Interaction of the Human Serine Protease Inhibitor Alpha-1-antitrypsin with Cryptosporidium parvum

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INTERACTION OF THE HUMAN SERINE PROTEASE INHIBITOR
ALPHA-1-ANTITRYPSIN WITH CRYPTOSPORIDIUM PARVUM

by

John R. Forney

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biology

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1997
ABSTRACT

Interaction of the Human Serine Protease Inhibitor Alpha-1-antitrypsin

with Cryptosporidium parvum

by

John Russell Forney, Doctor of Philosophy

Utah State University, 1997

Major Professor: Dr. Mark C. Healey, DVM, PhD
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The human serine protease inhibitor (serpin) alpha-1-antitrypsin (AAT) was studied for potential interaction with components of the protozoan parasite Cryptosporidium parvum. A homogenate prepared from C. parvum oocysts was incubated with purified human AAT, and complexes formed between the serpin and components of the homogenate were detected using an enzyme-linked immunosorbent assay (ELISA). Serpin:parasite interactions were effectively blocked by preincubating AAT with a cognate target enzyme, porcine pancreatic elastase, prior to performing the ELISA on the homogenate. Incubation of a mixture of C. parvum oocysts and sporozoites with AAT demonstrated preferential fluorescence labeling of the sporozoite surface membrane by indirect immunofluorescence assay. Localization of serpin complexes on sporozoites was confirmed by immunogold electron microscopy.
AAT was evaluated for in vitro anticryptosporidial activity in a bovine fallopian tube epithelial (BFTE) cell culture system using both oocysts and filter-purified sporozoites as inocula. Serial dilutions of AAT were mixed with oocysts (or sporozoites) and used to inoculate BFTE cell monolayers. Inoculated cells were maintained at 37°C/5% CO₂ and collected at 24-, 48-, 72-, and 96-hr post-inoculation intervals. The addition of AAT and other select protease inhibitors (i.e., antipain, aprotinin, leupeptin, soybean trypsin inhibitor, and phenylmethylsulfonyl fluoride) significantly inhibited parasite infection (P<0.01) in a concentration- and time-dependent manner when bleach-decontaminated oocysts were used in the inoculum.

The anticryptosporidial activity of AAT is postulated to be linked to an antagonistic effect on oocyst excystation and, putatively, the forced expenditure of bioenergetic reserves prior to host cell invasion. This postulate was supported by the observations that serpin activity had no statistically significant effect on reducing established in vitro infections (i.e., 24 hr post-inoculation prior to addition of AAT) and did not inhibit infection of BFTE cells when inoculated with sporozoite preparations. The combined application of AAT and the aminoglycoside paromomycin demonstrated a synergistic anticryptosporidial effect on in vitro infection and suggested the basis for a multi-agent therapeutic protocol in preventing cryptosporidiosis. These studies collectively demonstrated an anticryptosporidial potential for serine protease inhibitors, in particular for AAT, and suggest an alternative approach to conventional therapeutic strategies.
ACKNOWLEDGMENTS

I am thankful to the members of my supervisory committee, Drs. Mark C. Healey, Shiguang Yang, Stanley Allen, Reed Warren, and Bill Barnett. Their time, energy, and patience heightened the potential for success in my research endeavors. I am grateful for the collaborative insights and encouragement of Drs. Daryl DeWald and Dana Vaughan that propelled many of my “extracurricular” projects. I enthusiastically acknowledge the technical expertise of Chunwei Du and express my thanks for her support in the laboratory.

The studies detailed in this dissertation were funded in part by the Utah Agricultural Experiment Station and the Long-Term Health Education and Training Program, Office of The Surgeon General, United States Army Medical Department.

A special thanks is due to my family for willingly sacrificing holiday plans, summer vacations, weekends, and almost every evening while I studied and worked in the laboratory. Cinderella, Andy, and Luke best understand the eccentric character of this tour of duty. And finally, thanks to my “Point Man” for guiding my efforts and sustaining the pace.

John R. Forney (Russ)
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INTRODUCTION

In a span of less than 20 years, Cryptosporidium parvum has emerged from the initial reports of human infection in 1976 to world-wide recognition as an infectious agent of considerable morbidity in the AIDS patient population. This enteropathogenic parasite invades the intestinal epithelium and is capable of sustaining chronic patent infection in susceptible hosts, a condition exacerbated by the present lack of efficacious therapy. Research emphasizing a more thorough understanding of the parasite's developmental maturation and efforts to identify strategies for chemotherapeutic intervention are both timely and relevant.

This dissertation presents a succession of investigative studies that have enhanced the collective knowledge of the cellular physiology and immunobiology of C. parvum. The narrative is organized in a multiple-paper format supporting a common thematic focus; the functional interaction between a human serine protease inhibitor (serpin), alpha-1-antitrypsin (AAT), and the early developmental stages of the parasite. In addition to this introduction, the first chapter reviews the literature and summarizes the research objectives inherent to the studies presented in subsequent chapters. The literature review is topically arranged and chronicles the relevant findings of other investigators that influenced the current undertaking.
Chapter 2 describes the initial study identifying an interaction between AAT and *C. parvum* and provides an impetus for the evaluation of the in vitro anticryptosporidial activity of AAT (Forney et al., 1996b). The discussion section presents a hypothesized role for AAT in reducing the vulnerability of host cells to parasite protease activity during the early stages of infection. The implications of this study suggest several previously unexplored characteristics of the immunobiology of *C. parvum* and potentiate an innate defense mechanism against cryptosporidial infection in humans that is mediated, at least in part, by intrinsic serpin activity.

