In mice treated s.c. with DHEA (3, 6, and 12 mg) suspended in propylene glycol (Figure 17), there was no significant decrease in the oocyst shedding intensities when compared to the control mice. There was, however, a significant ($P \leq 0.05$) increase in shedding intensity in mice that received 6 mg DHEA compared to controls on day 14 of treatment. By this time, all mice receiving 3 and 12 mg DHEA had died. The propylene glycol vehicle was tissue toxic at the site of injection, resulting in scab formation and open sores as early as day 3 posttreatment. When the mice were necropsied following death or sacrifice, local deposits of DHEA were found under the skin near the injection sites of mice receiving 6 mg and 12 mg DHEA. Similar findings were reported by Loria et al. (67) when DHEA was administered s.c. in a suspension of dimethyl sulfoxide, suggesting that this local accumulation may lead to prolonged DHEA interaction with the lymphoid system.

The mice treated s.c. with DHEAS (3, 6, and 12 mg) in deionized water also failed to show any significant decrease in shedding intensity compared to control mice (Figure 18).

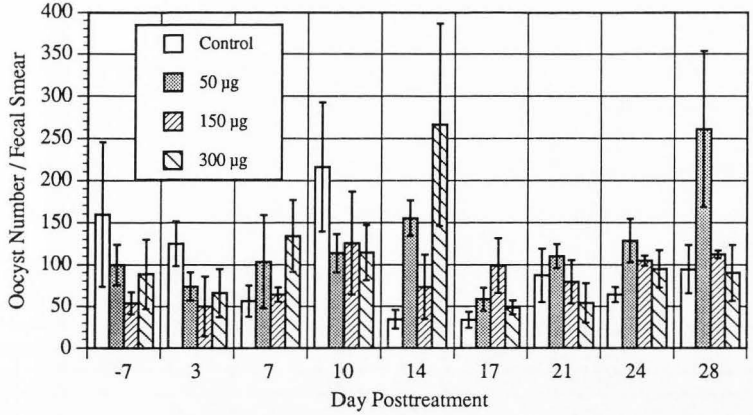

Figure 16. Oocyst shedding intensities of control mice compared to mice treated therapeutically with 50, 150, or 300 µg of dehydroepiandrosterone administered orally in 100 µl of a peanut-oil vehicle.

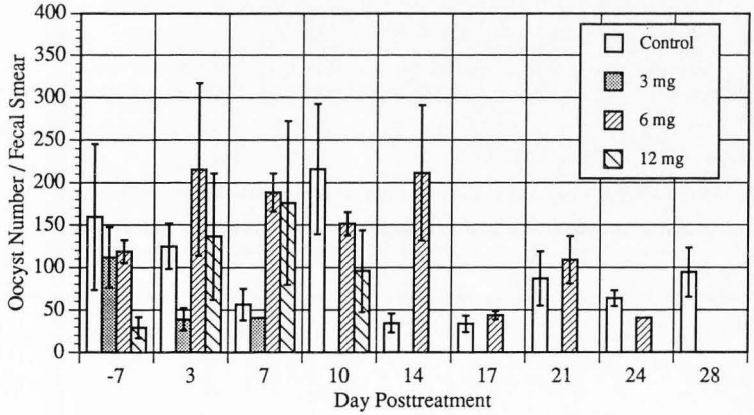

Figure 17. Oocyst shedding intensities of control mice compared to mice treated therapeutically with 3, 6, or 12 mg of dehydroepiandrosterone administered subcutaneously in 250 µl of a 100% propylene glycol vehicle.

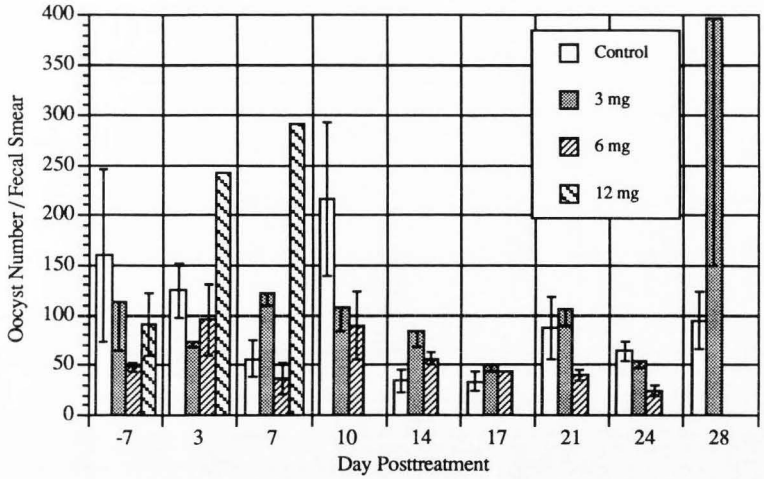

Figure 18. Oocyst shedding intensities of control mice compared to mice treated therapeutically with 3, 6, or 12 mg of dehydroepiandrosterone-sulfate administered subcutaneously in 250 µl of a sterile, deionized water vehicle.

As demonstrated in Table 9, two of the three mice receiving 12 mg of DHEAS died 1 day following treatment and the remaining mouse died 1 week later, suggesting possible DHEAS toxicity at this dosage. All mice receiving 3 and 6 mg DHEAS survived until the fourth week of treatment, with the exception of one mouse receiving 6 mg which died during the second week of treatment.

