Pharmacological and biochemical studies on *Cryptosporidium* in intestinal explants and suckling mice

by

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GENERAL INTRODUCTION

Cryptosporidium is a significant cause of diarrhea in both industrialized and developing countries (Ungar et al., 1990). Young children and immunocompromised patients usually have higher prevalence, in particular, those with acquired immunodeficiency syndrome (AIDS), with an incidence of about 10% (Laughon et al., 1991). Acute self-limiting diarrhea is the most common expression of infection in immunocompetent patients, whereas in immunocompromised patients, the disease can be prolonged and life-threatening (Fayer et al., 1986; Meisel et al., 1976). Waterborne outbreaks such as occurred in Milwaukee, Wisconsin, in April 1993, involving an estimated 370,000 individuals indicate the epidemic potential of this parasitic infection (Terry, 1993). A recent National Institutes of Health panel concluded that *Cryptosporidium* may now be one of the three most important enteropathogens causing diarrheal illness worldwide (Laughon et al., 1991).

Unfortunately, there is no anticryptosporidial therapy presently effective although over 150 drugs have been employed empirically in attempts to treat clinical or experimental infections (Reinemeyer, 1994). It is unclear whether this resistance is due to the unique intracellular but extracytoplasmic location of the parasite which limits drugs uptake or to unique metabolic processes of the organism. However, information on biochemical processes of the organism as potential targets for intervention action does not

exist. One of the factors that directs current biochemical research effort on protozoa is the availability of parasite material for study. For *Cryptosporidium,* it is the main limiting factor. Furthermore, there is an absence of satisfactory reproducible *in vitro* cultivation methods conducive to biochemical and physiological studies.

This thesis reports efforts to address some of these problems. The objectives were to establish the intestinal explant culture technique as a model for *in vitro* culture of *Cryptosporidium*, to study metabolic processes of *Cryptosporidium* as potential targets for drug intervention, and to evaluate the efficacy of some potential anticryptosporidial agents on the suckling mouse experimental model of *Cryptosporidium parvum* infection.

This thesis is presented as a general introduction, a review of literature, three separate studies, a general summary, a list of references and acknowledgements. Each of the three studies begins with an introduction, followed by materials and methods, results, and ends with discussion.

LITERATURE REVIEW

History

Cryptosporidium was first reported in 1907 by a well-known American parasitologist, Ernest Edward Tyzzer, when he found numerous tiny parasites within the gastric glands of a laboratory mouse. This protozoan parasite infected the gastric epithelium of laboratory mice used in Tyzzer's research program. Thus, it was named *Cryptosporidium muris* (Tyzzer, 1907). Three years later, in 1910, Tyzzer described the same organism in much greater detail and recognized many of the life cycle stages. In 1912, he identified and named a second species, *Cryptosporidium parvum*, which was found in the small intestine of laboratory mice. Once again he provided details of morphology and life cycle (Tyzzer, 1912). For over half a century after Tyzzer's work, cryptosporidial infection was not regarded as economically or medically important. Up to the 1970s, there were only sporadic reports of infection in species such as snakes (Triffit, 1925), turkeys (Slavin, 1955), and guinea pigs (Jervis et al., 1966). In general, this parasite was considered to be rare and of no pathogenic importance. The first cases of human cryptosporidiosis were reported in 1976 (Nime et al., 1976; Meisel et al., 1976), but subsequent reports were rare until several identification methods of oocyst stages in fecal samples were developed in the 1980s, which provided health care workers and

scientists with reliable techniques for noninvasive diagnosis. The severity of the infection in patients with acquired immune deficiency syndrome (AIDS) greatly stimulated research interest worldwide. Since then, extensive research has been conducted on the parasite and its disease effects. In the 1980s, over 900 papers were published (Laughon et al., 1991). During this period, the concept of cryptosporidiosis was transformed from that of a rare and largely asymptomatic infection to an important, widespread pathogen of enterocolitis and diarrhea in several animal species, including humans.

Taxonomic Position and Life Cycle

Taxonomy and host specificity

Cryptosporidium is one of several genera of protozoa in the phylum Apicomplexa that is referred to as coccidia. A total of 21 species of *Cryptosporidium* have been named from fish, reptiles, birds, or other vertebrate hosts (Angus, 1983; Fayer et al., 1986). But recent reviews and cross-transmission studies place the distinctiveness of many of these species in doubt. Only 6 of the isolates, including the two originally described by Tyzzer, are now accepted as valid species. A study of bovine cryptosporidial oocysts has revealed two species among mammals: *Cryptosporidium parvum*, small in size and very common, and *Cryptosporidium muris*, with large oocysts and much less common (Upton et al., 1985). On the basis of oocyst morphology, it is *C. parvum*, not *C. muris*, that is responsible for clinical illness in humans and other mammals. Cross-transmission studies

conducted by inoculating oocysts of *Cryptosporidium* obtained from animals of one species into animals of other species demonstrate a lack of host specificity for *Cryptosporidium*. Isolates from mammals are generally infective for other mammals, and the isolates from avians are generally infective for other avians (Fayer et al., 1986; O'Donoghue et al., 1987). However, transmission between avians and mammals has yielded mixed results. Oocysts of *C. parvum* from mammals produced mild respiratory infection in chickens inoculated intratracheally, but was not infective when given orally (Lindsay et al., 1987). At present, it is preferable to name a particular parasite obtained from a mammalian host as an isolate rather than a strain due to the present uncertainties in the taxonomy of *Cryptosporidium* spp. (Current, 1989).

Life cycle

Cryptosporidium completes its life cycle on intestinal and respiratory surface epithelia of its host. The parasite is monoxenous which means only one host is involved in the life cycle. Its reproduction is characterized by an alternating asexual and sexual stage. Briefly, the life cycle of *Cryptosporidium* is initiated when oocysts are ingested and sporozoites are released from oocysts (excystation) and invade host cells. In general, sporozoites parasitize the brush border of epithelial cells. They invade the apical portion of epithelial cells and establish themselves immediately beneath the host cell membranes, where the unique parasitophorous vacuoles are formed. This special parasitizing site is termed as intracellular but extracytoplasmic. Each intracellular stage of *Cryptosporidium* is

within a parasitophorous vacuole confined to the microvillous region of the host cell. After penetration, the sporozoite rounds up and differentiates into a spherical trophozoite with a prominent nucleus. Asexual multiplication referred to as merogony or schizogony results when this nucleus divides. Two morphologic types of meronts have been observed. As the meronts matures, each nucleus becomes incorporated into a merozoite, which can potentially invade adjacent host cell to form additional type I meront (recycling of type I meront) or to form type II meront. The merozoites from type II meronts are thought to initiate sexual multiplication (gametogony), in which micro- and macrogamonts develop. After fertilization, the macrogamont develops into an oocyst, which contains four sporozoites when sporogony is complete. Approximately 20% of the oocysts are surrounded only by a single unit membrane (thin-walled oocyst) and these have been implicated as the stage of autoinfection (Current et al., 1989). It has been suggested that thin-walled oocysts may undergo sporogony and excystation within the same host, releasing sporozoites, penetrating into the microvillous region of other epithelial cells and reinitiating the infection. Most oocysts (about 80%) form a thick, two-layered, environmentally resistant oocyst wall (thick-walled oocyst) and are discharged in the feces or excreted with respiratory or nasal secretion. It is the thick-walled oocyst that transmits the infection from one host to another.

Cryptosporidiosis in Humans

Historic background

The first case of human cryptosporidiosis was reported in 1976 in a 3-year-old boy from rural Tennessee (Nime et al., 1976). Two months later the second human case in a 39-year-old college administrator was described by Meisel and colleagues (Meisel et al., 1976). Subsequently to these reports, only a few cases were reported until 1982, when five cases in homosexual men with AIDS were documented (Ma et al., 1982). A survey of cryptosporidiosis by stool examination conducted at Vincent's Hospital & Medical Center of New York from 1981 to 1984 indicated that the rate of cryptosporidiosis increased exponentially both in immunocompromised and immunocompetent individuals (Ma et al., 1987). By 1986, the Centers for Disease Control (CDC) reported 697 (3.6%) of the first 19,187 AIDS patients were infected with *Cryptosporidium* (Ungar, 1990).

Prevalence

Cryptosporidium now has been generally accepted as an agent of diarrhea throughout the world. In most published surveys, C*ryptosporidium* was the most common parasite found, and was considered to be the most significant of all known enteropathogens causing diarrheal illness. Geographic surveys based on fecal examination for *Cryptosporidium* oocyst excretion revealed the distribution and mean prevalence of *Cryptosporidium* infection on various land areas. Human infection has been identified on

all six inhabited continents. In the more industrialized countries of Europe and North America, the prevalence is lower, generally between 1 and 3%. In contrast, mean prevalence from elsewhere in the world is between 4.9% in Asia and 10.4% in Africa (Ungar, 1990). The prevalence of cryptosporidiosis in children is significantly higher than that in adults although the age range for infection has been shown to be between 3 days to 95 years of age (Holten-Anderson et al., 1984). Most infections are reported for children under 2 years of age (Ungar, 1990). Infection are often seasonal, with a high prevalence during warmer and wetter months (Tzipori et al., 1983; Rahaman et al., 1984; Shahid et al., 1985; Montessori et al., 1985; Wolfson et al., 1985).

Clinical aspects

The clinical manifestations vary from one group of individuals to another and can range from subclinical to severe. Severity is mainly dependent on the age and immune status of the host and the intensity of exposure. Generally, young and immunologically immature individuals have a greater prevalence of infection and experience more severe illness than adults. In immunocompetent animals and human patients, the disease is non-life threatening and self-limiting with symptoms lasting a few days to a few weeks. These patients recover spontaneously, whereas immunodeficient individuals may become chronically or terminally ill. The clinical picture is dominated by watery, cholera-like diarrhea, which resists all treatment (Anon, 1982; Andreani et al., 1983; Pitlik et al., 1983). The very frequent defecation is accompanied by considerable loss of fluid and

electrolytes. It may contain mucus but rarely blood or leucocytes, and it is often associated with weight loss (Soave et al., 1984). Other less common clinical features include nausea and vomiting, abdominal pain and cramps, and low-grade fever (<39^{0}C) (Baxby et al., 1984; Wright et al., 1984; Holley et al., 1986). In the immune deficient patient, *Cryptosporidium* infections are not always confined to the gastrointestinal tract. A variety of respiratory problems (Kocochis et al., 1984; Ma et al., 1984; Goodstein et al., 1989; Hinnant et al., 1989), cholecystitis (Pitlik et al., 1983; Margulis et al., 1986; Kahn et al., 1987), hepatitis (Gross et al., 1986) or pancreatitis (Gross et al., 1986; Hawkins et al., 1987) has been reported.

Pathology

Although the parasites can colonize throughout the gastrointestinal tract on the mucosa of the stomach, duodenum, colon and rectum, the lesions are usually most severe in the distal jejunum and ileum (Meisel et al., 1976). Infections at extra-intestinal sites including the gallbladder (Pitlik et al., 1983; Margulis et al., 1986; Kazlow et al., 1986; Kahn et al., 1987), pancreatic duct (Gross et al., 1986; Hawkins et al., 1987; Hinnant et al., 1989; Kahn et al., 1987; Kocoshis et al., 1984), and respiratory tract (Forgacs et al., 1983; Kocochis et al., 1984; Ma et al., 1984; Goodstein et al., 1989) have been described. Microscopic lesions are similar among almost all host species infected. *Cryptosporidium* is found at the microvillus border of intestinal epithelial cells without penetration into the cytoplasm (intracellular but extracytoplasmic). Infected epithelial cells morphologically

change from tall columnar to short cylindrical or even to cuboid structure (Meisel et al., 1976). Scanning electron micrographs reveal rounded parasitic protrusions on the intestinal epithelial surface that disrupt the delicate microvilli (Heine et al., 1984). Reduced villus height combined with crypt hyperplasia have been reported in humans and several experimental models of *C. parvum* infection (Meisel et al., 1976; Soave et al., 1984; Isaacs et al., 1985; Heine et al., 1984; Fayer et al., 1986). Consistent with this morphological change, brush border enzyme levels are diminished in *C. parvum* infected intestinal epithelium (Guerrant et al., 1990; Gardner et al., 1994).