Chapter 3 characterizes the functional significance of the interaction between protease inhibitors and *C. parvum*. This chapter is based on the first published report demonstrating a definitive association between proteolytic activity and oocyst excystation and provides evidence of an antagonistic effect by serine protease inhibitors on excystation (Forney et al., 1996c). Further, it was demonstrated that *C. parvum* expressed multiple proteolytic enzyme activities during excystation; 1 protease component was inhibited by select serine protease inhibitors and the other component was susceptible to inhibition by the cysteine protease inhibitor L-trans-epoxysuccinyl-leucylamindo-(4-guanidino)-butane (E-64).

Chapter 4 provides a thorough and systematic evaluation of the antagonistic effect of AAT on oocyst excystation (Forney et al., 1997). Experiments were performed to discern the influence of oocyst wall permeability and the effect of inoculation source (i.e., oocysts or sporozoite)
on AAT activity. Further, in vitro efficacy was monitored over extended post-inoculation incubation periods and against established infections to evaluate serpin activity against asexual intermediates of *C. parvum*. An experimental protocol involved the reapplication of AAT at 24-hr intervals to determine whether bioavailability had a significant impact on in vitro activity. This final manipulation had no effect on in vitro efficacy, suggesting that the biological availability of the serpin in cell culture was not a limiting factor in its anticryptosporidial activity.

Chapter 5 incorporates a series of concise manuscripts (i.e., research notes, short communications) derived from studies investigating the anticryptosporidial potential of protease inhibitors in a bovine fallopian tube epithelial (BFTE) cell culture system. The first section reports the use of selected serine protease inhibitors to reduce *C. parvum* infectivity in BFTE cells (Forney et al., 1996a). The following section summarizes the results of the initial in vitro study evaluating the anticryptosporidial activity of AAT (Forney et al., 1996d). The final section of Chapter 5 describes the synergistic activity of AAT in combination with the aminoglycoside paromomycin. This final note presents a strategy for chemotherapeutic intervention that includes compounds targeting different developmental attributes of the parasite, such as oocyst excystation and propagation of asexual intermediates.

Concluding remarks in Chapter 6 summarily review the relative contributions of each of the individual studies and unify the collective results in a final discussion of *C. parvum* excystation and infectivity. Speculation is
made regarding the potential therapeutic application of protease inhibiting compounds in treating chronic cryptosporidiosis, in both single and multiple drug combinations. A final discussion includes recommendations for future research efforts.

LITERATURE REVIEW

Cryptosporidium parvum

E. E. Tyzzer (1907) is acknowledged with the first published report of a cryptosporidial organism when he described a sporozoan found in the peptic glands of laboratory mice. He further detailed the characteristic morphology and life-cycle stages of this parasite and established the basis for the species description of Cryptosporidium muris (Tyzzer, 1910). Two years later Tyzzer (1912) described a second species, C. parvum, in the small intestine of laboratory mice. The next 50 years witnessed very little in the way of scientific enlightenment regarding C. parvum; the parasite was generally considered to be a commensal organism in the intestinal tract of vertebrate hosts. Various reports of additional Cryptosporidium species were recorded during this period, primarily based on an assumption of unique host specificity. The majority of these reports have since been repudiated by cross-transmission studies that define C. parvum as a multi-host parasite exhibiting little, if any, host specificity (Current, 1986; Levine, 1986; Upton et al., 1989; Current and Garcia, 1991; O'Donoghue, 1995).
Levine (1986) systematically reviewed and updated the salient characteristics of the genus *Cryptosporidium* and validated its taxonomic classification in the phylum Apicomplexa, class Sporozoasida, order Eucoccidiorida. *Cryptosporidium* spp. are further classified into the suborder Eimeriorina based on their sexual reproductive stages (gamonts) and nonmotile zygote (oocyst). The genus is the sole representative of the family Cryptosporididiidae, distinguished by the presence of "free" sporozoites (i.e., lacking an intervening sporocyst) within the oocyst and the presence of an attachment organelle ("feeder") at the base of the parasitophorous vacuole (Johnson et al., 1990; Cai et al., 1992).

In many respects, *Cryptosporidium* exhibits a similar pattern of ontogeny to that observed in other coccidian genera, such as *Eimeria* and *Isospora*, with a monoxenous (single host) life cycle (Fayer et al., 1990; O'Donoghue, 1995). However, it is the differences in propagation and host cell infection that characterize this enigmatic parasite. The infective stage is the oocyst, a small spherical to elliptical shaped form approximately 4-7 mm in diameter, each one containing 4 sporozoites. Sporozoites excyst in the lumen of the small intestine and invade the apical surface of epithelial cells lining the microvilli. Within an intracellular, but extracytoplasmic, parasitophorous vacuole, the sporozoite differentiates into a trophozoite and undergoes multiple asexual reproduction (merogony) producing meronts (Gobel and Brandler, 1982; Current, 1986; Rosales et al., 1992, 1993).
The formation of an intramembranous vacuole by *C. parvum* appears to be a unique evolutionary characteristic of this parasite. A thorough search of the literature failed to reveal any phylogenetically related organism that forms a similar developmental residence within an infected host cell. There are many examples of microbial pathogens that occupy a more conventional intracellular niche, within an intracytoplasmic vacuole. Such is the case for obligate intracellular bacteria represented by the genera *Shigella*, *Listeria*, *Neisseria*, and *Vibrio* as well as for the protozoan parasites *Plasmodium* and *Toxoplasma* spp. The biochemical and molecular mechanism that fosters the formation of the cryptosporidial parasitophorus vacuole is a confounding puzzle to the parasitology community.