At sacrifice, mice were examined internally and externally for gross abnormalities. Those mice receiving DHEA or DHEAS had almost no fatty deposits around their kidneys and terminal colon. In contrast, the control mice had large accumulations of fatty

material at both of these sites, which is a common finding in mice receiving prolonged GC therapy.

At the time of sacrifice, spleens were collected and weighed as a general representation of the immune status of the mice. Table 10 presents the average spleen and body weights of each respective group at the time of sacrifice. Groups-3b and 4a had significantly higher spleen weights when compared to group-1 ($P \leq 0.05$) and group-3a ($P \leq 0.01$). Group-3b also had a significantly ($P \leq 0.05$) higher spleen weight compared to group-3c. Furthermore, group-2b were the only mice with body weights significantly ($P \leq 0.05$) lower than group-1.

Table 10. Spleen and body weights of control mice and mice treated therapeutically with dehydroepiandrosterone (DHEA) or dehydroepiandrosterone-sulfate (DHEAS) for chronic cryptosporidiosis.

Group	n	Treatment (dose)	Route	Spleen Weight (mg)[a]	Body Weight (g)[a]
1	4	None (controls)	None	27.5 ± 4.8	17.2 ± 0.8
2a	4	DHEA (50 µg)	p.o.	32.5 ± 4.8	17.1 ± 1.2
2b	4	DHEA (150 µg)	p.o.	25.0 ± 5.0	13.6 ± 0.6[b]
2c	4	DHEA (300 µg)	p.o.	26.9 ± 2.4	17.1 ± 0.8
3a	4	DHEA (3 mg)	s.c.	20.0 ± 4.1	16.5 ± 0.3
3b	4	DHEA (6 mg)	s.c.	45.0 ± 11.9[bcd]	17.2 ± 1.9
3c	4	DHEA (12 mg)	s.c.	20.0 ± 7.1	16.6 ± 0.7
4a	3	DHEAS (3 mg)	s.c.	50.0 ± 11.5[bc]	15.4 ± 0.2
4b	3	DHEAS (6 mg)	s.c.	35.0 ± 8.7	16.3 ± 0.6
4c	3	DHEAS (12 mg)	s.c.	27.5 ± 0.0	15.1 ± 0.2

[a] values represent those data obtained on day of sacrifice.
[b] difference compared to group-1 is significant ($P \leq 0.05$).
[c] difference compared to group-3a is significant ($P \leq 0.01$).
[d] difference compared to group-3c is significant ($P \leq 0.05$).
p.o = per os; s.c = subcutaneous; DHEA = dehydroepiandrosterone; DHEAS = DHEA-sulfate.

DISCUSSION

Characterization of Chronic Cryptosporidiosis in Immunosuppressed Mice. The results of the present study suggest that the DEX-immunosuppressed C57BL/6N mouse will provide a reliable laboratory animal model for evaluating chronic *C. parvum* infections. The utility of DEX as an effective immunosuppressent inducing susceptibility to *C. parvum* has been supported by Rasmussen et al. (92, 94) and Rheg et al. (98). Furthermore, the adult C57BL/6N mouse has been described previously as being susceptible to *C. parvum* following immunosuppression (92). Our results show that the C57BL/6N mouse can be successfully immunosuppressed with DEX, be infected with *C. parvum* oocysts, and maintain an infection for at least 4 months. The chronicity of the infection was determined by evaluating infection intensity as represented by oocyst excretion in the feces, parasite distribution in the tissues, and parasite induced-pathology.

The infection intensity was very sensitive to the amount of DEX administered, a finding that has been reported previously (98). As the DEX regimen was reduced from daily dosing to dosing ever other day, the infection intensity decreased rapidly (Figure 2). This indicates that 1) the DEX was rapidly removed from the circulation of the mice, and 2) the immune system was not totally ablated by the first 2 months of DEX administration. The idea that DEX was removed quickly from circulation would need to be confirmed by specific assays used to determine plasma levels and excretion rates of DEX over a given time interval in this particular animal model. These assays were not carried out in the present study and merit further investigation. The decrease in DEX that was administered and consequent reduction in infection intensity emphasizes the important role DEX plays in a properly immunosuppressed mouse model for studying chronic cryptosporidiosis.

The presence of high levels of DEX in the circulation stimulates the synthesis of excess concentrations of corticosteroid binding globulin (CBG) (70). This protein

globulin binds with very high affinity to GC's in the blood, thus inhibiting the migration of the GC's to their target tissues. Since only unbound GC's readily gain access to their target tissue receptors, the elevated CBG levels not only make it possible to have high circulating GC's without incurring toxic effects, but also increase the availability of the GC's for degradation by the liver.

Specifically, DEX is known to be an inducer of the cytochrome P-450 system in liver cells, which is responsible for the detoxification and degradation of foreign substances like exogenous drugs (17) such as DEX. The P-450 system converts the lipid-soluble DEX into a water-soluble substance that has little or no biological activity (70). This substance is then readily excreted via the bile and/or urine. Furthermore, because the P-450 system was activated by the DEX and the liver cells had an increased capacity to degrade the DEX, it is not surprising that when the DEX-administration was decreased, the efficiency of the P-450 system resulted in a rapid clearance of the DEX from the mice.