Pathophysiology

Little is known of the pathophysiology of cryptosporidiosis. The morphological changes and the diminished absorption of nutrients suggest that malabsorption plays a role in the pathophysiology of diarrhea resulting from *C. parvum* infection. However, the available data on the electrical resistance of infected epithelium are conflicting. In a porcine experimental model, the tissue electrical resistance increased despite the villus destruction observed (Argenzio et al., 1990). However, in another study, the electrical resistance of monolayers of the immortalized human crypt epithelial cell line, T84, was reduced in the presence of *C. parvum* infection (Adams et al., 1994). It is also puzzling that *C. parvum* infected rodents do not develop diarrhea even with obvious morphological changes of the intestinal mucosa. The host response to infection may also play a role in the pathophysilolgy of cryptosporidial diarrhea. In a porcine model of *C. parvum*,

prostaglandin-mediated inhibition of Na^+-Cl^- cotransport contributed to the diarrhea found in cryptosporidiosis (Argenzio et al., 1993). Glutamine stimulated prostaglandin-sensitive Na^+-H^+ exchange (Argenzio et al., 1994).

A recent study indicated the presence of a heat-labile, calcium-dependent, reversible, and mucosally active factor in the stools of calves infected with *C. parvum* that rapidly stimulates a chloride-dependent increase in short-circuit current in human jejunum mounted in Ussing chambers *in vitro*. The chloride dependence of the short-circuit current observed suggests that active secretion of chloride (which could result in diarrhea) was stimulated by the factor present in the stools of infected calves. This enterotoxic activity was dose-dependent, saturable, and may be responsible for secretory diarrhea in humans (Guarino et al., 1994). However, the source of enterotoxin is not clear. Whether it is produced by *Cryptosporidium* itself, or synthesized by parasite-infected intestinal epithelial cells, or due to the stimulation of host's enteric or systemic immune system or enteric nervous system is not known.

Diagnosis

The earliest cases of *Cryptosporidiosis* in humans were diagnosed by identification of endogenous stages of *C. parvum* by microscopic examination of intestinal biopsy or necropsy specimens. In 1980, this invasive and time-consuming procedure was replaced by a variety of techniques of identifying oocysts in fecal specimens. Acid-fast staining is usually the method of choice for the clinical microbiology laboratory (Garcia et al., 1983;

Ma et al., 1983). Negative staining (Current, 1983) and Sheather's sugar flotation (Current, 1986) are also useful in the research laboratory. The use of serodiagnostic techniques for monitoring exposure to *Cryptosporidium* has thus far been limited to a few laboratories. Antibodies specific to *Cryptosporidium* in sera obtained from persons recovered from confirmed infection (Campbell et al., 1983) and from eight other species of animals (Tzipori et al., 1981) have been detected by indirect immunofluorescent antibody (IFA) techniques. Specific anti-*Cryptosporidium* IgG, IgA and/or IgM were also detected by an enzyme-linked immunosorbent assay (ELISA). Although useful for epidemiologic evaluation, serologic studies have no role in the diagnosis of acute illness (Ungar et al., 1986; Current et at., 1988).

Cryptosporidiosis in Calves

Historical perspective

The first described case of bovine cryptosporidiosis in an 8-month-old heifer with chronic diarrhea appeared in 1971 (Panciera et al., 1971). The significance of this finding was not fully appreciated because the infection was accompanied by *Sarcocystis* infection. This report was soon followed by numerous reports of sporadic case in which similar infection was found. Co-infection with other enteropathogens, particularly rotavirus, coronavirus, enterotoxigenic *Escherichia coli*, or *Clostridium perfringens* was frequently observed (Pohlenz et al., 1978; Tzipori et al., 1982; Angus, 1983; Fayer et al., 1985;

Current, 1985). In 1980, field studies demonstrated that the parasite was capable of causing clinical diarrhea in calves in the absence of concurrent infection with common viral or bacterial agents. This established *Cryptosporidium* spp. as a primary entero-pathogen of calves (Tzipori et al., 1980).

Prevalence

Infection is most common in calves less than a month old, with a peak incidence at approximately 2 weeks of age (Meuten et al., 1974; Pohlenz et al., 1978; Moon et al., 1982). All the calves on a dairy farm may become infected. Subclinical infection has been confirmed in adult cattle. Beef cows, though not proven, may be potential sources of infection for their calves (Anderson, 1987). Most infection results from horizontal transmission among calves or from oocysts persisting in the environment. The most common source of infection for a calf is contact with infected penmates. Calves may serve as a reservoir host for transmitting cryptosporidiosis to other farm animals (Tzipori et al., 1982). Although the economic impact of cryptosporidiosis has not been accurately estimated, the high morbidity, weight loss, growth retardation, and occasional mortality likely represent a significant loss to the cattle industry.

Clinical features

The most common clinical sign of cryptosporidiosis in calves is yellow, watery, malodorous diarrhea which is associated with profuse shedding of infective oocysts (10^6 to

10^7/g of feces) (Current, 1985). The duration of diarrhea is determined by various factors such as host susceptibility, intensity of exposure, and age at the time of infection. In most cases, the diarrhea lasts 2 to 14 days, accompanied by varying degree of dullness, anorexia, depression, fever and loss of weight (Tzipori et al., 1980; 1981; 1983; Heine et al., 1984). Diarrhea associated with cryptosporidiosis is usually self-limiting. Mortality is typically low unless the calf is concurrently infected with other enteric pathogens.

Pathology

Only two species of *Cryptosporidium* have been identified in ruminants. The intestinal species, *C. parvum* is the one which most commonly causes ruminant cryptosporidiosis. The abomasal species, *C. muris*, found in cattle in the west central U.S. in 1985 (Anderson, 1987), is not considered a major parasite at this time. Parasites and lesions are most commonly concentrated in the distal small intestine (Pohlenz et al., 1978; Person et al., 1982; Sanford et al., 1982; Anderson, 1984). Mild to moderate villous atrophy, villous fusion, and crypt hyperplasia are also commonly observed. Changes in the surface epithelium include metaplasia to a low columnar or cuboidal form, degeneration and sloughing of enterocytes, shortening and disruption of microvilli, and increased numbers of lysosomes in parasitized cells (Pohlenz et al., 1978). Microscopic lesions appear to be consistent whether or not other pathogens are present.

Diagnosis

The demonstration of oocysts in the feces of diarrheic animals is indicative of infection. As with human cryptosporidiosis, sucrose flotation (Anderson, 1981), acid-fast (Ma et al., 1983) and Giemsa staining (Pohenz et al., 1978) are applied. Histologic or immunocytochemical examinations of freshly fixed and/or frozen intestinal tissue may identify agents undetected in fecal smears (Hall et al., 1988).

Cryptosporidiosis in Lambs

Historical perspective

Ovine cryptosporidiosis was first diagnosed in Australia in 1974 in diarrheic lambs, 1 to 3 weeks of age (Barker et al., 1974). Endogenous stages of *Cryptosporidium* were demonstrated by electron microscopy. Subsequently, several outbreaks of diarrhea in lambs associated with excretion of cryptosporidial oocysts were reported (Tzipori et al., 1981; Anderson, 1982). In transmission experiments, specific pathogen-free lambs developed severe diarrhea following oral administration of calf feces containing cryptosporidial oocysts (Tzipori et al., 1981). Similar results were obtained when germ-free lambs were inoculated with calf cryptosporidial oocysts that were free of viruses and bacterial contaminants. These studies confirmed that *Cryptosporidium* was pathogenic for neonatal lambs (Snodgrass et al., 1984).

Clinical sign and lesion

As in calves, the primary clinical sign is diarrhea, which is most severe if complicated by concurrent infection with other enteric pathogens. Numerous oocysts are excreted in diarrheic stools. Infected lambs are often depressed and anorexic. (Tzipori et al., 1981; Casemore et al., 1985). Lesions may be found in the entire small intestine and much of the large intestine. Villous atrophy, villous fusion, absence of mucus-secreting cells and epithelial cross bridging between contiguous villi were frequently described (Tzipori et al., 1981; Angus, 1983). The infections were age-dependent with more severe infection in the newborn (Tzipori et al., 1981). The presence of oocysts in feces or gut contents can be diagnosed by examining Giemsa-stained smears.

Cryptosporidiosis in Birds

Historical background and prevalence

Two species of *Cryptosporidium* have been confirmed in avian species. *C. meleagridis* was named by Slavin in 1955 when he found the parasite in the ileum of a turkey (Slavin, 1955), thirty three years after Tyzzer's first description (Tyzzer, 1929). In 1986, Current and colleagues named *C. baileyi* to the species of *Cryptosporidium* isolated from broiler chickens (Current et al., 1986).

Cryptosporidium infections of avain species have been reported in chickens (Dhillon et al., 1981; Itakura et al., 1984; Goodwin et al., 1988; Latimer et al., 1988;

Nakamura et al., 1988), turkeys (Hoerr et al., 1978; Glisson et al., 1984; Tarwid et al., 1985; Ranck et al., 1987), quails (Tham et al., 1982; O'Donoghue et al., 1987), ducks (O'Donoghue et al., 1987), geese (Porctor et al., 1974; Current et al., 1986), pheasants (Whittington et al., 1985; O'Donoghue et al., 1987), and peacocks (Mason et al., 1980). However, information on the prevalence of infection in these avian species is scarce.

Clinical feature and lesion

Naturally occurring cryptosporidiosis in birds manifests itself in three clinical forms: respiratory disease, enteritis, or renal disease. Usually only one form is present in an outbreak, but combinations of the three have occurred (Nakamura et al., 1988). Concurrent bacterial infections were also observed. Studies conducted with *C. baileyi* revealed that the route of inoculation was the major factor that influenced the infectivity and site of development of the parasites in experimentally infected chickens (Lindsay et al., 1986; 1987). Little is known about the site specificity of *C. meleagridis.* Clinical respiratory signs consisting of coughing, sneezing, and dyspnea were associated with infection with this species. Hypertrophy and hyperplasia of the epithelial surface and loss of cilia of the tracheal epithelium are the common histological findings. Numerous cryptosporidia were found within the ciliated border of the tracheal epithelium, on the luminal surface of the mucous glands, and on the respiratory epithelium of nasal passages (Goodwin, 1989). Diarrhea is the major clinical sign in cryptosporidial enteritis in turkeys (Slavin, 1955; Goodwin et al., 1988; Wages et al., 1989). Gross lesions are confined to the

small intestine and occasionally the cecum. Intestinal distention due to mucoid intestinal contents and gas is commonly seen. Clinical signs of renal cryptosporidiosis are often masked by respiratory and/or gastrointestinal signs of the disease in chickens (Nakamura et al., 1988). The major microscopic findings include hyperplasia, hypertrophy of the epithelial cells and the presence of *Cryptosporidium* in collecting ducts, collecting tubules, and rarely in the distal convoluted tubules.

Transmission of *Cryptosporidium*

C. parvum infection is spread through fecal-oral contamination of oocysts, a fact confirmed by numerous transmission experiments. Cross-transmission studies discussed previously, demonstrated that *C. parvum* is not host specific. Isolates from humans are infectious for a variety of mammals, and isolates from one mammalian species are infectious for others (Fayer et al., 1986; O'Donoghue et al., 1987). In persons closely associated with animals, the most *Cryptosporidium* infections are a result of zoonotic transmission (Mann et al., 1986; Pohjola et al., 1986; Rahamán et al., 1984; Anderson et al., 1982). The high incidence of cryptosporidiosis in veterinary workers, farmers, animal handlers and caretakers has been well documented (Crawford et al., 1988). Many reports implicate cattle or other domestic animals as sources of human infection. At least 20 cases of infected laboratory workers have been reported (Anderson et al., 1982; Anon, 1982;

Reese et al., 1982; Blagburn et al., 1983; Current, 1983; Levine et al., 1988; Pohjola et al., 1986).