Two distinct types of meronts exist in the cryptosporidial life-cycle (Vetterling et al., 1971; Current, 1986; Fayer et al., 1990; Ostrovska and Paperna, 1990). The mature type I meront develops 8 merozoites that are released from their intramembranous location and recycle the infection in the intestinal epithelium. Type II meronts contain 4 merozoites that, upon release, infect other intestinal epithelial cells and initiate a sexual reproductive cycle (syngamy). Merogony amplifies parasite numbers and the type I meront can putatively undergo multiple propagatory cycles before differentiating into the type II meront stage. Separate macrogamonts and microgamonts develop from type II merozoites, and microgametes are released to fertilize mature macrogamonts (Gobel and Brandler, 1982). The resulting zygote undergoes sporogony within the host epithelial cell and, upon maturation, is released
into the intestinal lumen as an infective oocyst (Bird and Smith, 1980; Yoshikawa and Iseki, 1992). Approximately 80% of the sporulated oocysts are contained within a thick, multi-layered, environmentally resistant wall and pass from the host in fecal excrement. The remaining oocysts are characterized by a single, thin oocyst wall and are thought to excyst during luminal passage. Thin-walled oocysts perpetuate an autoinfective cycle in the chronically infected host (Current, 1985; Current and Reese, 1986; Fayer et al., 1990). In light of the multiple cycling of type I meronts and the autoinfective potential of thin-walled oocysts, it is not difficult to visualize the sustained proliferation of *C. parvum* that accompanies patent infection.

The role of surface adhesin molecules in the recognition and attachment of *C. parvum* to the membrane of susceptible epithelial cells is not clearly defined. Similarly, the sequence of biochemical and physiological events that result in hypertrophy of microvilli at the site of parasite attachment, fusion of extended microvillar membranes, and retention of the parasitophorous vacuole within the host cell membrane is poorly understood. Investigators have reported a putative role for surface-expressed glycoproteins or lectin-associated membrane affinity to aid in the initial attachment of *C. parvum* sporozoites to host cells (Bonnin et al., 1991a, 1991b; Tilley et al., 1991; Petersen et al., 1992; Thea et al., 1992; Hamer et al., 1994; Joe et al., 1994; Mitschler et al., 1994; Tilley and Upton, 1994). The role of specific receptor-ligand interactions between invasive *C. parvum* stages (i.e., sporozoites, type I and II merozoites, microgametes) and the host cell has not
been thoroughly investigated. It is conceivable that cryptosporidial protease-like components are involved in interactions at the host cell membrane level such as has been demonstrated with related coccidians (Adams and Bushell, 1988; Fuller and McDougald, 1990). Bristow et al. (1995) reported a cell surface adhesion and fusion mechanism between the human immunodeficiency virus and host cells that involved recognition between serine protease-like receptors and corresponding structures analogous to serpin reactive sites. The initial adherence of sporozoites to host cell membranes may facilitate the spatial orientation of parasite proteases that further enhance cell attachment and, potentially, contribute to the parasite’s unique intramembranous location within infected cells.

Panciera et al. (1971) reported the initial incidence of disease associated with *C. parvum* when he cited the parasite as the causative agent of diarrhea in calves. Twenty-five years later, the literature abounds with reports of diarrheal illness in many different animals attributed to *C. parvum*. Veterinary medicine recognizes this protozoan as a significant etiology of neonatal diarrhea in calves and lambs, with a concomitant economic impact on the domestic animal industry. A multitude of reports, citing cryptosporidiosis in 170 different animal species and a well documented record of pathogenesis in over 50 countries worldwide, clearly expands Tyzzer’s initial description of a benign commensal to a ubiquitous enteric pathogen (Fayer et al., 1990; Current and Garcia, 1991; Powell, 1994).
Human cryptosporidiosis

Despite the association between *C. parvum* and disease in animals for over two decades, it was the initial reports of human cryptosporidiosis that kindled the greatest spark of international scientific interest in the parasite. In 1976, *Cryptosporidium* spp. was independently reported in two immunocompromised patients as the primary etiology of profuse diarrhea (Meisel et al., 1976; Nime et al., 1976). Reports of clinical illness from cryptosporidiosis in both immunocompetent and immunocompromised patient populations appeared intermittently in the medical literature over the next decade. Ungar et al. (1990a) and Petersen (1992) have reviewed the increased incidence of human cryptosporidiosis in the immunocompromised host, a trend that parallels the rise in HIV infection noted in the mid-1980s and persists in the current medical and scientific literature (Brandonisio et al., 1993; Cotte et al., 1993; Georgiev, 1993; Goodgame et al., 1993, 1995; Moroni et al., 1993; Giang et al., 1994; Mifsud et al., 1994; Molbak et al., 1994; Sorvillo et al., 1994). The conclusive association between *C. parvum* infection and a chronic, watery diarrheal illness in patients with AIDS has substantiated interest in the immunology and biology of this protozoan (Current et al., 1983; Tzipori, 1983; Casemore, 1990; Ungar et al., 1991; Gellin and Soave, 1992).