The half-life of DEX is significant when considering excretion rates in different hosts. Studies in mammals immunosuppressed with DEX suggest a half-life of 3 to 9 hours, with a duration of action lasting up to 72 hours (20, 51). The short half-life supports our findings that the DEX was rapidly excreted when given every other day. It also indicates that under the conditions of the present study, administering DEX at 125 µg/mouse/day on a daily basis was an appropriate regimen for inducing a chronic infection without inducing acute signs of toxicity. If one intends to examine cryptosporidiosis over an extended period (4 months) in the C57BL/6N mouse model, it is recommended that the initial dose of DEX be decreased rather that altering the amount in the middle of the experiment. Further studies in our laboratory suggest that decreasing the DEX dosage may yield more optimal results and thus increase the utility of this animal model for evaluating chronic cryptosporidiosis.

It is probable that the chronic use of DEX does not totally destroy the host's immune system. This is an important consideration when attempting to relate this mouse model to individuals harboring *C. parvum* who also have AIDS or are receiving chemotherapy. The mice in the present study were able to reduce the oocyst shedding intensity and the parasite colonization in the tissues when the DEX was decreased (Figures 2 and 4). This finding indicates that the immune cell populations responsible for clearing *C. parvum* infections were still able to be restored once the immunosuppressive effects of DEX were decreased. This suggests promise for AIDS patients and other immunocompromised individuals exhibiting fulminate cryptosporidiosis. In these patients, the use of immunomodulators to boost the immune system may facilitate a decrease in intensity and/or a more rapid clearance of the infection. The infection intensity in human hosts harboring *C. parvum* is dependent upon the immune status of the host. Patients receiving exogenous immunosuppression and exhibiting chronic cryptosporidiosis quickly cleared the infection once the source of immunosuppression was withdrawn (39). Because of the inhibitory effects DEX has on antigen presentation by the macrophage and lymphocyte proliferation, it is likely that when the immunosuppression is removed or decreased, the cascade of events associated with antigen presentation is reinitiated (51).

The cascade of events inhibited by DEX includes the release of interleukin-1 (IL-1) and tumor necrosis factor from macrophages, the release of interleukin-2 (IL-2) and gamma-interferon from activated T lymphocytes, and the actions of migration-inhibitory factor (MIF) and gamma-interferon on macrophages (51). Previous studies suggest an important role for T lymphocytes, IL-2, and gamma-interferon in fighting cryptosporidiosis (55, 75, 122, 123). A decrease in exogenous immunosuppression as was seen in the present study, and the subsequent decrease in oocyst shedding intensity and tissue colonization by *C. parvum*, correlate well with previous findings describing

the interrelationships between immune status and persistent *C. parvum* infections in animals and humans. Our findings agree with other research which has shown that if the source of immunosuppression is removed or overcome in patients with chronic cryptosporidiosis, the infection intensity decreases (39, 98).

We used spleen weights as a general representation of the overall immune status of the mice. In a recent study, Pabst et al. (89) found that more lymphocytes migrate to the spleen than any other lymphoid organ. Whether or not there is a direct correlation between spleen weights and immune status of the host has not been described to our knowledge. We found that the more active mice were those that generally had the largest spleens, which indicates a possible correlation between overall health status and spleen weights. Our observations also suggested that the mice with the greatest oocyst shedding intensities and tissue colonization by *C. parvum* had the smallest spleens. In both the pilot and follow-up studies examining chronic cryptosporidiosis, mice challenged with *C. parvum* without being immunosuppressed had the greatest spleen weights at the time of sacrifice. Although the difference compared to normal mice was not significant, it indicates that *C. parvum* may be initiating an active immune response by the non-immunosuppressed host, resulting in an increase in lymphocyte numbers. This response may be useful in identifying the immune parameters important for inducing resistance to *C. parvum* or in examining a latent infection. Relating this response to the function of memory cell involvement in inducing resistance is another area requiring further investigation. An increase in immune status was also evident as was determined by the spleen weights of mice sacrificed before, during, and after the DEX regimen was altered. The mice receiving DEX alone and mice receiving DEX and *C. parvum* challenge had a 1.5-fold and a 2.5-fold increase in spleen weight, respectively, when the DEX was reduced. Although specific lymphocyte populations responsible for the increased spleen weights were not assessed, studies using immunomodulators to boost immune

parameters in mice infected with *C. parvum* have demonstrated a significant increase in total T lymphocyte populations in the spleen (unpublished data). This finding further emphasizes the protective importance of the CMI branch of the immune system in cryptosporidial infections. Because the C57BL/6N mouse has a fully intact immune system, using this mouse as a model for chronic cryptosporidiosis would readily facilitate the elucidation of specific immune responses involved in resistance to cryptosporidiosis.