Infections as a result of contamination of the environment have been increasingly recognized. In 1987, *Cryptosporidium* was conclusively recognized as an agent of human waterborne disease (Rose, 1988; Hayes et al., 1989). Contaminated water for drinking or swimming has been implicated as a source of cryptosporidial oocysts from clearly documented waterborne outbreaks. In 1984, Braun Station, a suburb of San Antonio, Texas, experienced two outbreaks of cryptosporidial gastroenteritis caused by sewer leakage into the community's well water (D'Antonio et al., 1985). An outbreak of cryptosporidiosis was recorded in Sheffield, England in 1986. Cryptosporidial oocysts were identified first from affected people, and subsequently from the drinking water supplied from a common reservoir. Laboratory investigations confirmed that cattles on a farm adjacent to the reservoir complex were a possible source of contamination (Rush et al., 1987). Two similar outbreaks have been reported in Ayshire, Scotland, and Oxfordshire-Swindon, England in 1988 and 1989, respectively (Anon, 1989; Milne, 1989; Smith et al., 1990). Consumption of untreated surface water appeared to be the primary risk factor linked to the outbreaks of cryptosporidial illness in 1986 in a day care center in New Mexico (Gallaher et al., 1989). In January 1987, an estimated 13,000 case of gastroenteritis associated with a waterborne outbreak of cryptosporidiosis occurred among 64,900 residents of Carroll County, Georgia (Hayes et al., 1989). The latest waterborne outbreaks occurred in

Milwaukee, Wisconsin, in April 1993, involved an estimated 370,000 individuals (Terry, 1993).

Person-to-person transmission has been documented in day-care centers, households and hospitals (Crawford et al., 1988). These reports suggested that close personal contact may be an important source of transmission. Thus, transmission can be either direct (by contact with an infected subject) or indirect (drinking water, food, bedding etc.). Airborne transmission is considered as a possible source of respiratory cryptosporidiosis in poultry (Dhillon et al., 1981; Hoerr et al., 1978; Itakura et al., 1984), but has not been documented in other species.

Treatment and Prevention

A total of over 150 agents have been tested for curative and preventive efficacy against cryptosporidiosis in humans or animals (Reinemeyer, 1994). These include antimicrobial agents, coccidiostats and other antiprotozoal compounds, and ionophorous. To date, no specific treatment has been found which consistently cures cryptosporidiosis. Only a few chemotherapeutic agents have shown any efficacy. Lasalocid, a polyether antibiotic which acts as an ionophore, was found to have dose-dependent prophylactic effects in an immunosuppressed rat model (Rehg, 1993). Oocyst production of prednisolone-immunosuppressed mice was markedly reduced when lasalocid was administered prior to infection (Kimata et al., 1991). A

prophylactic effect of lasalocid against experimental *C. parvum* infection in calves was reported, but the required dosage was toxic (Moon et al., 1982). Prophylactic administration of cyclosporin A, alborixin or maduramycin were effective in suppressing oocyst excretion in a suckling mouse model (Weikel et al., 1986). Spiramycin, a macrolide antibiotic, has been reported to provide symptomatic improvement in some patients with cryptosporidiosis (Portnoy et al., 1984; Connolly et al., 1988; Moskovitz et al., 1988), but not in others (Gross et al., 1986; Lazo et al., 1986; Wittenberg et al., 1989). Arprinocid reduced the lever of *C. parvum* recovered from neonatal hamsters, but not from mice (Kim, 1987).

At present, treatment of cryptosporidiosis is limited to supportive therapy with oral or intravenous hydration, parenteral nutrition, and treatment for concurrent infections. Supportive therapy is often required by both immunodeficient and immunocompetent patients, especially the young with severe cryptosporidial diarrhea. Immunocompetent individuals who have cryptosporidiosis respond well to supportive therapy. In contrast, immunocompromised patients are refractory to all treatments unless the cause of immunosuppression can be resolved or ameliorated.

Since chemotherapy and chemoprophylaxis are ineffectual, the prevention of cryptosporidiosis has become more important. *Cryptosporidium* oocysts are widely distributed in the environment, particularly in water, but food or milk products may be potential sources (Ungar et al., 1990). The infective oocysts survive for long periods in the environment. As long as the thick, two-layered wall remains intact, the

oocysts are remarkably resistant to most common disinfectants and can survive for months if kept cold and moist (Tzipori, 1983; Sundermann et al., 1987). Hence, hygienic precautions must be taken to eliminate or reduce the infective source from the environment or avoid contact with known sources.

INTESTINAL EXPLANTS AS A MODEL FOR *CRYPTOSPORIDIUM* INFECTION

Introduction

Cryptosporidium parvum is a significant cause of diarrhea in animals and man. It causes moderate to severe diarrheal illness in immunocompetent individuals and life-threatening disease in immunocompromised individuals (Fayer et al., 1986; Meisel et al., 1976). Cryptosporidiosis is also a frequent complication of humans with AIDS (Laughon et al., 1991). There are currently no effective drugs for the treatment of established infections of *Cryptosporidium* although over 150 drugs have been employed empirically in attempts to treat clinical or experimental infections (Reinemeyer, 1994). It is unclear whether this resistance is due to the unique intracellular but extracytoplasmic location of the parasite which limits drugs uptake or to unique metabolic processes of the organism. Studies of biochemical processes of the organisms as potential targets for drug action would be facilitated by an *in vitro* culture model. Although nearly 25 papers have been devoted to studying development of *C. parvum* in cell culture, there continues to be a need for a good, reproducible and practical *in vitro* model system. The intestinal mucosal explants developed by Nietfeld et al. (Nietfeld et al., 1990) may provide the basis for short-term culture of *Cryptosporidium* on ileal epithelium and the means for rapid

screening of anti-infective drugs. However, the ability of *Cryptosporidium* to grow on these explants has not been evaluated.

The objectives of this study were to establish the intestinal explant culture technique as a model for *in vitro* cultivation of *Cryptosporidium* and to develop a system for rapid pharmacological screening of anticryptosporidial drugs.

Materials and Methods

Culture medium

RMPI 1640 medium with HEPES buffer and L-glutamine, supplemented with 5% heat-inactivated fetal bovine serum (FBS), 0.25% lactalbumin hydrolysate, 0.2 μg of hydrocortisone/ml, and 0.1 μg of insulin/ml was used. Penicillin G (100 U/ml), streptomycin (100 μg/ml), amphotericin B (0.1 μg/ml), and gentamicin (50 μg/ml) were added to the medium to inhibit bacterial and fungal growth. Ascorbic acid (0.3 mg/ml), sodium butyrate (10 mM), and propionic acid (10 mM) were also added to improve mucosal preservation and cellular morphologic features.

Preparation of explants

Pigs intestinal explants

Pigs, 1 to 4 days old, from SPF sows were used. A laparotomy was performed following anesthesia with halothane. A section of ileum was isolated and washed by

injection of 0.9% cold saline. The serosa and muscularis were removed and explants prepared as described (Nietfeld et al., 1990). After the ileal specimens were soaked for 0.5 hours in cold 0.04% sodium hypochlorite solution to reduce microbiological contamination, they were cut into 2.5 cm long sections, and rinsed three times in cold RMPI 1640 medium (GIBCO Laboratories, Grand Island, NY). The sections were then opened along the mesenteric border and cut into 3 x 5 mm pieces. The ileal pieces were then placed villi uppermost on 1 x 1 cm gelfoam sponges (Upjohn CO, Kalamazoo, MI) presoaked with medium. Two explants were placed in each 60-mm plastic petri dish and 3.0 ml cell culture medium was added. The medium just reached the base of the explants, and capillary action pulled the medium over the villus surface. The cultures were incubated on a rocking platform set at 6 cycles/min in an atmosphere of 5% CO_2 and 95% air at 37^{0}C. Medium was changed at 24 hours intervals.

Mouse intestinal explants

Mouse intestinal explants were prepared from CF-1 suckling mice as described above.

Preparation of inoculum

Purified calf isolate of *C. parvum* oocysts were obtained from Dr. James A. Harp of the National Animal Disease Center (NADC). Excystation was initiated by preincubating oocysts in phosphate-buffered saline (PBS) for 1 hour at 37^{0}C (Dubey et al., 1990). An equal volume of excystation fluid consisting of 1.5% sodium taurocholate

(w/v in PBS) with 0.5% trypsin (w/v in PBS) was added, and incubated for 45 minutes at 37^0C on a shaker. Sporozoites were washed free of excystation fluid by centrifugation twice at 1200 x *g* for 12 minutes in PBS. The pellet was then resuspended with PBS and adjusted to desired concentration.

Infection and assessment

Two methods were used to infect explants. For *in vitro* infection, intestinal explants were infected directly with excysted sporozoites by exposure of explants to culture medium containing 10^6 *C. parvum* sporozoites/dish. For preinfected explants, CF-1 suckling mice were inoculated orally with 10^5 *C. parvum* oocysts of calf origin at 6 days of age. Sections of ileum were collected at peak infection, and ileal explants prepared as above as flat explants on gelfoam sponges. Both *in vitro* infected and pre-infected explants were removed at 0, 24, 48, 72, 96, and 120 hours of culture, fixed in 10% buffered formalin, processed for direct immunofluorescence assay.

Specimens were tested with Merifluor *Cryptosporidium-Giardia* direct immunofluorescence (DIF) assays (Meridian Diagnostics, Inc. Cincinnati, OH). Formalin-fixed explants were dehydrated and embedded in paraffin blocks. The explants were then sectioned at 5 μm and placed on treated slide wells. One drop of detection reagent containing fluorescein isothiocyanate (FITC) labeled anti-*Cryptosporidium* and anti-*Giardia* monoclonal antibodies were added to each well, followed by one drop of counterstain. The reagents were then mixed with an applicator stick. After a 30 min

incubation at room temperature, the slides were rinsed with wash buffer (PBS) to remove excess detection reagent and counterstain. A drop of mounting medium was added to each well. A coverslip was applied and the slides were examined by fluorescent microscopy (160x) .

For quick assessment, mouse explant samples were fixed with 10% buffered formalin for 30 min at room temperature. A drop of intestinal tissue suspended in fixative was placed on a treated slide well, spread over the entire well, allowed to completely air dry (about 30 min) and subjected to DIF test procedure as described above.

Drug screening

Experimental explants were exposed to fresh medium containing experimental drugs (azithromycin or novobiocin) at a concentration of 10^{-3} M, beginning at 24 hours post-infection (PI). Both experimental explants and infected but untreated explants were sampled every 24 hours for 5 days and processed for DIF assay.

Results

Morphologically, the epithelia of explant were well preserved and retained a normal microvillus border through 72 hours of culture (Figure 1, Figure 2). Although in most cases, between 96 and 120 hours of culture, a loss of villus architecture was evident, the explants still contained viable villus epithelium at 120 hours (Figure 3). It was

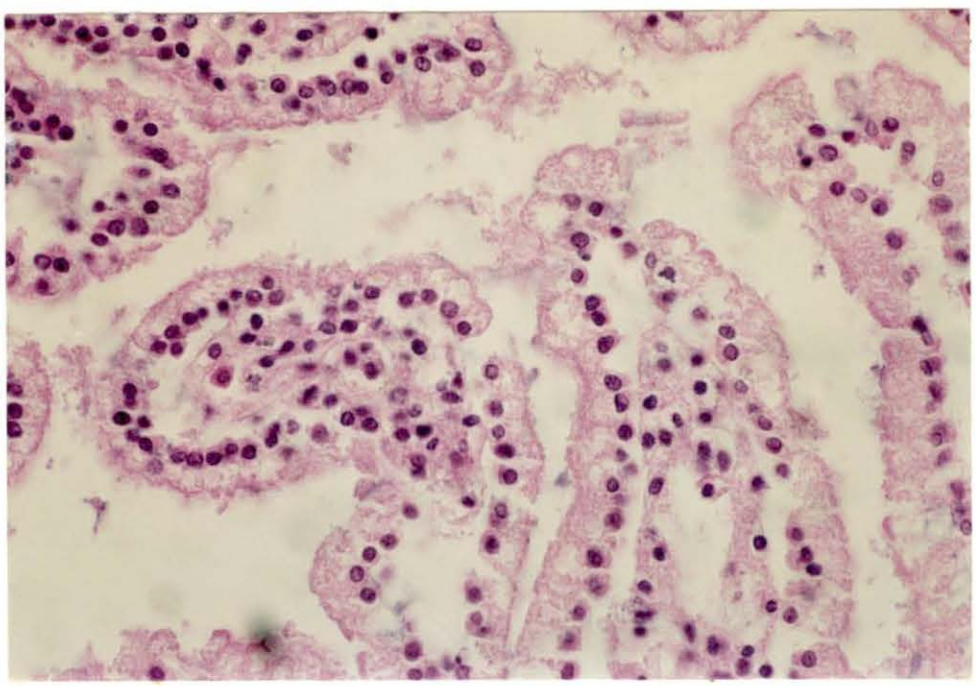

Figure 1. Pig ileum mucosal explant after 24 hours of incubation. Sections of ileum (3 x 5 mm) were placed on gelfoam sponges and incubated in RMPI 1640 medium. The villus epithelia are intact and retain normal, tall, columnar morphologic features (H. & E. stain) (400x).