The epithelial lining of the microvilli in the small intestine is the preferential site of parasite infection, and incubation periods ranging from 2-24 days are typically noted in human hosts (Tzipori et al., 1981a, 1981b; Goodgame et al., 1995). The ensuing diarrhea is generally self-limited in an
otherwise healthy individual with a spectrum ranging from asymptomatic, subclinical cases to acute diarrhea, abdominal cramps and nausea of 1-3 wk duration (Phillips et al., 1992). Spontaneous eradication of the parasite from the intestinal mucosa and protective acquired immunity is the general outcome of infection in the immunocompetent individual. The most severe consequence of human cryptosporidiosis occurs in the immunodeficient host. These individuals frequently develop a profuse, protracted diarrheal illness which progresses to a chronic patent infection exhibiting colonization of the intestinal epithelium and, potentially, dissemination into the alveolar and tracheal epithelium, stomach, pancreatic duct, and gall bladder (Garone et al., 1986; Ramsden and Freeth, 1989; Travis et al., 1990; Petersen, 1992; Giang et al., 1994; Mifsud et al., 1994). Fluid loss from voluminous diarrhea can become life-threatening for the chronically infected patient. This emerging clinical scenario has established C. parvum as a significant opportunistic pathogen and a cause of severe morbidity in susceptible patients (Davis et al., 1987; Kahn et al., 1987; Petersen, 1992; Sun, 1994). Investigators have further reported an association between severe cryptosporidiosis and profound malnutrition that, if unresolved, results in a clinically apparent illness in children closely resembling that seen in AIDS patients (MacFarlane and Horner-Bryce, 1987; Sallon et al., 1988; Kuhls et al., 1992; Guarino et al., 1993).

The underlying pathophysiology of cryptosporidial diarrhea is unknown. Histologically, C. parvum infection is characterized by blunted, fused microvilli in the small intestine with a concomitant crypt hyperplasia
(Tzipori, 1983; McDonald and Bancroft, 1994; Goodgame et al., 1995; Moore et al., 1995). The voluminous, secretory nature of the resulting diarrhea, frequently termed “cholera-like,” suggests an enterotoxogenic etiology, but studies have yet to define an operative agent (Brown et al., 1992; Chui and Owen, 1994; Eggleston et al., 1994; Guarino et al., 1994).

Cryptosporidiosis has emerged dramatically as a significant water-borne infection, best illustrated by the massive outbreak of cryptosporidiosis in Milwaukee, Wisconsin in 1993 that totaled an estimated 403,000 cases of infection (MacKenzie et al., 1994). In Milwaukee and related outbreaks, the infective stage of the parasite (i.e., oocyst) has been recovered from treated municipal water supplies (Peeters et al., 1989; Korich et al., 1990; Robertson et al., 1992; MacKenzie et al., 1994; Aldom and Chagla, 1995). A growing body of evidence suggests that cryptosporidial contamination of run-off water from commercial livestock operations and human sewage treatment facilities perpetuates a significant parasite reservoir in the environment (Jokipii et al., 1985; Madore et al., 1987; Current and Garcia, 1991; Hansen and Ongerth, 1991; LeChevallier et al., 1991; MacKenzie et al., 1994). In both laboratory and environmental settings, C. parvum oocysts demonstrate exceptional resistance to chemical treatment and disinfection, particularly at those levels of treatment determined to be safe for human consumption (Campbell et al., 1982, 1993; Fayer et al., 1990; Fayer, 1994, 1995). The Centers for Disease Control (1994) now consider cryptosporidiosis a major public health concern for the United States and a significant emerging disease threat.
The severity and chronicity of human cryptosporidiosis is accentuated by the present lack of clinically efficacious therapy and an incomplete understanding of the immunobiology of the parasite (Flanigan and Soave, 1993; Moroni et al., 1993; Flanigan, 1994; O'Donoghue, 1995). Reports of efficacious anticryptosporidial agents are largely based on anecdotal evidence that has not been sustainable in controlled clinical trials (Wittenberg et al., 1989; Zu et al., 1992; Flanigan and Soave, 1993). Additionally, in vitro evidence of potential therapeutic efficacy against cryptosporidial infection has not been successfully transferred to in vivo laboratory models or to infected patients in a reproducible fashion (Wittenberg et al., 1989; Zu et al., 1992; Flanigan and Soave, 1993; Verdon et al., 1994). Attempts to employ conventional chemotherapeutic agents with established antimicrobial, antifungal, or antiviral efficacy have not been consistently and reproducibly successful in preventing or abrogating C. parvum infection in humans.

The immune status of the host appears to be the most significant determinant of C. parvum infection severity. Predictably, immunotherapy and immunological intervention highlight current research efforts to develop an effective anticryptosporidial protocol. Intervention in the form of passive oral transfer from hyperimmune colostrum and colostral derivatives has shown encouraging results in the treatment of the immunocompromised host (Mata, 1986; Tzipori et al., 1986; Ungar et al., 1990b; Plettenberg et al., 1993; Shield et al., 1993). Lactogenic transfer, however, continues to be plagued by an inconsistent clinical response and the inevitable recrudescence of infection.
following cessation of therapy (Tzipori, 1983; Moon et al., 1988; Arrowood and Sterling, 1989; Moody et al., 1991; Tachibana et al., 1991). Strategies to enhance immune responsiveness with immunomodulating substances (i.e., dehydroepiandrosterone, azidothymidine, dialyzable leukocyte extracts) have shown preliminary success in reducing the severity of infection and indicate a potential route for continued therapeutic research (Chandrasekar, 1987; Greenberg et al., 1989; McMeeking et al., 1990; Rasmussen et al., 1992; Rasmussen and Healey, 1992a, 1992b). Likewise, cryptosporidiosis in the iatrogenically immunosuppressed host is frequently resolved once the suppressive therapy is discontinued and immune responsiveness regains normal efficacy (Miller et al., 1983; Foot et al., 1990).