The body weights of the mice in the pilot and follow-up studies were compared as was the overall appearance and activity of the mice. Normal mice and mice challenged with *C. parvum* without DEX-immunosuppression had a gradual increase in body weight throughout the experiments. Mice in these two groups remained active and were quite aggressive when handled. Mice immunosuppressed with DEX and challenged with *C. parvum* were more unhealthy and less active when compared with the other treatment groups. The body weights of DEX-treated mice (with or without *C. parvum*) were generally lower than the body weights of non-DEX-treated mice, but this observation was not always the case (Tables 5 and 8). When mice were initially immunosuppressed, their overall body weights decreased by approximately 20% (3 to 4 grams) during the first 2 to 3 weeks of immunosuppression. Toward the end of the experiment, particularly in the follow-up study, mice receiving DEX without *C. parvum* had body weights comparable to normal controls. This increase in body weight was attributed to obesity and water retention induced by the DEX and was not an indication of increased health status. Chronic DEX administration resulted in accumulation of fat deposits in the face, neck, and peritoneal cavity of the mice. This finding is described as a common side effect of long-term, high-dose DEX-immunosuppression in mammals, causing a condition known as Cushing's Syndrome (70). As a consequence, some of the mice receiving DEX had larger body weights due to excess accumulation of fat. Mice that received DEX and *C. parvum* maintained significantly lower body weights throughout

the study when compared with the other treatment groups. This indicates that the presence of the parasite in an immunosuppressed host, rather than the immunosuppression alone, results in deterioration of the mice. Mice with DEX and *C. parvum* had very little fat deposition compared to mice treated with DEX alone. The DEX-treated mice infected with *C. parvum* were more anorexic and their fat deposits were probably depleted by the energy expended in combating the parasitic infection.

The route of DEX-administration (intraperitoneal vs. oral) yielded different results with respect to animal health, mortality, infection intensity and tissue colonization. Intraperitoneal administration (125 µg/mouse/day) and oral administration (8 µg/ml in the drinking water *ad libitum*) were both successful in inducing susceptibility to *C. parvum* after 12 days of immunosuppression. The DEX administered i.p. was the only route successful in maintaining a 4-month chronic infection once the mice were inoculated with infective oocysts, as determined by excretion of oocysts in the feces and tissue colonization by the parasite (Figures 2, 9 and 10; Table 6). Monitoring clinical signs and examining shedding intensities in the feces were possible for a longer period of time when DEX was administered i.p. Moreover, a more consistent shedding pattern in mice given DEX i.p. over a longer course of infection will enable better delineation and characterization of therapeutic interventions under the experimental conditions employed in this study. The utility of administering DEX i.p. in the assessment of chronic cryptosporidiosis is further supported by the lack of significant evidence indicating toxicity following chronic therapy (Table 7). Follow-up studies in our laboratory have shown that fewer than 12 days of i.p. DEX administration are required to induce susceptibility to *C. parvum* in this mouse model (unpublished data). These findings support the C57BL/6N mouse model for its use in screening therapeutics. Continuing studies in our laboratory are examining different formulations and routes for administering DEX to this mouse model.

Daily i.p. injections are labor intensive and cause undue stress to mice which may result in stress-induced immunosuppression. Oral administration of the DEX was assessed in the present study as a possible alternative to i.p. administration. Oral administration of DEX to induce susceptibility to *C. parvum* in rats has been described previously (94, 98). It is much more practical and less time consuming to administer the DEX orally. The majority of human patients receiving DEX-induced immunosuppressive therapy are administered DEX orally because the drug is resistant to degradation by the intestinal enzymes (51). Oral DEX administration resulted in higher numbers of parasites detected in sections of the duodenum, jejunum, cecum, and colon (Figure 10) and an increase in the number of mice with pancreatic infections compared to mice given DEX i.p. The DEX given orally may have a detrimental effect directly on the intestinal mucosa, thus making the mucosa more susceptible to invasion by *C. parvum* (1, 106). Subcutaneous administration of DEX has been shown to cause an increase in bacterial adherence to intestinal cells (1). The DEX administered orally may also destroy the intestinal microflora, increasing the susceptibility of the enterocytes to invasion by *C. parvum*, a finding that has been previously reported (53). Damage to intestinal mucosa and/or destruction of intestinal flora resulting in an increase in infection intensity would explain the increased parasite colonization in the intestinal sections and would agree with previous findings suggesting the involvement of non-specific immune responses to *C. parvum* (53). Pathologic findings comparing mice immunosuppressed with DEX i.p. to mice immunosuppressed orally failed to show any significant difference in lesions or decrease in lymphoid regions between the two groups. Cytochemical analysis of intestinal cells from mice receiving DEX orally compared with mice receiving DEX i.p. may further explain the direct effects of DEX on intestinal cells.

In the present study, oral administration of DEX was associated with a number of disadvantages. It is difficult, if not impossible, to control the amount of DEX each

mouse receives on a daily basis. Because the mice had free access to the water and evidence suggests that mice in the same cage drink different amounts of water (unpublished data), it was concluded that mice in the same cage were getting different amounts of DEX. Also, polydipsia (excessive drinking) is a common side effect of GC administration (62). As a result, the mice would drink increased amounts DEX, the DEX would induce more water consumption, and the mice would, in a sense, overdose on the DEX. This could explain the acute signs of deterioration as represented by the significantly lower body weights (Table 8) and much higher mortality rates (Table 7) over a shorter period observed in mice receiving DEX orally . Furthermore, it is also probable that some of the mice would drink excess DEX in the water, become moribund, have decreased activity, and consequently decrease their water consumption. This, in turn, would decrease the immunosuppression of the mice and the infection intensity would rapidly diminish as is already evident from the pilot study and other studies (98). Consequently, the mice would have such large fluctuations in infection intensity that it would be difficult to use this type of a model in drug screening studies.