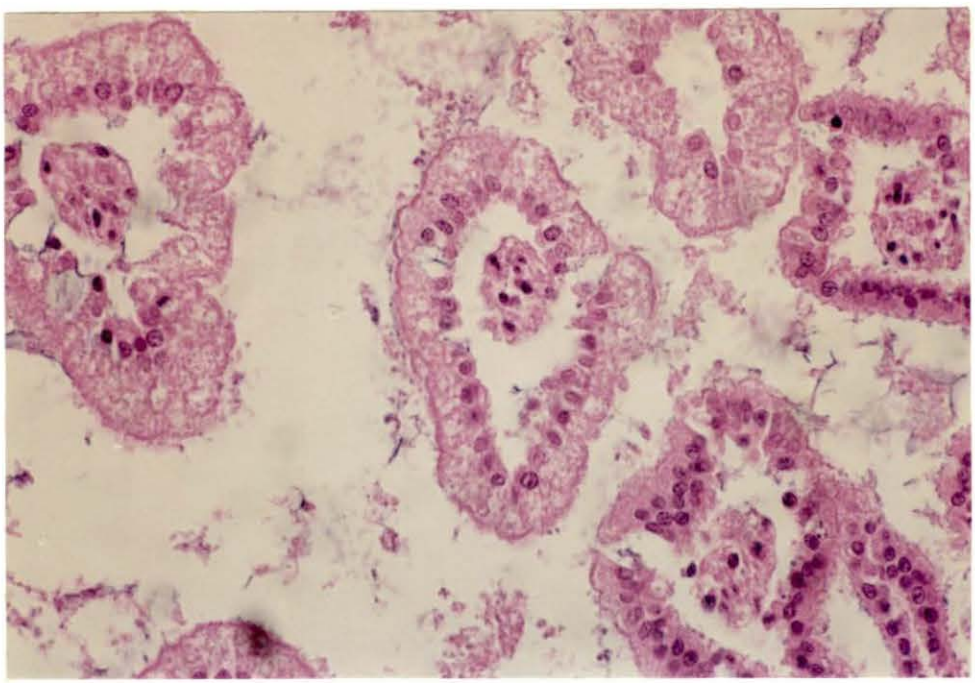

Figure 2. Pig ileum mucosal explant after 72 hours of incubation. Sections of ileum (3 x 5 mm) were placed on gelfoam sponges and incubated in RMPI 1640 medium. The villus epithelia are intact and retain normal, tall, columnar morphologic features (H. & E. stain) (400x).

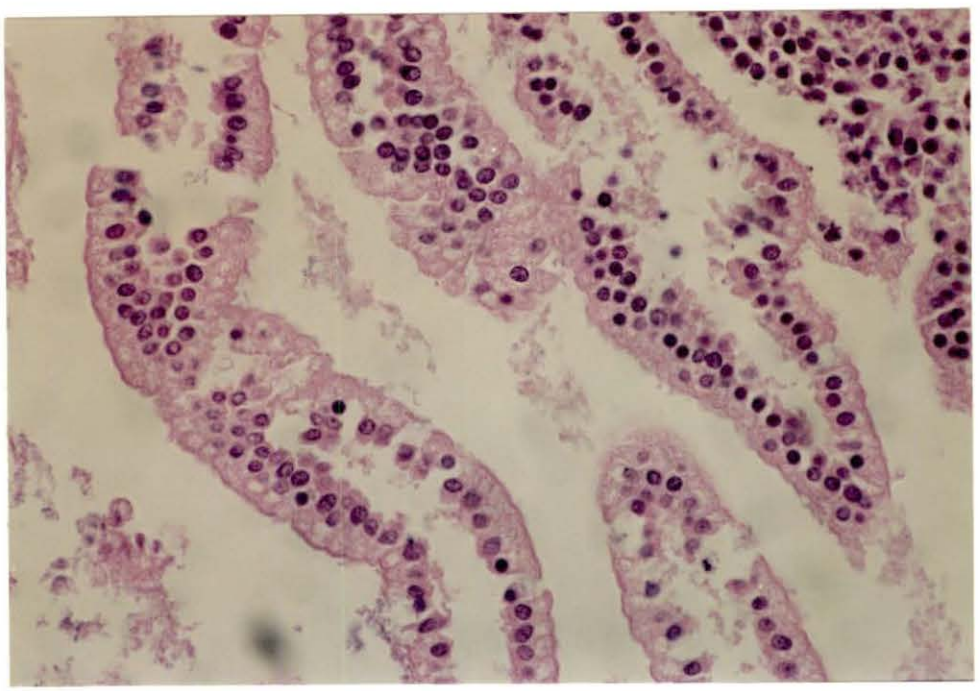

Figure 3. Pig ileum mucosal explant after 120 hours of incubation. Sections of ileum (3 x 5 mm) were placed on gelfoam sponges and incubated in RMPI 1640 medium. The villus epithelia are intact and retain normal, tall, columnar morphologic features (H. & E. stain) (400x).

apparent that both pig and mouse intestinal explants were susceptible to the *in vitro* infection of *C. parvum* by exposure to culture medium containing sporozoites. Infection on pig explants at 24 and 96 hours of culture (Figure 4, Figure 5), and infection on mouse explants at 48 and 72 hours of culture were confirmed by direct immunofluorescent test (Figure 6, Figure 7). Immunofluoresence was observed on explants though 120 hours of culture. *C. parvum* appeared round to slightly oval in shape, with a bright apple green color.

In preinfected mouse intestinal explants, infections of *Cryptosporidium* were detected on all explants during the entire 120 hours of cultivation (Figure 8, Figure 9).

However, the infective stages of the parasite in culture were not demonstrated by H. & E. staining. This may be due to the loss of villus architecture which obscures the diagnosis by conventional light microscopy.

In infected but untreated mouse explants, *Cryptosporidium* infections peaked at 48 to 72 hours PI. When azithromycin or novobiocin was added to culture medium, neither of them eliminate the infection of *Cryptosporidium* on explants (Data not shown).

Discussion

By modification of culture techniques and using flat explants we have observed the establishment of *Cryptosporidium* infection on intestinal explants.

Organ culture of intestinal explants provides a method for preserving normal

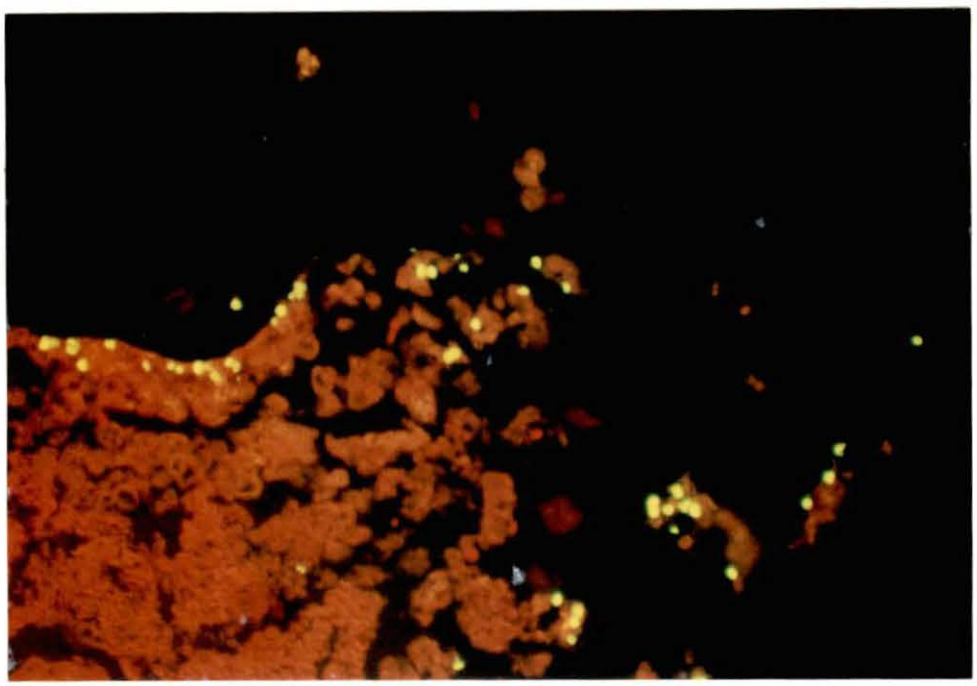

Figure 4. Fluorescent micrograph showing specificity of direct immunofluorescence test for antibodies to *Cryptosporidium* on *in vitro* infected pig intestinal explant after 24 hours of culture. Explants were infected by the addition of 10^6 sporozoites to the culture medium. *Cryptosporidium* appears round to slightly oval in shape with a bright apple green color (160x).

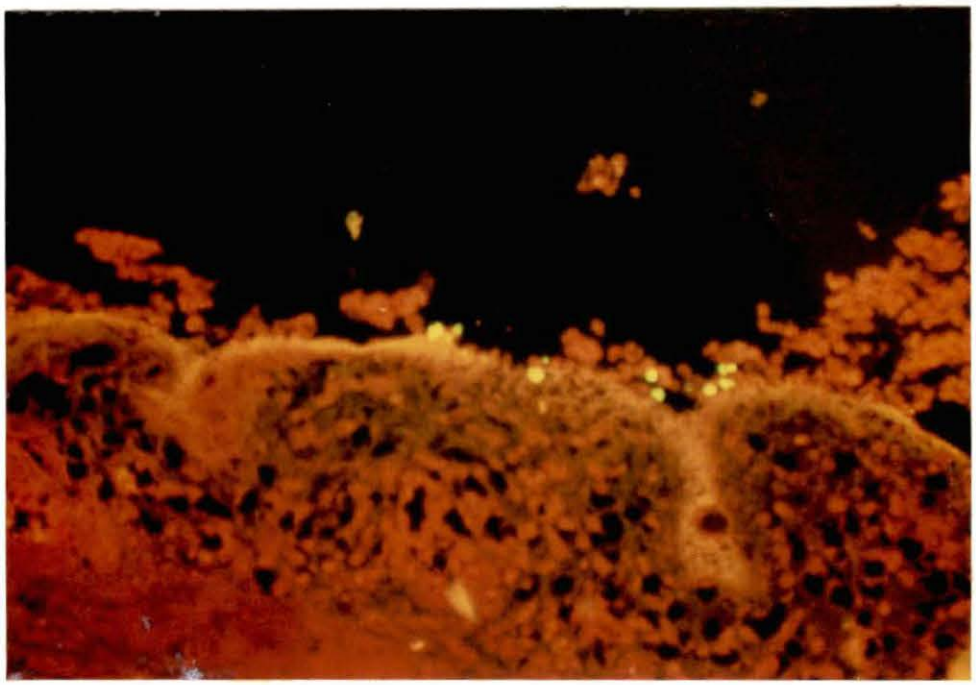

Figure 5. Fluorescent micrograph showing specificity of direct immunofluorescence test for antibodies to *Cryptosporidium* on *in vitro* infected pig intestinal explant after 96 hours of culture. Explants were infected by the addition of 10^6 sporozoites to the culture medium. *Cryptosporidium* appears round to slightly oval in shape with a bright apple green color (160x).