The human immune response to *C. parvum* infection has been an area of considerable research. Studies have demonstrated a significant humoral response to *C. parvum* and have reported the production of antibodies directed against antigens expressed in one or more developmental stages (Taghi-Kilani et al., 1990; Fayer et al., 1991; Mosier et al., 1992; Nina et al., 1992; Georgiev, 1993; Moss et al., 1994; Sorvillo et al., 1994). In vitro neutralization of surface-expressed epitopes by both monoclonal and polyclonal antibodies has been shown to reduce sporozoite and merozoite infectivity, implicating a role for acquired antibodies in protection from repeated exposure (Riggs and Perryman, 1987; Harp et al., 1989; Bjorneby et al., 1990; Tilley et al., 1991; Uhl et al., 1992; Perryman et al., 1993; Tilley and Upton, 1994; Harp et al., 1995). The putative role of the cell-mediated immune system, and its various cytokine
mediators, in the adaptive immune response to *C. parvum* has received considerable attention in the literature. Reconstitution studies in nude and SCID mice have shown an ameliorating effect on patent infection following the adoptive transfer of human T lymphocytes, bone marrow cells, or cells of lymph node origin (Mead et al., 1991; Whitmire and Harp, 1991; Harp et al., 1992; Moss and Lammie, 1993; Cozon et al., 1994). Several studies have shown that the depletion of differentiated effector cells such as NK cells, CD8 T lymphocytes, and CD4 T lymphocytes alters the course of infection in laboratory models (Ungar et al., 1990b; Harp and Moon, 1991; Ungar et al., 1991; Kuhls et al., 1992; Boher et al., 1994; McDonald et al., 1994; Mead et al., 1994; Perryman et al., 1994). There is, however, evidence to suggest an absence of measurable effects on resistance following selective depletion of effector cells (Harp and Whitmire, 1991; Ungar et al., 1991; Rasmussen and Healey, 1992b; Chen et al., 1993a; Harp et al., 1995). Reduced levels of interferon gamma (IFN-γ) and interleukin-2 (IL-2) have been shown to contribute to enhanced murine infection (Ungar et al., 1991; Chen et al., 1993a, 1993b). Clearly, the mutual contributions of both the humoral and cell-mediated adaptive immune responses are intimately associated with eradication of cryptosporidial infection and formation of protective immunity to subsequent challenge (Heyworth, 1990; Tachibana et al., 1991; Zu et al., 1992; McDonald and Bancroft, 1994; Mead et al., 1994; Harp et al., 1995).
It is also apparent that the immune response to cryptosporidiosis is a complex, and largely unresolved, process.

Anecdotal evidence suggesting an age-related susceptibility to cryptosporidiosis in humans most likely reflects a lack of immunologic maturity of the host at the time of primary exposure (MacFarlane and Horner-Bryce, 1987; Daoud et al., 1990; Tangermann et al., 1991; Kuhls et al., 1992). Notwithstanding the significance of underlying physiological changes during the first several years of human development, it is probable that maturation of the neonatal immune system accounts for the progressively enhanced resistance to *C. parvum* infection observed in adolescence and adults (Fripp et al., 1991; Zu et al., 1994).

The innate, nonspecific, nonadaptive defense mechanisms of the human system are largely unexplored in regard to *Cryptosporidium* infection. Reports of the beneficial effects of normal gut microflora in resisting *C. parvum* infection in mice and the observation of anticryptosporidial activity in nonimmune colostrum and bovine milk suggest that one or more nonspecific factors, outside the parameters defined by the adaptive immune response, potentially reduce host vulnerability and morbidity associated with *C. parvum* infection (Brown et al., 1992; Harp et al., 1992; Garber et al., 1994). At present, the dynamics of early cryptosporidial infection and host defenses against invasive stages of the organism are not fully understood.
Alpha-1-antitrypsin

Alpha-1-antitrypsin (AAT) is a single chain glycoprotein composed of 394 amino acid residues with a molecular weight of approximately 56 kDa (Travis and Salvesen, 1983). AAT is one of a group of human serine protease inhibitors (serpin) found in peripheral circulation and tissue fluids. Collectively, the serpin family provides intrinsic regulation of proteolytic activity from endogenous proteases (Baumstark, 1967; Joslin et al., 1993). These inhibitors are particularly active during periods of acute inflammation and are substantive components of the “acute phase” plasma protein response elicited during the inflammatory process (Travis and Salvesen, 1983; Carrell and Travis, 1985; Whicher et al., 1993; Potempa et al., 1994). In the human system, AAT inhibits excessive protease-mediated tissue destruction from neutrophil elastase and provides systemic protection from enzymatic degradation in adjacent tissues (Cohen, 1975; Beatty et al., 1980; Carrell, 1986; Castellucci et al., 1994).