Oocyst shedding intensities were affected by the route of immunosuppression. Oral administration of DEX was initially associated with a delay in oocyst shedding and then a rapid increase in shedding until all mice died (Figure 9). Some mice did not begin shedding until day 6 postinfection, indicating that it would take longer to screen drugs using this model. Fluctuations in shedding intensities yielded much higher variability between individual mice immunosuppressed with DEX in the drinking water compared with mice immunosuppressed with DEX i.p. To account for the variability in shedding, and to compensate for the rapid death of mice receiving DEX orally, it would be necessary to dramatically increase the number of mice used for a chronic study, thus decreasing the practical use of an animal model receiving DEX in the drinking water.

The prepatent period for *C. parvum* in the C57BL/6N mouse model ranged from 3 to 6 days, depending upon the route of immunosuppression. A prepatent period of 3 to 6 days is similar to the prepatent period reported by Tzipori et al. (120) for neonatal mice and rats. There appeared to be a correlation between the oocyst shedding intensity in the feces and the parasite colonization in the ileum. The oocyst shedding pattern over time (Figure 2) paralleled the pattern of parasite colonization in the ileum (Figure 4). This finding supports the use of the oocyst shedding pattern as a good representation of infection intensity.

Histopathologic findings in the C57BL/6N mouse model demonstrated parasite colonization throughout the entire intestinal tract as well as in extraintestinal sites. Parasites were present in the upper duodenum near the pyloric ring adjacent to the mucus secreting Brunner's glands. However, very few parasites were found in the terminal duodenum. A similar finding involving the pyloric ring was described by Ungar et al. (122). There may be some secretory products associated with the upper duodenum that facilitate the survival of *C. parvum* in this region but not in the terminal duodenum. It may be that the mucus secretions from the Brunner's glands or other secretory cells alter the intestinal environment enough to allow the oocysts to excyst in the upper duodenum and initiate the life cycle of *C. parvum*. It is also possible that pancreatic and/or bile secretions released into the lumen of the duodenum create an environment compatible for *C. parvum* growth because of a decrease in pH. *In vitro* studies have shown that *C. parvum* is sensitive to pH under some conditions and resistant under others (39). The development of a reliable *in vitro* culture system may help to answer questions regarding the sensitivity of *C. parvum* to various environmental conditions. The jejunum was seldom colonized heavily unless the parasites in the terminal small intestine were quite numerous. This indicates that *C. parvum* may have an ability to migrate to other sites when the competition for intestinal cells in the terminal small intestine is too demanding.

Intestinal tropism of *C. parvum* in rats has been described previously (97). The ileum and terminal ileum were the most heavily colonized intestinal sections in the present study. Similar results in mice and rats infected with *C. parvum* have been reported by others (75, 94, 98).

During the early stages of infection, *C. parvum* was found predominantly along the sides and tips of the villi. As the infection progressed, more parasites were found deep within the crypts. The parasites would destroy the epithelial cells in the ileum so rapidly that the villi became fused and shortened, while the crypts became deeper and hyperplastic. This ultimately resulted in a decrease in the villus-to-crypt ratio and more parasites had access to the epithelial cells of the crypts. Villar fusion and crypt hyperplasia were seen only in the ileum and terminal ileum, undoubtedly due to the infection intensity in this region compared to other intestinal sites. The reason *C. parvum* colonizes this region of the intestine in greatest numbers is not known. Anatomical obstruction at the ileal-cecal junction may play a role, but this has not been described previously. It may be that receptors for *C. parvum* are present in the intestines and are much more numerous in the terminal small intestine compared to other intestinal sites. In the present study numerous parasites were found associated with Peyer's patches in mice receiving DEX-treatment, while fewer parasites were found more distal to the Peyer's patches. This may be a preferred site for receptors specific for *C. parvum*. Intact and partially digested *C. parvum* have been identified in macrophages beneath M-cells (membrane-like epithelial cells overlying Peyer's patches) (26). Peyer's patches and adjacent M-cells are believed to play a role in introducing *C. parvum* to the immune system of the host (26, 69). The fact that the mice in our studies were immunosuppressed may explain why the lymphocytes associated with Peyer's patches and the M-cells were not functioning in the removal of *C. parvum* from these sites in the ileum. The immunological response to *C. parvum* in the ileum may be mediated by other

unknown factors not found in other regions of the intestines. These factors may be immobilized by toxins produced by the parasite, enterotoxins originating in the intestines, or by the exogenous immunosuppression, whether it be DEX, HIV-virus, or some other source (39). The phenomena regarding the immunological response to *C. parvum* are not clear and deserve further investigation. This mouse model should provide a reliable model for investigating the immune response to *C. parvum.*

In the two chronic studies conducted, *C. parvum* was found in the lungs, pancreatic ducts, and gallbladder. Respiratory, pancreatic, and biliary involvement by *C. parvum* as seen in this mouse model has been documented in humans (49, 82) and only recently in other animal models (75, 122). Only one mouse in the present study had detectable parasites in the lungs (Table 6). The parasites could have been inhaled during the initial intragastric inoculation of oocysts. This finding does suggest, however, that the mouse model as described in these studies could be beneficial in examining respiratory infections, an important condition in AIDS patients with chronic cryptosporidiosis (82). The presence of *C. parvum* in the pancreatic ducts and gallbladder indicates that the parasite must be able to migrate to these extraintestinal sites. The mode of migration may be mediated by mobile cells of the immune system, such as macrophages, that have specific receptors which recognize *C. parvum* and facilitate transport. Furthermore, the environment associated with pancreatic and bile secretions appears to be conducive for the growth and development of *C. parvum*. This observation is based on our findings which demonstrated large numbers of parasites associated with almost every available epithelial cell in some gallbladder and pancreatic duct infections. An earlier study suggests that chronic immunosuppression with GC's alters the electrolyte balance in bile secretions, which may also play a role in creating an environment that supports *C. parvum* development in the gallbladder (18).