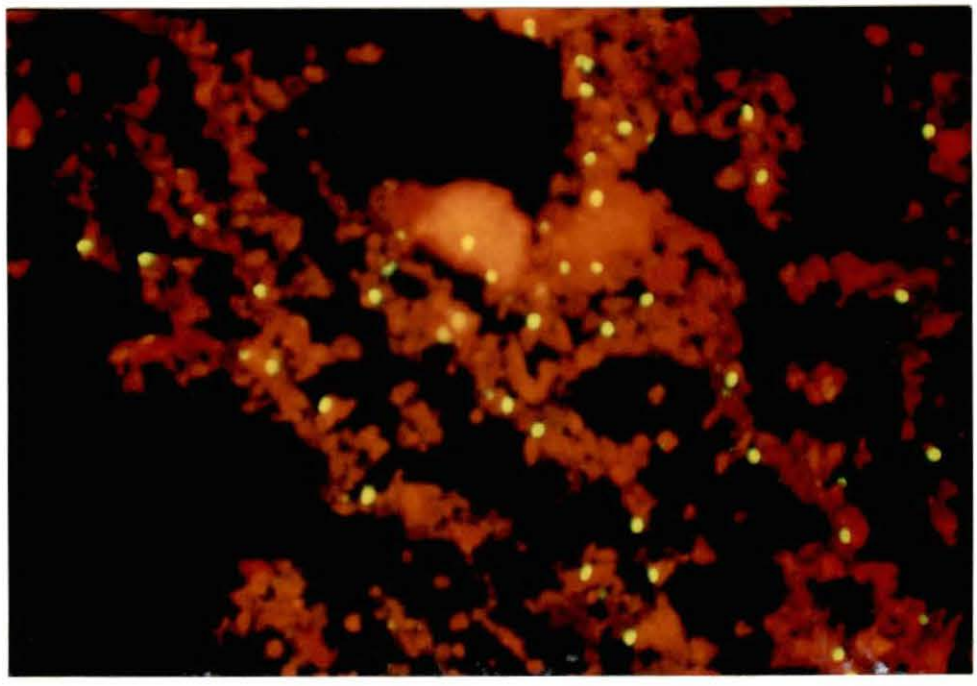

Figure 6. Fluorescent micrograph showing specificity of direct immunofluorescence test for antibodies to *Cryptosporidium* on *in vitro* infected mouse intestinal explant after 48 hours of culture. Explants were infected by the addition of 10^6 sporozoites to the culture medium. *Cryptosporidium* appears round to slightly oval in shape with a bright apple green color. Intestinal explants in formalin suspensions were used for the immunofluorescent detection of *C. parvum* (160x).

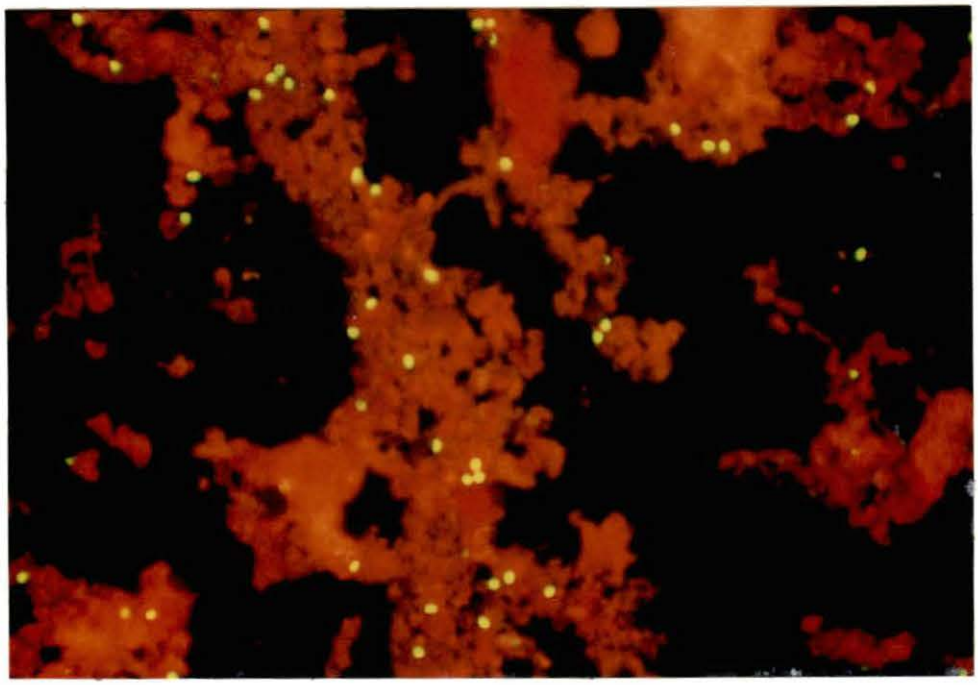

Figure 7. Fluorescent micrograph showing specificity of direct immunofluorescence test for antibodies to *Cryptosporidium* on *in vitro* infected mouse intestinal explant after 72 hours of culture. Explants were infected by the addition of 10^6 sporozoites to the culture medium. *Cryptosporidium* appears round to slightly oval in shape with a bright apple green color. Intestinal explants in formalin suspensions were used for the immunofluorescent detection of *C. parvum* (160x).

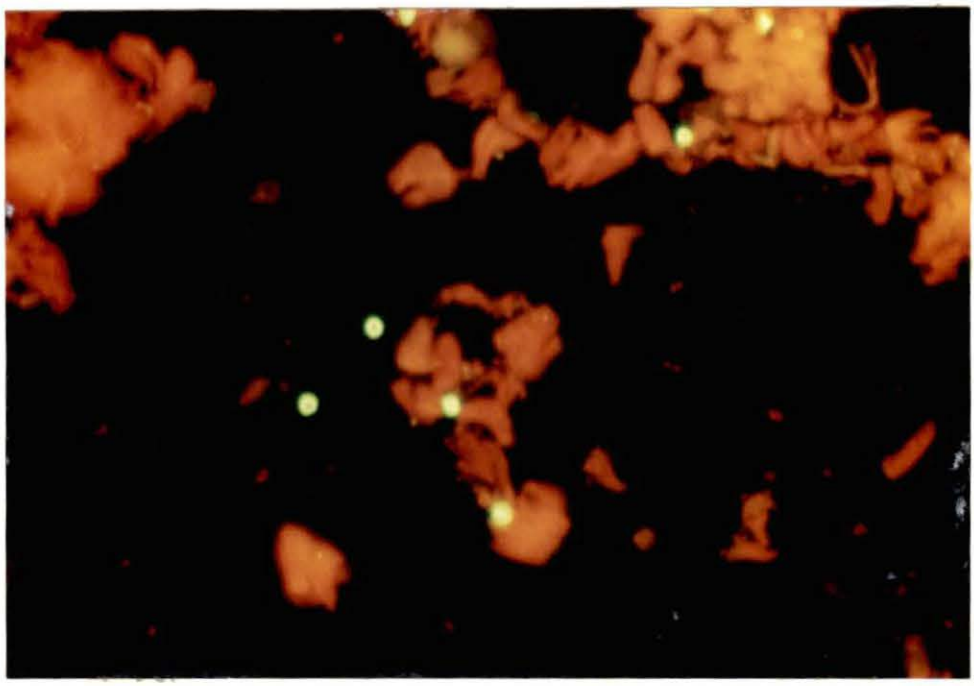

Figure 8. Fluorescent micrograph of *C. parvum* preinfected mouse intestinal explant after 48 hours of culture. Explants were prepared from CF-1 suckling mice orally inoculated with 10^5 oocysts. Intestinal explants in formalin suspensions were used for the immunofluorescent detection of *C. parvum* (250x).

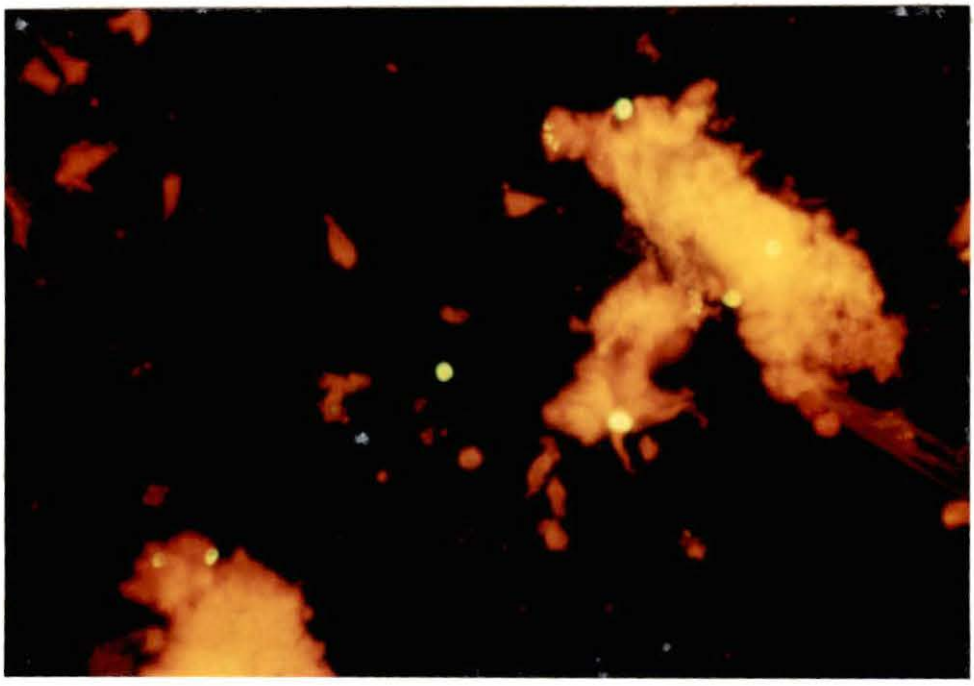

Figure 9. Fluorescent micrograph of *C. parvum* preinfected mouse intestinal explants after 96 hours of culture. Explants were prepared from CF-1 suckling mice orally inoculated with 10^5 oocysts. Intestinal explants in formalin suspensions were used for the immunofluorescent detection of *C. parvum* (250x).

histologic structure and mucosal viability *in vitro* (Nietfeld et al., 1990). Explant cultures provide a solution to some of the disadvantages of other animal models of cryptosporidiosis. Large numbers of explants can be prepared from a single animal, thus greatly reducing the number of animals required for a given study. The testing environment can be precisely controlled, thus reducing the variables created by the presence of extra-intestinal secretions, normal intestinal flora, and possibly other copathogens. Furthermore, test materials can be easily added or withdrawn from the culture medium to study their effects on the mucosa. Explant methods also prevent the contamination of the environment from oocysts shed by infected animals. Also, the effects of several test materials can be tested on explants derived from the same animal, thus reducing individual variation.

Cryptosporidium infection of explants can be easily detected by direct immunofluorescent antibody. The assay is simple to perform and has higher sensitivity than traditional staining procedures (Garcia et al., 1987), which make it useful as a detection method for quick pharmacological screening of anticryptosporidial agents on the intestinal explants. Although the architecture of villi cannot be demonstrated under the florescence microscope, intestinal epithelial cells of explants are visible. The commercial fluorescent kit was originally designed for detecting oocysts in fecal stools by direct monoclonal antibody reaction against the cell wall antigen of *Cryptosporidium* oocysts. The results of this study suggest that *Cryptosporidium* infection on intestinal specimens can be detected by monoclonal antibody.

Like other *in vitro* culture models, the extent of multiplication and the number of parasites produced on intestinal explants are much lower than in experimentally infected suckling mice or in chorioallantoic membranes of chicken embryos. The reduced proliferation in culture was attributed to the absence of the autoinfective oocysts that develop in the mouse intestine and in the chorioallantoic membranes of chicken embryos (Dubey et al, 1990).