The AAT glycoprotein undergoes a dramatic increase in hepatocellular synthesis during acute inflammation. This episodic production is primarily mediated by the cumulative interactions of interleukin-1 (IL-1) and interleukin-6 (IL-6) and manifests itself in serum concentrations several magnitudes greater than those observed in the normal, noninflammatory state (Koj et al., 1993; Perlmutter, 1993; Potempa et al., 1994; Kanakoudi et al., 1995). While the primary source of systemic AAT is of hepatic origin, increasing evidence supports the synthesis and release of supplemental AAT
from extrahepatic sources. Reports of enhanced AAT synthesis in response to inflammatory cytokines have been attributed to mononuclear cells from peripheral circulation and intestinal epithelial cell lines in culture (Perlmutter et al., 1989, 1990; Molementi et al., 1993). Perlmutter and his colleagues (1989) first demonstrated the synthesis and secretion of AAT in response to IL-6 stimulation in a human adenocarcinoma colonic epithelial cell line (Caco-2). Molementi et al. (1993) confirmed IL-6-induced AAT production in the differentiated Caco-2 cell line and also measured AAT synthesis in the undifferentiated enterocytic form of the Caco-2 cell line. These investigators further demonstrated AAT synthesis in the human intestinal epithelial cell line T84, another undifferentiated (crypt-like) cell line, induced by the additive and synergistic action of IL-1 and IL-6. These findings potentiate an in vivo mechanism for AAT synthesis and secretion in the human intestinal tract independent of crypt-to-villus differentiation.

Alpha-1-antitrypsin performs its inhibition action through the formation of a 1:1 molecular complex with a serine protease (Baumstark, 1967; Carrell, 1986). The reactive site of AAT, Met358:Ser359, interacts with the active site of the target enzyme and blocks proteolytic activity by the bound enzyme. In effect, AAT acts as a substrate analog for the cognate enzyme. The serpin:enzyme complex (SEC) formed by the reactive site interactions demonstrates high affinity in its binding association under physiological conditions, primarily as a result of covalent attractions between
the serpin and its cognate enzyme (Perlmutter et al., 1990; Herve and Ghelis, 1991; Joslin et al., 1993). The AAT reactive site loop imparts a degree of functional specificity to the molecule; this portion of the molecule must closely mimic the physiochemical properties of an appropriate substrate to achieve serpin:protease recognition (Perlmutter, 1993). The SEC is rapidly cleared from circulation and both molecules are physically eliminated from further enzymatic activity (Carrell, 1986; Joslin et al., 1993).

There is considerable evidence of a minor structural rearrangement of the AAT molecule following complex formation with neutrophil elastase. Cleavage of the complexed serpin at the methionine reactive site releases the stressed configuration of the reactive site loop and exposes a small (pentapeptide) sequence of the AAT protein (Perlmutter et al., 1990; Joslin et al., 1991; Perlmutter, 1993). This exposed peptide fragment includes the carboxyl terminal portion of the protein and has been attributed a functional role in signaling upregulation of AAT synthesis in cells bearing a specific SEC receptor (Perlmutter et al., 1989). This mechanism of enhanced serpin synthesis following inhibition of a target protease (i.e., elastase) and recognition of the resulting complex by a SEC-specific receptor is a homeostatically relevant mechanism in the balance of elastase and anti-elastase activity in humans. Additionally, the SEC possesses neutrophil chemotactic properties and serves as an adjunct to other migration/attraction signals expressed during inflammation or tissue injury (Travis and Salvesen, 1983; Banda et al., 1988). Thus, the native AAT molecule demonstrates
functionally specific inhibitory activity and the structurally altered molecule in the SEC formation, specifically the carboxyl terminus associated pentapeptide, exhibits a relatively broad range of biological effector properties that enhance the process of inflammation in humans.

The literature contains very few previous examples of interactions between AAT and parasitic pathogens. Lushbaugh et al. (1981) reported the inhibition of *Entamoeba histolytica* cytotoxin, a proteolytic enzyme thought to account for the amoeba’s aggressive tissue invasion, by AAT and the alpha-2-macroglobulin (A₂M) protein complex. A₂M and, to a lesser extent, AAT were shown to inhibit tissue invasion of *E. histolytica* trophozoites. Further analysis revealed the isolated protein cytotoxin-enterotoxin to be a cysteine protease with a high sequence homology to papain-like enzymes; the trivial name “amiebapain” has been suggested for the enzyme (Stanley et al., 1995).

Modha and Doenhoff (1994a) reported the formation of complexes between human AAT and surface membrane components of *Schistosoma* spp cercariae. Their results substantiate previous reports of serine protease expression in schistosomes. The nature of the reported interaction, presumably AAT binding to surface protease enzymes, suggests the basis for a nonspecific host defense mechanism against exogenous protease activity introduced by the invading schistosomule. The AAT:schistosome interactions described by these investigators were further implicated as an example of immune evasion by antigenic mimicry; the formation of AAT complexes with surface-expressed parasite proteases blocks the
conformational availability of these immunogenic proteins to immune effector cells. A parasite coated by host endogenous serpin proteins could potentially evade host immune recognition (Modha and Doenhoff, 1994b).