The DEX-immunosuppressed C57BL/6N mouse shows promise as a reliable model for studying chronic cryptosporidiosis. The pathogenesis of infection and the immune response of the mouse can readily be examined using this model. The disease in humans is comparable to the disease described in this mouse model in that the distribution of the parasite in both hosts involves similar intestinal and extraintestinal sites. In AIDS patients, histological documentation of extraintestinal cryptosporidiosis associated with respiratory, pancreatic, and biliary infestations by *C. parvum* have been increasingly reported (50, 122). Similar findings have also been reported in immunocompetent patients (122). Because of the chronic infection that is established in DEX-immunosuppressed C57BL/6N mice, and because of the close resemblance *C. parvum* infection in this model has to human cryptosporidiosis, evaluation of possible anticryptosporidial drugs as well as the development of a vaccine against this disease is probable. The mouse model described herein for chronic infection will also be useful in further defining immune defects responsible for establishment of chronic cryptosporidiosis in patients with compromised immune systems. Furthermore, this model can be used for the assessment of potential immunomodulators for treating chronic *C. parvum* infections.

Effects of *Cryptosporidium parvum* and Dexamethasone on B and T Lymphocyte Proliferation. This experiment compared normal mouse splenocytes to the effects that DEX alone, *C. parvum* alone, and DEX + *C. parvum* had on B and T lymphocyte functionality. The anti-inflammatory and immunosuppressive actions of DEX are inseparably linked because they both largely result from inhibition of specific lymphocyte functions (51). In most instances, the effects on lymphocytes are a consequence of glucocorticoid-induced inhibition of the action of lymphokines, including IL-2 through IL-6 and gamma-interferon (22, 51). Specific assays to determine lymphokine inhibition were not performed in these studies and would be required for an

in depth characterization of the immune response to *C. parvum*. However, the proliferative responses were assessed by the ability of splenic lymphocytes to respond to the mitogens LPS (B lymphocyte stimulant) and ConA (T lymphocyte stimulant) at 1 and 2 weeks postinfection with *C. parvum*. To our knowledge, no one using DEX in rodents infected with *C. parvum* has looked at the immune status of the animals prior to an earlier study done in our laboratory involving *C. parvum*-infected rats (94) and the present study in C57BL/6N mice.

In the present study, all three concentrations of LPS (10, 5 and 2.5 μg/ml) resulted in similar trends in proliferation. The two lower concentrations of ConA (2.5 and 1.25 μg/ml) resulted in similar trends. The greatest stimulation (though not significant) occurred at 2.5 μg/ml for both mitogens. The finesse required in performing these lymphocyte proliferation assays, as well as the lack of experience on my part, may account for discrepancies in these results. Nevertheless, trends were observed and are discussed herein.

Spleen cells collected from normal mice at 1 and 2 weeks p.i. had the greatest proliferative response to all three LPS concentrations when compared to the other treatment groups (Figure 14). Lymphocytes from normal mice had the greatest response to ConA at the two lower concentrations for the first week p.i. (Figure 15A) and the highest and lowest concentration at 2 weeks p.i. (Figure 15B) compared to the other treatment groups. Lymphocyte responses from groups-2, 3 and 4 (Cp only, DEX only, and DEX + Cp, respectively) were compared for statistical significance to normal mice (group-1). Although the difference compared to normal mice was not significant, a general trend indicated that mice in group-2 had a decreased lymphocyte response to mitogen stimulation. Antigen stimulation (*C. parvum* in this case) would be expected to result in an increased lymphocyte response. In our experiments, the parasite may be inducing an immunosuppressive effect on the lymphocytes, a phenomenon that has been

described in association with *Trypanosoma cruzi* (61). Parasite-induced immunosuppression, if present, may be inducing alterations in lymphocyte activation by interfering with lymphokine production or lymphokine-receptor interactions. Investigators have reported a decrease in IL-2 in association with the development of chronic *Trypanosoma cruzi* infections in mice (61) and in association with *C. parvum* infections (39). Decreased IL-2 levels have also been reported in AIDS patients with cryptosporidiosis. Because IL-2 is important for effective lymphoproliferation in response to antigens, a decrease in this lymphokine would result in a decreased immune response. The use of IL-2 as a prophylactic/therapeutic agent against *C. parvum* infections has been examined and has demonstrated promise as a treatment for cryptosporidiosis (39, 123). Furthermore, *C. parvum* may be releasing a toxic substance that alters the expression of surface molecules on lymphocytes. A variety of cell membrane molecules that serve as receptors for chemicals such as IL-2 are involved in the activation of lymphocytes, and are critical for the clonal expansion of immune cells. It is possible that the immunosuppressive effects of *C. parvum* are mediated by a secretion product that destroys membrane molecules and ultimately interferes with lymphocyte activation. If a toxin were involved, it would indicate that the heavier the infection (or the larger the dose of parasites ingested), the more immunosuppressed the host would become. This could explain why the present study and others have seen a dose-response effect with regard to the number of parasites inoculated and the immune response of the host (9, 19). Many other immune effector mechanisms could be involved and a more representative study examining these mechanisms is merited.