The highly controllable environment of the intestinal explants may be suited to the study of parasite-enterocyte interaction and for the development or evaluation of anticryptosporidial chemotherapeutic agents. Further studies will attempt to refine the model to include methods such as electron microscopy to confirm the presence of developmental stages of the parasite on explants and to quantify the mucosal infection.

LACTATE PRODUCTION, AMYLOPECTIN CATABOLISM AND MANNITOL MEASUREMENT IN *CRYPTOSPORIDIUM*

Introduction

Cryptosporidium infections are remarkably resistant to antiprotozoal drugs and there is currently no effective therapy for established infections (Reinemeyer, 1994). Information on metabolic processes of the organism which might be amenable to pharmacologic attack is lacking. One of the factors that limits current biochemical research on *Cryptosporidium* is the availability of satisfactory methods of cultivation of the organism *in vitro*. In addition to its role as the infective stage, the sporozoite is one of the few life cycle stages not associated with host cells. For this reason, the sporozoite may be a stage at which pharmacologic attack may yield clinically useful drugs.

The objective of this study was to characterize selected metabolic processes in *Cryptosporidium* sporozoites as potential targets for drug intervention. Like other coccidia, amylopectin granules are stored in the developmental stages in *Cryptosporidium* and may serve as source of energy. However, there is no information on glycolytic rates in *Cryptosporidium*. In this study, lactate production and amylopectin catabolism in freshly prepared sporozoites were measured. A new metabolic pathway, known as the mannitol cycle, has been identified in the coccidian parasite *Eimeria tenella* (Schmatz et

al., 1989). It may be important for the production and utilization of energy reserves for the parasite. This pathway has not been found in any other animal system and was previously thought to be unique to fungi. The amount of mannitol in oocysts was measured in this study.

Materials and Methods

Preparation of sporozoites

The excystation procedure was described on page 26. Viability assessment was made on excysted sporozoite samples placed on an indentation slide at 0, 30, 60, 90, and 120 min following excystation. The number of sporozoites was quantitated by using a phase hemacytometer. The infectivity of sporozoites was determined by intrarectum inoculation of suckling mice with 10^5 *C. parvum* sporozoites.

Measurement of sporozoite glycolysis

Lactate production and intrasporozoite amylopectin concentration in 10^6 sporozoites were determined at 0, 30, 60, 90, and 120 min following excystation and incubation at 37^0C in glucose-free Hank's balanced salt solution (HBSS). For lactate assay, aliquots of sporozoite suspension were mixed with equal volumes of 8% perchloric acid and quickly frozen at -80^0C until assayed. Lactate concentration was determined by the increased absorbance at 340 nm due to NADH formation following oxidation of

lactate to pyruvate by lactate dehydrogenase (Sigma Chemical Co, St Louis, MO). For the amylopectin assay, half volumes of 0.6 N perchloric acid were used. Amylopectin concentration was measured as described by Keppler and Decker, 1974. Briefly, amylopectin was hydrolysed to glucose by amyloglucosidase. Enzymatic phosphorylation and dehydrogenation of liberated glucose to 6-phosphogluconolactone and NADPH resulted from the addition of hexokinase and glucose-6-phosphate dehydrogenase, respectively. NADPH was measured spectrophotometrically at 340 nm and was proportional to the glucose liberated from amylopectin (Keppler et al., 1974).

Mannitol assay

A 2 ml aliquot of oocyst suspension (10^7 oocysts/ml) was homogenized to break the cell walls. The suspension was then dried under a stream of nitrogen. The dried material was resuspended in 4 ml chloroform/methanol (1:1, v/v) and left overnight. The following day the samples were again dried under a stream of nitrogen gas, resuspended in 0.5 ml distilled water, centrifuged at 12000 x *g* for 5 min to remove particulates, and the supernatant passed over a C18 Sep-Pak filter (Waters Associates, Milford, MA) in water to remove lipids. Ethanol was added to the eluent to a final concentration of 80% (v/v). The precipitate was removed by microcentrifugation and the supernatant containing the polyols was dried under nitrogen. Acetic anhydride (200 µl) and pyridine (200 µl) were then added, left overnight and dried under nitrogen gas. Methylene chloride (100 µl)

was added before analyzing on a gas chromatograph (GC). Acetate-derivatived samples were analyzed on Bendix GC (series 2300) fitted with a packed column (1.5 m x 4 mm i. d., 4% SE 30, 6% OV 210, chromosorb-W-HP) (Alltech Associates Inc, Deefield, IL) and a flame ionization detector (FID). The column temperature was 240^{0}C, and the detector temperature was 250^{0}C. Concentrations of mannitol were determined by comparison of peak areas to standard curves of the mannitol standards (Schmaltz et al., 1988).

Results

The infectivity and the viability assessment on sporozoites revealed that sporozoites were infective to suckling mice and their motility was maintained during the incubation.

Lactate production and amylopectin concentrations of 10^6 sporozoites incubated in glucose-free HBSS at 37^{0}C are shown in Figure 10 and Figure 11. The data are expressed as percentage of zero-time controls to standardize between trials. Lactate concentration increased slightly only at 90 and 120 min following excystation. In some of the trials (2 out of 5), the concentration remained the same during the whole incubation period. There were no significant changes in amylopectin concentrations in sporozoites at 30, 60, 90, or 120 min of incubation (Figure 11). In some experiments, 10^5 or 10^7 sporozoites were incubated for 4 hours with similar results (data not shown).

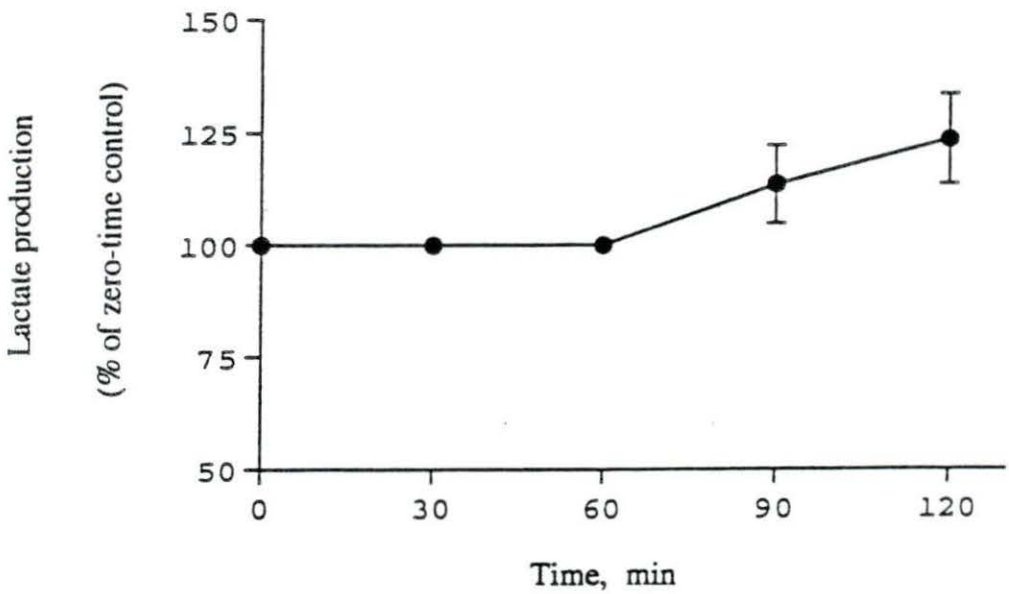

Figure 10. Lactate production by *C. parvum* sporozoites (10^6) incubated in glucose-free HBSS for 2 hours. Lactate concentrations were determined with lactate dehydrogenase. Results are the mean (± SE) of 5 experiments. Data are expressed as percentage of zero-time controls.

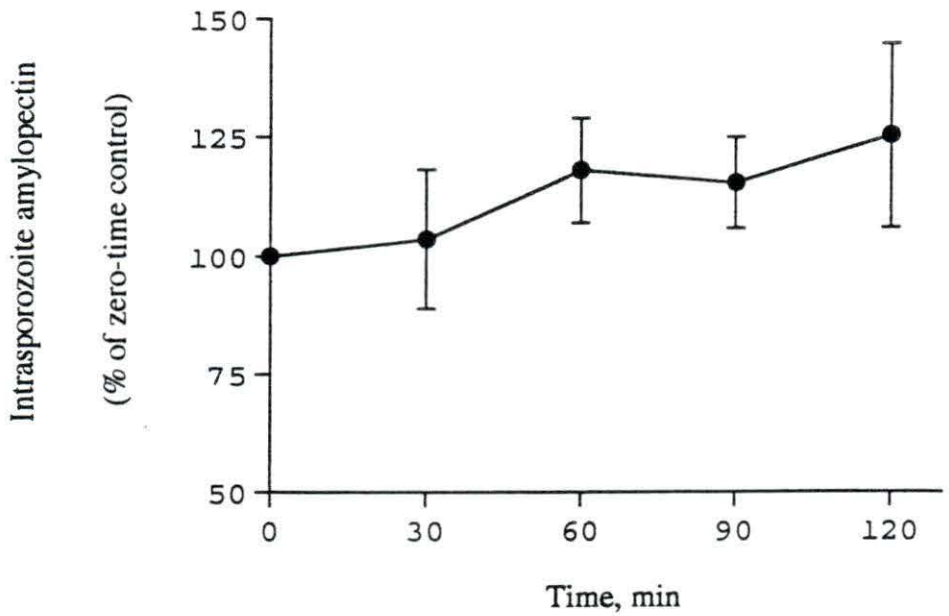

Figure 11. Amylopectin concentration in *C. parvum* sporozoites (10^6) incubated in glucose-free HBSS for 2 hours. Amylopectin concentrations were determined with amyloglucosidase. Results are the mean (±SE) of 6 experiments. Data are expressed as the percentage of zero-time controls.

No mannitol could be detected in oocyst preparations. The sensitivity limit of the method was 0.014 mM.

Discussion

Glucose as a source of energy is essential to the survivability of protozoa. Amylopectin is a branched polysaccharide found as granules in almost all stages of the life cycle of coccidian parasite *E. tenella*. Metabolic studies on extracellular *E. tenella* sporozoites revealed a linear increase in lactate production during a two hour incubation in a glucose-free medium. Amylopectin concentration in *E. tenella* sporozoites decreased by 50% over the same time period indicating a high rate of glycolysis (Smith et al., 1983). For a long time, amylopectin was thought to be the parasite's only carbohydrate reserve (Ryley et al., 1969). However, the consumed amylopectin during the early stages of sporulation may be followed by resynthesis of the same polysaccharide prior to completion of the process (Wilson et al., 1961). Recent studies with *E. tenella* have now identified mannitol as accounting for 25% of the oocyst dry weight. Mannitol is present at very high concentrations in the unsporulated oocyst, approaching levels as high as 300 mM. After sporulation is complete, the concentration of mannitol falls to approximately 10 mM suggesting that it is being used as energy source (Schmatz et al., 1988). A similar mannitol cycle as that found in fungi has now been identified in *E. tenella*. All of the required enzymes have been found in both sporulated and unsporulated oocyst and

mannitol-1-phosphatase (M1Pase) has been found in *Eimeria* sporozoites (Schmatz et al., 1989). Two of the enzymes in the mannitol cycle, mannitol-1-phosphate dehydrogenase (M1PDH) and mannitol-1-phosphatase (M1Pase) were also found in *C. parvum* (Schmatz et al., 1989). However, in our study, mannitol was not detected in oocysts and the presence of the mannitol cycle in *C. parvum* could not be confirmed. This may due to the lack of sensitivity of the method or to the fact that, unlike other true coccidia (e.g., *Eimeria* and *Isospora* spp.), *C. parvum* oocysts undergo sporogony while they are within the host cells. Mannitol may be used up during sporulation so very low levels of mannitol would be present in oocysts released in feces.