**Proteases in coccidian parasites**

The role of natural and synthetic inhibitors in mediating the effects of exogenous parasitic proteases is an area of active investigation. Key to this research interest is the essential role of proteolysis in living cells and the potential parasiticidal effects that could be achieved by interfering with parasite protease activity. Proteolytic activity has been described in the invasion, infectivity, and pathogenicity of many parasitic pathogens. Protease-mediated events facilitate host cell attachment (Arroyo and Aldrete, 1989), membrane penetration (Hadley et al., 1983; Adams and Bushell, 1988; Fuller and McDougald, 1990), tissue migration (Modha et al., 1988; Modha and Doenhoff, 1994a; Morris and Sakanari, 1995; Yenbuter and Scott, 1995), and degradation of host proteins for use as a nutrition source (Chappell and Dresden, 1986), and serve as a potential mechanism to elude host immune responses (Marikovski et al., 1988; Malzels et al., 1993; Modha and Doenhoff, 1994b).

The majority of protozoan proteases described in the literature are of the cysteine protease class (Coombs and North, 1991; North, 1992; McKerrow et al., 1993). Serine protease activity has been demonstrated in several parasitic species, but isolation and characterization of this class of enzymes
have not been as frequently reported. Further, it is quite reasonable to speculate that many protozoa may express multiple proteolytic enzymes of various classes, either simultaneously or in a developmental-stage specific fashion. A particularly difficult obstacle to overcome in working with protozoan enzymes is the low levels of protein recovery from organisms and the diverse number of life-cycle stages found in some species. Latency (i.e., expression of precursor enzyme forms) and autocatalytic properties further hamper efforts to isolate and characterize enzyme activity.

Within the phylogenetic scheme Apicomplexa:Coccidia:Eucoccidiida, protease enzyme identification and characterization have primarily focused on *Plasmodium* spp. A number of malarial proteases have been described, both by isolation as well as by the demonstration of proteolytic activity. The initial report of protease activity in malaria appeared 50 years ago (Moulder and Evans, 1946), and now over 15 distinct proteolytic enzymes have been reported (reviewed in Coombs and North, 1991). Proteases have been reported as critical factors in erythrocyte invasion/reinvasion (Rosenthal et al., 1987; McKerrow et al., 1993), cell rupture (Bernard and Schrevel, 1987; Grellier et al., 1989), and hemoglobin degradation for nutrition (Sherman and Tanigoshi, 1981, 1983; Rosenthal et al., 1989). Of particular interest is the report of a malarial protease that exhibits an inhibitor sensitivity pattern suggesting serine and cysteine protease properties in a single enzyme (Dluzewski et al., 1986).
Michalski et al. (1994) isolated and partially characterized a serine protease from homogenized sporulated *Eimeria tenella* oocysts. This study cited the presence of both serine and cysteine protease activity in *E. tenella* oocysts. Multiple proteases have been reported in a number of parasitic genera, including *Entameoba*, *Plasmodium*, *Trichomonas*, *Trypanosoma*, and *Leishmania* (McKerrow et al., 1993). The phylogenetic relationship between *Eimeria* and *Cryptosporidium* spp. suggests an analogous pattern of proteolytic enzyme activity may exist in cryptosporidial oocysts.

Only recently has enzymatic activity been described in *C. parvum*. Okhuysen et al. (1994) demonstrated arginine aminopeptidase (AP) enzyme activity associated with the sporozoite membrane and suggested a role for aminopeptidase activity in facilitating oocyst excystation. Nesterenko et al. (1995) reported cysteine protease activity associated with solubilized membrane fractions of excysted sporozoites and proposed an association between enzyme activity and host cell infection. Inhibition of proteolytic enzymes in coccidian parasites, notably in *Eimeria* spp and *Plasmodium* spp., by natural and synthetic serine protease inhibitors has shown a significant in vitro reduction in cell infectivity (Hadley et al., 1983; Adams and Bushell, 1988; Fuller and McDougald, 1990). It has now been shown that *C. parvum* expresses protease activity, evidenced by azocasein hydrolysis, during peak periods of excystation (Forney et al., 1996c). Further, serine protease inhibitors significantly antagonize excystation of oocysts and reduce in vitro infectivity
Unlike Nesterenko’s report of a cysteine protease, cryptosporidial serine protease(s) has yet to be isolated and characterized.

**RESEARCH OBJECTIVES**

The studies comprising this dissertation hypothesized the expression of serine protease activity in one or more of the developmental stages of *C. parvum*. It was further hypothesized that AAT, as the major serine protease inhibitor in human circulation, could be an active component of an innate, nonspecific defense mechanism against *C. parvum* infection. The specific emphasis of these related postulates generated four research objectives:

1. Evaluate *C. parvum* for the expression of serine protease-like activity.
2. Determine if serine protease expression is a stage-specific characteristic of *C. parvum*. If so, identify the developmental stage(s) that express serine protease activity.
3. Locate the site(s) of serpin complex formation on, or within, the *C. parvum* organism.
4. Evaluate the in vitro anticryptosporidial potential of protease inhibitors.

These objectives represented an opportunity to expand the current knowledge of the immunobiology of *C. parvum* and explore the relationship between protease activity and infectivity. Localization of AAT interactions with specific developmental stages of the parasite demonstrated the expression of serine protease-like activity on the surface of freshly excysted...
sporozoites. Further, the capability of AAT, and other protease inhibitors, to antagonize oocyst excystation and alter the infection dynamics of *C. parvum* has provided a scientific basis to support the investigation of natural and synthetic protease inhibitors as potential chemotherapeutic agents in the treatment of cryptosporidiosis.