At the optimal LPS and ConA concentration (2.5 μg/ml), DEX-administration alone significantly decreased functionality of B lymphocytes at 1 and 2 weeks p.i. (Figure 14) and T lymphocytes at 2 weeks p.i. (Figure 15) when compared with normal mice. This trend was observed with both mitogens at all concentrations, with the exception of 5

μg/ml ConA at 1 week p.i. This decrease in lymphocyte response to mitogen stimulation following DEX-administration supports findings which suggest that the mechanism of DEX therapy involves lymphocytolysis (22, 62). In GC-sensitive species like rodents, DEX-treatment is believed to result in an immediate lymphopenia (22). This profound decrease in circulating lymphocytes results from cell lysis mediated by the activation of an endonuclease which destroys cellular DNA (22). In the present study, the mice immunosuppressed with DEX without *C. parvum* challenge had lower stimulation at 2 weeks p.i. than at 1 week p.i. This is an expected finding if DEX results in a gradual immunosuppression. However, as described earlier, DEX is believed to result in a immediate lymphopenia. Mice had been receiving the DEX for 21 and 28 days prior to spleen collection at 1 and 2 weeks p.i., respectively. If the immunosuppressive effect due to lymphocytolysis was immediate, one would expect to find fewer splenocytes at 2 weeks p.i. than at 1 week p.i. Absolute lymphocyte counts were actually higher at 2 weeks p.i. than at 1 week p.i. in mice receiving DEX-treatment alone (unpublished data). This indicates that even though the lymphocytes were present, their ability to respond to mitogen had been diminished by DEX. It may be that in the mouse, a combination of lymphocytolysis and lymphocyte redistribution results in lymphopenia. In GC-resistant species, lymphopenia results from redistribution of lymphocytes to other lymphoid regions (22). Studies done in rats showed that DEX treatment resulted in a significant drop in B and T lymphocyte response to LPS and ConA, respectively (94). Additionally, bovine lymphocyte responses to mitogens were suppressed by DEX *in vivo* and *in vitro* and the authors suggested a selective depletion of B lymphocytes (87). In contrast, other investigators have shown that cattle treated with DEX had an enhanced lymphocyte response to mitogen stimulation (22). Cattle are considered GC-resistant (as are humans), while mice are considered GC-sensitive. These contrasting observations not only support the fact that DEX-treatment results in a diversity of effects in different

species, but also emphasizes the importance of using caution when extrapolating results between species.

Mice in group-4 (DEX + Cp) had a significant decrease in B lymphocyte function at 5 ($P \leq 0.05$) and 2.5 ($P \leq 0.01$) μg/ml LPS at two weeks p.i. Group-4 mice also had a significant decrease in T lymphocyte function at 2.5 μg/ml of ConA at 2 weeks p.i. A general trend of the experiment demonstrated that mice in group-4 had the lowest stimulation compared to the other treatment groups. This experiment also demonstrated a decrease in both B and T lymphocyte proliferation when results at 2 weeks p.i. were compared to results at 1 week p.i. However, the difference was only significant ($P \leq 0.05$) for group-3 at 10 μg/ml LPS and group-2 at 2.5 μg/ml ConA. These findings further support the hypothesis that *C. parvum* may be inducing its own suppressive effects on the immune system, with the immunosuppressive effects becoming more pronounced as the infection progresses. In patients with AIDS and other related immune dysfunctions, cryptosporidiosis can result in death without symptomatic treatment. Treatment resulting in an up-regulation of the humoral and/or CMI system can be expected to prolong the life of the patient. It is still not clear as to which branch of the immune system is responsible for clearing chronic cryptosporidiosis. It is probable, however, that an interaction of both B and T lymphocyte functions is required to eliminate an infection. In the present study, both B and T lymphocyte functions were depressed by DEX alone, *C. parvum* alone, and DEX + *C. parvum*. Because of the interactions of B and T lymphocyte populations, macrophages, cytokines, and other factors in initiating a successful immune response, and because of the controversy concerning this subject, further investigation of the immunological response to *C. parvum* infections is paramount. The use of the C57BL/6N mouse model in further defining the immune parameters involved in chronic *C. parvum* infections should be pursued.

Effects of Dehydroepiandrosterone on Oocyst Shedding Intensities of Mice Infected with *Cryptosporidium parvum*. The therapeutic utility of the immunomodulator DHEA was investigated for its ability to reduce chronic *C. parvum* infections in C57BL/6N mice. The mice were immunosuppressed with DEX and then infected intragastrically with 10^6 *C. parvum* oocysts. Following the establishment of a 35-day chronic infection, mice were treated with DHEA or its sulfated conjugate DHEAS for 28 consecutive days. Infection reduction was determined by significant decreases in oocyst shedding.