Large amounts of amylopectin granules have also been found in the developmental stages of *Cryptosporidium* by transmission electron microscopy (TEM) (Dubey et al., 1990). Amylopectin may provide an energy source for developmental stages of parasite *in vivo*. However, in present study the glycolysis pattern of *E. tenella* was not found in *C. parvum* sporozoites incubated in HBSS at 37^{0}C. Lactate concentrations did not significantly increase with time. There was no decrease in amyloglucosidase release of glucose in excysted sporozoites for up to 4 hours. The results suggest that amylopectin may not be the major source of energy for *Cryptosporidium* sporozoites. This may also explain why antibiotics such as nojirimycin and deoxynojirimycin which specifically inhibit glucosidase I and II (enzymes necessary for the breakdown of glycogen and amylopectin) are not effective for *Cryptosporidium* infection. It is also possible, although unlikely, that *Cryptosporidium* resynthesize amylopectin while they are using it as an

energy source. Further studies are required to determine the metabolic energy source for *Cryptosporidium.*

EFFECTS OF POTENTIAL ANTICRYPTOSPORIDIAL AGENTS ON *CRYPTOSPORIDIUM* INFECTIONS OF SUCKLING MICE

Introduction

Cryptosporidiosis, an enteric disease of humans and animals caused by the protozoan *Cryptosporidium*, occurs worldwide (Ungar, 1990). The disease may be fatal in immunocompromised individuals, and is a frequent complication of humans with AIDS (Laughon et al., 1991). Data regarding the diagnoses, clinical signs and epidemiology of the disease are well documented, but no effective chemotherapy or immunotherapy is available (Casey, 1991). With the expanding epidemic of AIDS, there is a critical need for effective anticryptosporidial therapy.

In this study, several potential anticryptosporidial agents were screened on the suckling mouse experimental model of *C. parvum* infection.

Materials and Methods

Infection of mice

Groups of 7 to 11 CF-1 suckling mice were housed with their dams in sterilized cages. Mice were inoculated orally with 10^5 purified *C. parvum* oocysts of calf origin

(courtesy of Dr. James A. Harp, National Animal Disease Center, Ames, IA) at 6 days of age.

Medication

Potential therapeutic agents including novobiocin (4 mg/ml), a DNA gyrase inhibitor; nojirimycin (4 mg/ml), a glucosidase inhibitor; and griseofulvin (5 mg/ml), a fungistatic antibiotic which inhibits spindle formation in mitosis; azithromycin (20 mg/ml), a macrolide antibiotic; were administered. Medication was started at 8 days of age (48 hours-post infection) and given daily by gavage (0.05 ml per mouse) in experimental groups during the entire experimental period.

Assessment

Mice were sacrificed at 24 hour intervals. Portions of ileum (5 mm) were immediately fixed in 10% buffered formalin and processed for histologic evaluation. Hematoxylin-eosin (H. & E.) stained sections of the distal ileum were examined microscopically at 400x under bright field illumination. Infections of *Cryptosporidium* were scored as 1+ to 4+ according to the scale: < 5/field = 1+; 5 to 25/field = 2+; 25 to 50/field = 3+; > 50/field = 4+. The mean infection scores of experimental groups were compared with that of the infected but untreated groups.

In some experiments, fecal smears and mucosa smears of ileum were prepared and examined for oocysts by acid-fast staining.

Results

Uninoculated mice remained negative for *Cryptosporidium* throughout the experiment.

Using H. & E. stain, *C. parvum* appeared nearly round with a pink color, and were confined within the microvillus border of infected villus epithelial cells (Figure 12).

The score of infections was averaged among trials both in experimental groups and infected but untreated groups and plotted over time (hours post-infection). *Cryptosporidium* infections appeared as early as 72 hours post-infection (PI), with peak infections at 96 to 144 hours PI in infected but untreated groups (Figure 13). The severity of infection correlated well with the degree of fecal shedding.

No anticryptosporidial activity was found in suckling mice treated with novobiocin (50 mg/kg/day), nojirimycin (50 mg/kg/day) or griseofulvin (100 mg/kg/day). None of them diminished the degree or duration of infection (Figure 14, Figure 15, Figure 16). However, novobiocin and griseofulvin slightly delayed the onset of infection.

Only light infections (+1) were detected at 144 and 168 hours PI (Figure 17) in azithromycin (400 mg/kg/day) treated mice, which was significant lower than untreated mice or mice treated with other three drugs ($P < 0.01$).

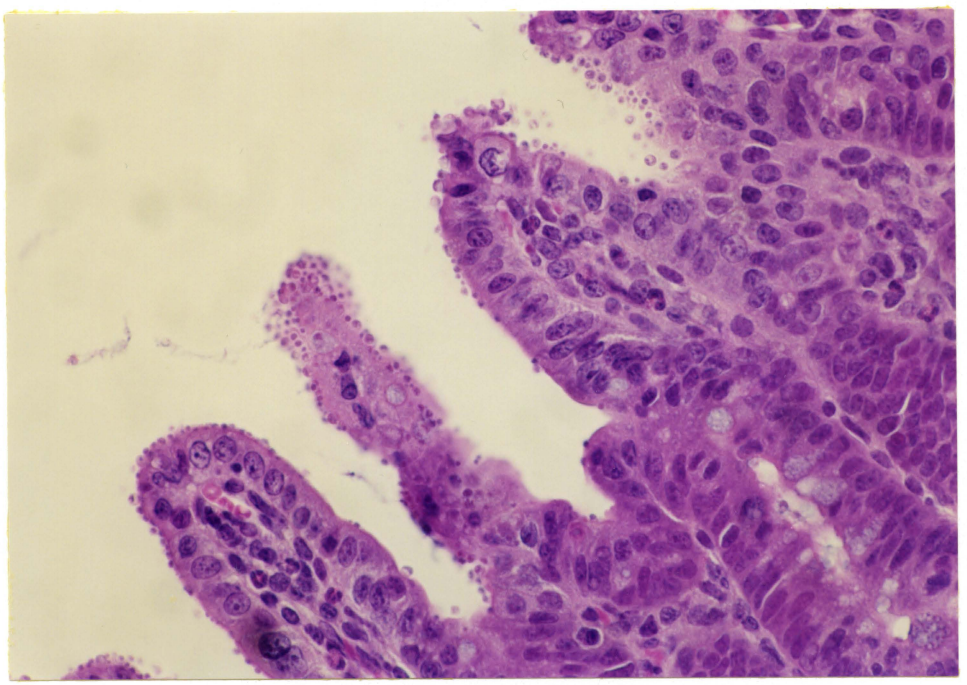

Figure 12. Histologic section of ileum from mouse orally infected with 10^5 *C. parvum* oocysts. Note the numerous parasites with different sizes within the brush border of the enterocytes (H. & E. stain) (x400).

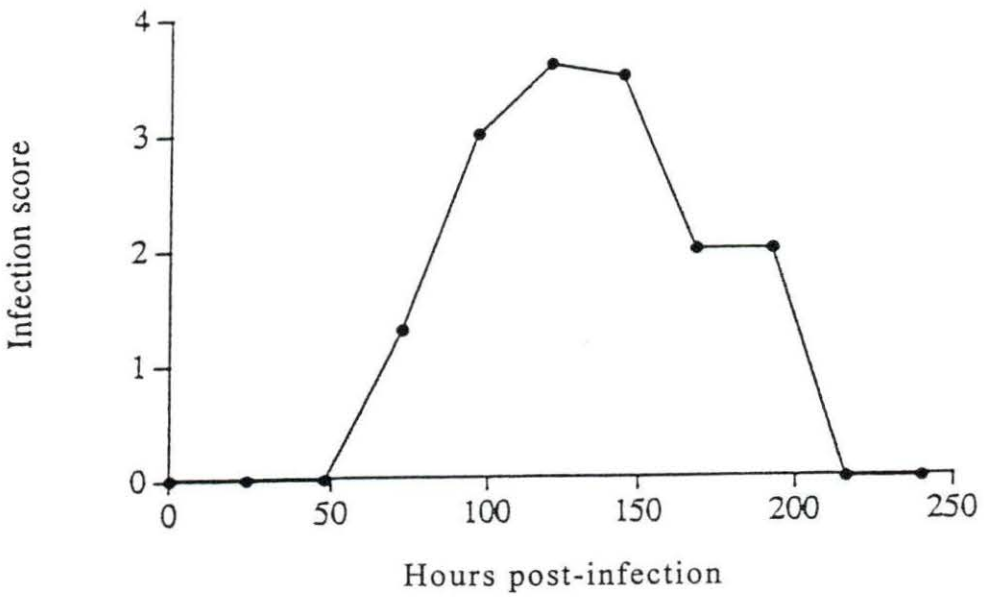

Figure 13. Intensity of *C. parvum* infection of suckling mice in infected but untreated groups. Mice were infected orally with 10^5 oocysts at 6 days of age. Results are the means of 7 mice at each time period. Infection score: < 5/field = 1+; 5 to 25/field = 2+; 25 to 50/field = 3+; > 50/field = 4+. Note that infection appears as early as 72 hours post-infection, and peaks at 96 to 144 hours post-infection.

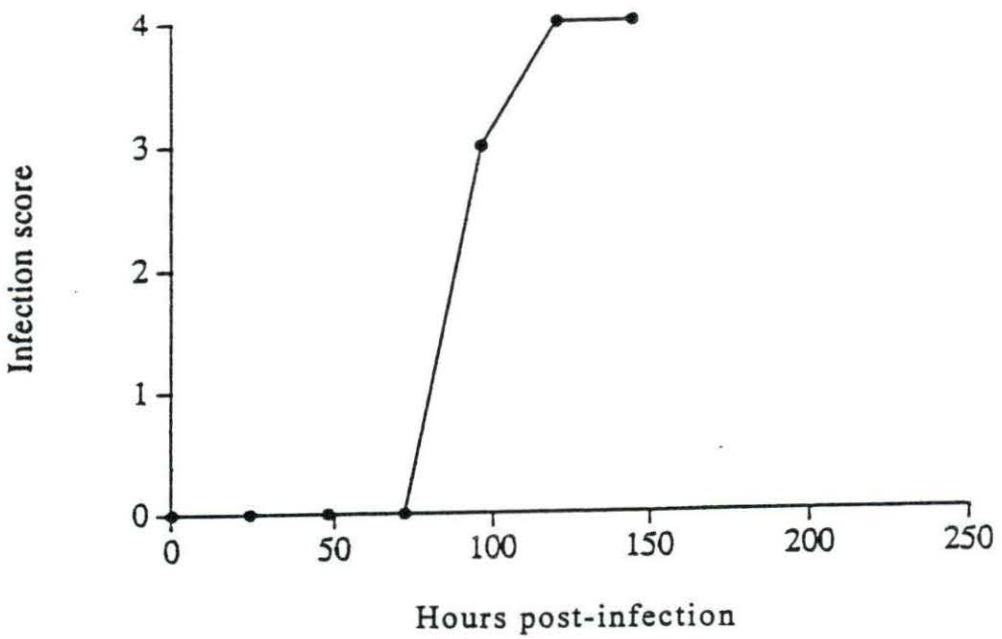

Figure 14. Effect of novobiocin (50 mg/kg/day) on experimental *C. parvum* infection of suckling mice. Mice were infected orally with 10^5 oocysts at 6 days of age. Medication was started at 8 day of age and given daily by gavage. Results are the means of 3 mice at each time period. Infection scale is the same as described in Figure 13.

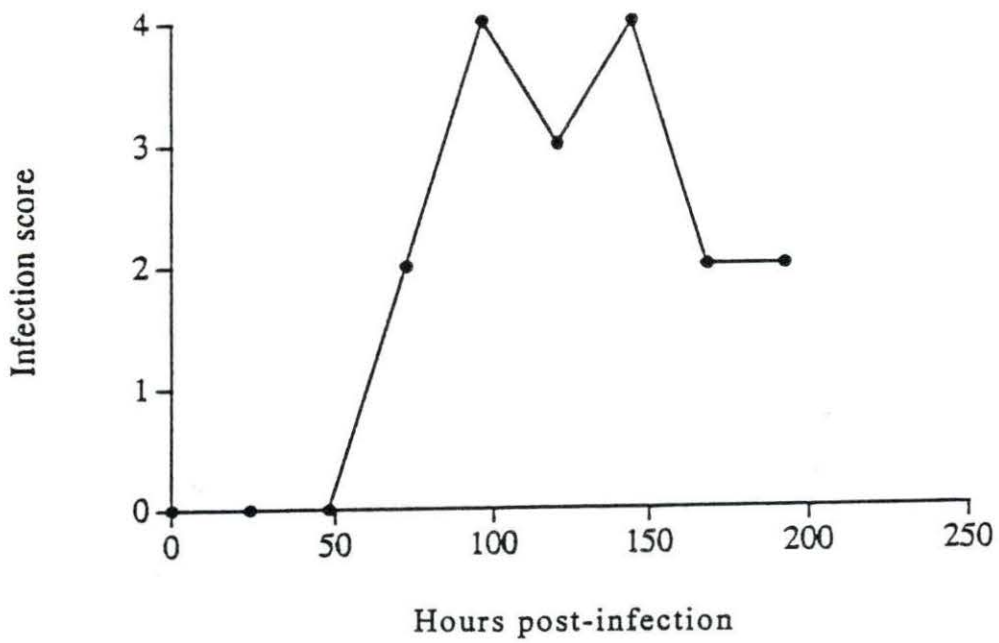

Figure 15. Effect of nojirimycin (50 mg/kg/day) on experimental *C. parvum* infection of suckling mice. Mice were infected orally with 10^5 oocysts at 6 days of age. Medication was started at 8 days of age and given daily by gavage. Results are the means of 3 mice at each time period. Infection scale is the same as described in Figure 13.

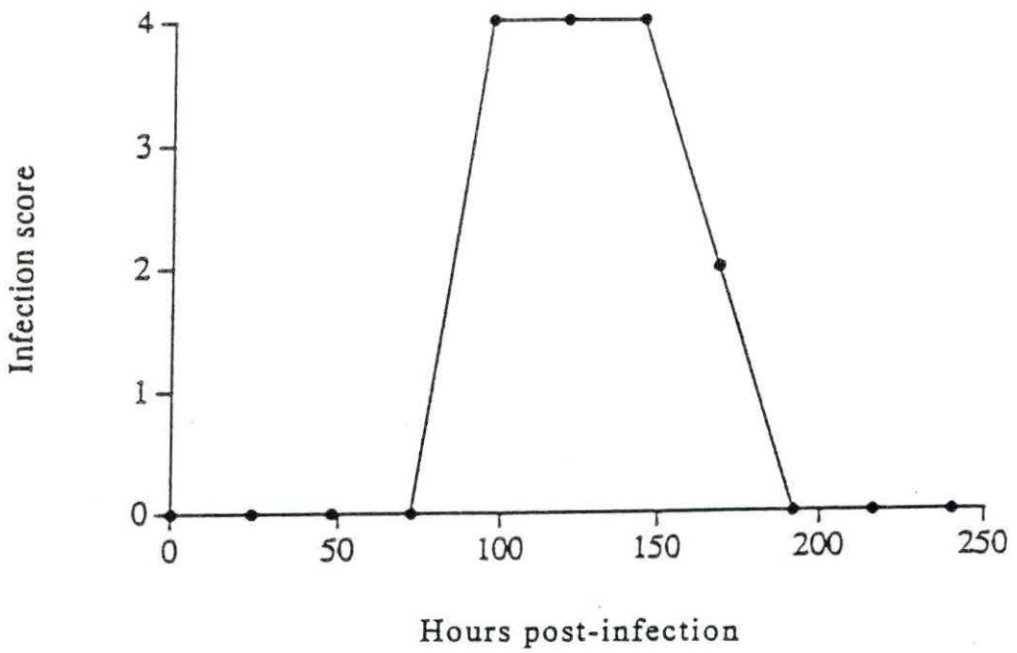

Figure 16. Effect of griseofulvin (100 mg/kg/day) on experimental *C. parvum* infection of suckling mice. Mice were infected orally with 10^5 oocysts at 6 days of age. Medication was started at 8 days of age and given daily by gavage. Results are the means of 3 mice at each time period. Infection scale is the same as described in Figure 13.

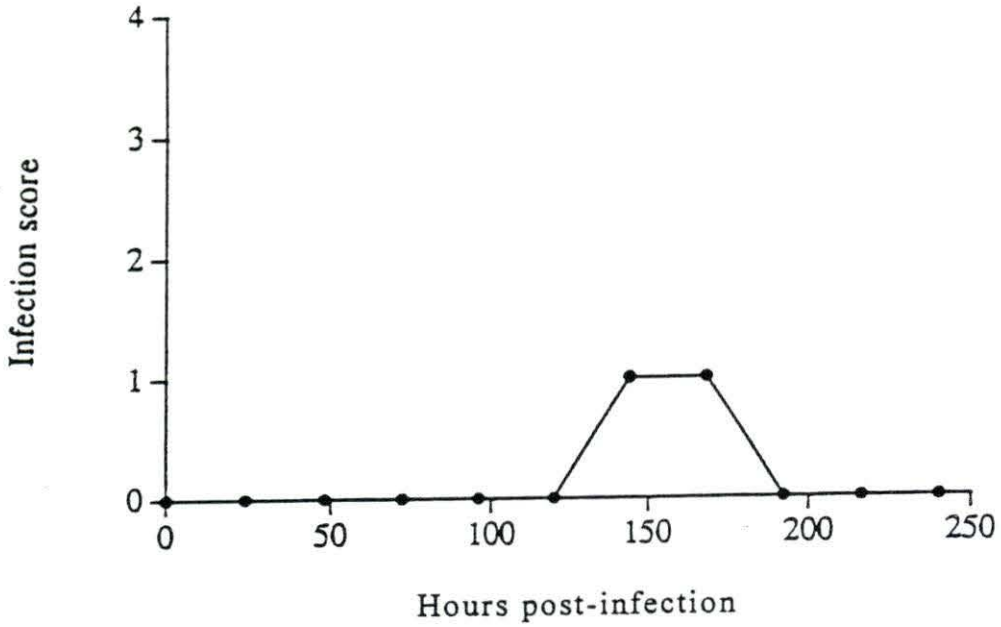

Figure 17. Effect of azithromycin (400 mg/kg/day) on experimental *C. parvum* infection of suckling mice. Mice were infected orally with 10^5 oocysts at 6 days of age. Medication was started at 8 days of age and given daily by gavage. Results are the means of 3 mice at each time period. Infection scale is the same as described in Figure 13.

Discussion

The rationale for selection of these agents was based on their mechanism of action and the absence of previous studies (novobiocin, nojirimycin, griseofulvin) or reported efficacy in other models of cryptosporidiosis (azithromycin). Novobiocin inhibits the beta subunit of DNA gyrase to block supercoiling and chain formation in bacteria and thus prevents replication. Nojirimycin inhibits glucosidase thus prevents the breakdown of amylopectin to glucose (Romero et al.,1985). If amylopectin serves as an energy source for the organism, this antibiotic might inhibit growth. Griseofulvin is an antifungal antibiotic which binds to microtubules and inhibits mitotic spindle formation in fungal hyphae. Its action is similar to that of colchicine which inhibits mitosis in mammalian cells, but is too toxic for therapeutic use. Colchicine inhibited *C. parvum* infectivity in a human enterocyte cell culture (Wiest et al., 1993). Azithromycin suppressed *Cryptosporodium* oocyst shedding in immunosuppressed adult mice (Kimata et al., 1991). The mechanism of action of azithromycin in bacteria is inhibition of protein synthesis, but the mechanism of its action on protozoa is unknown.

Anti-cryptosporidial activity was only found in azithromycin-treated suckling mice. None of the drugs tested in this study totally suppressed *Cryptosporidium* infection.

Nojirimycin is a glucosidase inhibitor thus prevents the breakdown of amylopectin. In an earlier phase of our study, amylopectin concentration in sporozoites did not decrease with time up to 4 hours following excystation, which indicated that

amylopectin may not be the only energy source. This may explain the lack of effectiveness of nojirimycin against *Cryptosporidium*.

No anticryptosporidial activity was observed when using novobiocin and griseofulvin, expect for a slight delay (24 hours) of onset of infection.

Azithromycin showed marked anticryptosporidial activity, but did not eliminate infection completely even at a dosage (400 mg/kg/day) which was toxic to one-third of the mice. Similar results were observed with an immunosuppressed rat model, in which cryptosporidial infection was completely prevented by treatment with 200 mg/kg/day azithromycin for 9 to 10 days, but withdrawal of the drug after treatment was associated with recurrence of infection (Rehg, 1990). In prednisolone-immunosuppressed mice treated with azithromycin starting at 13 hours PI, the number of oocysts discharged was less than 1/100 of the control mice on 4 to 12 days PI (Kimata et al, 1991).

To date, no specific treatment for cryptosporidiosis has been found although over 150 agents have been tested (Reinemeyer, 1994). It is likely that any developments in this field will be made with drugs with novel mechanisms of action rather than with any existing antiprotozoal agents.

GENERAL SUMMARY

To date, no effective treatment for cryptosporidiosis has been reported although more than 150 agents have been tested (Reinemeyer, 1994). Experimental work has been hampered by a lack of information on biochemical processes of the organism as potential targets for drug intervention and the absence of satisfactory *in vitro* cultivation methods. This thesis reports efforts to address some of these problems.

Flat explant culture techniques were used in an attempt to grow *C. parvum* on pig and mouse intestinal mucosal explants. Two methods were used to infect explants. *Cryptosporidium* infections on both *in vitro* infected and pre-infected explants during the entire 120 hours of culture were demonstrated by direct immunofluorescent antibody. It is apparent that both pig and mouse explants were susceptible to *in vitro* infection of *Cryptosporidium* by exposure of explants to culture medium containing sporozoites. The DIF assay is simple to perform and has higher sensitivity than traditional staining procedures (Garcia et al., 1987), which make it useful as a detection method for *Cryptosporidium* infection. This culture model may provide a system for rapid pharmacological screening of anticryptosporidial drugs. However, the extent of multiplication and the number of parasites produced on intestinal explants are much lower than in experimentally infected mice or in chicken embryos. Further studies will attempt

to refine the model to include methods such as electron microscopy to confirm the presence of developmental stages of the parasite on explants and to quantify the mucosal infection. Several biochemical processes of *C. parvum* were investigated as potential targets for drug intervention. A similar mannitol cycle as that found in fungi has been recently identified in *E. tenella*. Two of the enzymes in mannitol cycle, M1PDH and M1Pase were also found in *C. parvum*. However, in our study no mannitol was detected in oocyst preparation and the presence of the mannitol cycle in *C. parvum* could not be confirmed. This may due to the lack of sensitivity of the method or to the fact that, unlike other coccidia (e.g., *Eimeria* and *Isospora* spp.), *C. parvum* oocysts undergo sporogony while they are within the host cells. Mannitol may be used up during sporulation so very low levels of mannitol would be present in oocyst excreted from feces. Like *Eimeria,* Large amounts of amylopectin granules have also been found in developmental stages of *Cryptosporidium* by transmission electron microscopy (Dubey et al.,1990). However, the glycolysis patten of *E. tenella* was not found in *C. parvum* sporozoites incubated in glucose-free HBSS at 37^{0}C. Lactate concentrations did not significantly increase with time. There was no decrease in amyloglucosidase release of glucose in excysted sporozoites for up to 4 hours. This results suggest that amylopectin may not be the major source of energy for *Cryptosporidium*. Further studies are required to determine the metabolic energy source for *Cryptosporidium*.

The efficacy of potential anticryptosporidial drugs on *Cryptosporidium* infection of suckling mice was evaluated in the third phase of the study. No anticryptosporidial

activity was found in suckling mice treated with novobiocin (50 mg/kg/day), nojirimycin (50 mg/kg/day) or griseofulvin (100 mg/kg/day). None of them diminished the degree or duration of infection. Only light infections (+1) were detected at 144 and 168 hours post-infection in mice treated with azithromycin (400 mg/kg/day), which was significantly lower than untreated mice or mice treated with other three drugs. Although a marked reduction of infection was observed in azithromycin treated mice, there was no complete elimination of *Cryptosporidium* infection. The efficacy of azithromycin confirms results obtained in other studies. It is likely that any developments in this field will be made with drugs with novel mechanisms of action rather than with any existing antiprotozoal agents.

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