Based on the results of this experiment, the therapeutic administration of DHEA or DHEAS had no significant effect on decreasing *C. parvum* infection. Our laboratory has shown that DHEA resulted in a significant decrease in infection when administered to rats at the same time (prophylactic) as *C. parvum* inoculation (94). In the present study, mice were used as opposed to rats and the infection was allowed to progress before DHEA or DHEAS was administered. The different animal models used (rats vs. mice) may account, in part, for the contrasting effects observed. Moreover, the fact that the mice were allowed to establish a chronic infection before DHEA or DHEAS therapy was initiated is a more plausible explanation for the lack of cryptosporidial reduction. It may be that the infection had progressed too far for DHEA or DHEAS to reverse the immunosuppressive effects of DEX. The DHEA and DHEAS have been reported to act by reversing glucocorticoid-induced suppression of lymphocytes (13, 16, 71, 113). Rasmussen et al. (94) suggested that DHEA may act by potentiating immune parameters suppressed by DEX. Even though an effect of DHEA or DHEAS on decreasing parasite intensities was not demonstrated in the present study, the effect of the immunomodulators on specific immune parameters was not assessed and requires further investigation.

The oral doses of DHEA and DHEAS selected for this experiment were within the same range as the concentration of DEX that was used to immunosuppress the mice. The

amount of DEX given (125 µg/mouse/day) was equivalent to approximately 7 mg/kg. Likewise, the concentrations of oral DHEA (50, 150, and 300 µg/mouse/day) were equivalent to 2.7, 8.3, and 16.6 mg/kg, respectively. The amount of DEX administered (7 mg/kg) was within the range of the three DHEA concentrations (2.7 to 16.6 mg/kg). If DHEA is an antagonist of DEX (competing for the same receptor), then an effect may be expected if both drugs were given at approximately equivalent doses. However, recent evidence suggests that although DHEA is an anti-glucocorticoid, it is not an antagonist of DEX because it utilizes its own receptors (76).

If DHEA truly acts as an anti-glucocorticoid in up-regulating the immune system, decreased cryptosporidial activity may be more readily observed if both drugs (DEX and DHEA) are administered via the same route. In this study, DEX was administered i.p. and DHEA was administered orally. The route alone may have such a dramatic effect on bioavailability that little DHEA had access to its effector tissues, which would have decreased its immunomodulator effect. The route of administration not only affects the bioavailability of these drugs (51), but has been shown to be an important factor influencing DHEA's effects on the immune system (101). The fact that the *C. parvum* infection was chronic prior to DHEA treatment is also a reasonable hypothesis explaining the lack of parasite reduction following oral therapeutic administration. Utilizing DHEA in a prophylactic manner may prove to be more effective against *C. parvum* infection (2).

Based on more recent findings in our laboratory (unpublished data), it is recommended that the DHEA administered orally be given at a concentration several fold greater than the dose administered in the present study. The mice given oral DHEA may have received too low of a dose to enable any reversal of the effects on the immune system caused by the DEX-immunosuppression.

Subcutaneous administration of DHEA and DHEAS also failed to show any significant reductions in infection with *C. parvum*. Decreased cryptosporidial activity in

rats and hamsters has been associated with DHEA administered subcutaneously (93, 94). Moreover, DHEA and DHEAS given subcutaneously have yielded significant increases in humoral and CMI responses as well as increased lymphokine production in rodents (36, 37, 94). In AIDS patients, circulating levels of DHEA and DHEAS are suppressed while GC levels are elevated (42, 78). Dehydroepiandrosterone is presently undergoing evaluation for treatment of AIDS patients and has had minimal side effects (42). Numerous reports continue to support the use of DHEA and DHEAS as agents for potentiating the immune response against opportunistic infections such as cryptosporidiosis.

As indicated by the spleen weights in Table 10, some doses of DHEA and DHEAS appeared to be boosting the immune system of the mice. Mice receiving DHEA administered orally had spleen weights comparable to controls. Subcutaneous administration of DHEA at 6 mg (group-3b) and DHEAS at 3 mg (group-4a) resulted in spleen weights significantly ($P \leq 0.05$) higher than control mice. Although secondary infections were not observed in mice from groups-3b and 4a, it is possible that their higher spleen weights resulted from an undetected secondary infection. The mice in group-3b and group-4a survived longer than the mice with lower spleen weights that were receiving the same DHEA or DHEAS regimen at a different dose (e.g., those mice in the same group but different subgroup). This finding suggests that the spleen weights may have a correlation to the overall health of the mice. The body weights (Table 10) gave very little indication as to the health of the mice. All but one group had body weights comparable to the controls, which further suggests that the doses of DHEA and DHEAS administered in these experiments were not high enough to elicit a significant reduction in cryptosporidial infection.

Since this study was completed, follow-up experiments in our lab have utilized the results of the present study to determine an optimal dose for DHEA in mice. These

follow-up results concluded that a higher dose of DHEA and a different dosing regimen induced more profound effects on decreasing cryptosporidial activity (unpublished data).

Because of the apparent association between GC, stress, and disease, evidence suggests that DHEA can enhance the ability of the immune system to guard against GC-induced immunosuppression and pathogen-induced illness (67, 100). Utilizing the C57BL/6N mouse as a model to determine the efficacy of DHEA and other immunomodulators against cryptosporidiosis deserves further investigation.

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