**LITERATURE CITED**


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CHAPTER 2

INTERACTION OF THE HUMAN SERINE PROTEASE INHIBITOR
ALPHA-1-ANTITRYPSIN WITH CRYPTOSPORIDIUM PARVUM

ABSTRACT: The protozoan parasite Cryptosporidium parvum was studied for interaction with a human serine protease inhibitor (serpin), alpha-1-antitrypsin (AAT). A C. parvum homogenate (CPH) prepared from oocysts was incubated with purified human AAT, and complexes formed between the serpin and CPH were detected using an enzyme-linked immunosorbent assay (ELISA). The optical density read at 450 nm of AAT:CPH reactivity was significantly increased ($P < 0.001$) relative to CPH in the absence of AAT treatment. Additionally, ELISA reactivity was blocked by incubating AAT with a cognate target enzyme, porcine pancreatic elastase (PPE), prior to treatment of the CPH. Incubation of a partially excysted sample of C. parvum with AAT (37°C X 60 min) demonstrated preferential fluorescence labeling of sporozoites by indirect immunofluorescence assay; AAT complexes were not detected on intact oocysts. Localization of AAT interactions with C. parvum sporozoites was visualized by transmission immunoelectron microscopy. Collectively, these data suggest that C. parvum sporozoites express a protease-like component that is recognized by human AAT. The ability to block ELISA reactivity with PPE suggests that the AAT interactions we detected are functionally similar to the serpin:enzyme complex AAT forms with a protease target.

INTRODUCTION

_Cryptosporidium parvum_ (Apicomplexa: Cryptosporidiidae) is a parasitic agent of diarrheal illness. This protozoan preferentially invades the epithelial cells lining the microvilli of the small intestine and causes a generally self-limited diarrhea in otherwise healthy individuals (Fayer et al., 1990). Spontaneous eradication of the parasite from the intestinal mucosa and protective acquired immunity is the general outcome of infection in the immunocompetent individual (O'Donoghue, 1995). The most severe consequence of cryptosporidiosis occurs in the immunodeficient host. These individuals frequently develop a profuse, protracted diarrheal illness which progresses to a chronic patent infection of the intestinal epithelium (Gellin and Soave, 1992; Phillips et al., 1992) and, potentially, cryptosporidial dissemination into the alveolar and tracheal epithelium (Petersen, 1992; Giang et al., 1994; Mifsud et al., 1994).

Protease activity has been described in the invasion, infectivity, and pathogenicity of several parasitic diseases. Protease-mediated events facilitate host cell attachment (Adams and Bushell, 1988; Arroyo and Aldrete, 1989), membrane penetration (Hadley et al., 1983; Fuller and McDougald, 1990), degradation of host proteins for use as a nutrition source (Chappell and Dresden, 1986), tissue migration (Modha et al., 1988; McKerrow et al., 1990; Modha and Doenhoff, 1994b; Morris and Sakanari, 1995; Yenbuter and Scott, 1995), and parasite maturation and development (Richer et al., 1993), and
provide a potential mechanism to elude host immune responses (Malzels et al., 1993; Modha and Doenhoff, 1994b).

Alpha-1-antitrypsin (AAT) is one of a group of human serine protease inhibitors (serpin) found in peripheral circulation and tissue fluids. Collectively, the serpin family provides an intrinsic defense mechanism to regulate proteolytic activity from endogenous proteases (Baumstark, 1967; Carrell and Travis, 1985; Joslin et al., 1993). These inhibitors are particularly active during periods of acute inflammation and are a substantive component of the “acute phase” plasma protein response elicited during the inflammatory process. AAT undergoes a dramatic increase in hepatocellular synthesis that manifests itself as serum concentrations several magnitudes greater than normally present in peripheral circulation (Travis and Salvesen, 1983; Whicher et al., 1993; Potempa et al., 1994). In the inflammatory setting, AAT inhibits excessive protease-mediated tissue destruction from leukocyte elastase and provides systemic protection from enzymatic degradation in adjacent tissues (Beatty et al., 1980; Travis and Salvesen, 1983; Huber and Carrell, 1989).

The specific objective of the present study was to examine *C. parvum* for a serine protease-like component capable of recognition and functional interaction with human AAT. Immunolocalization of the resulting interactions was performed to determine if AAT-recognized protease-like activity was expressed as a constitutive component of both oocysts and sporozoites of *C. parvum* or if differential expression limited detection to
only one of these stages. Here we report the detection of AAT complexes formed with components of a cryptosporidial homogenate by an enzyme-linked immunosorbent assay (ELISA). Further, a combination of indirect immunofluorescence labeling and colloidal gold immunoelectron microscopy localized AAT complexes on the sporozoite stage of C. parvum.

MATERIALS AND METHODS

Parasite propagation and isolation

Oocysts were maintained by passage in experimentally infected Holstein calves and purified from feces using discontinuous sucrose and isopycnic Percoll gradients (Arrowood and Sterling, 1987). Purified oocysts were stored in 2.5% potassium dichromate (K₂Cr₂O₇) at 4°C until used. Oocysts, stored less than 90 days, were decontaminated with a 10% (v/v) bleach solution for 10 min at 4°C and washed thoroughly with sterile Hanks' balanced salt solution (HBSS) to remove residual bleach and K₂Cr₂O₇. Decontaminated oocysts were harvested following centrifugal concentration and resuspended in sterile HBSS or phosphate buffered saline (PBS) prior to use in assay protocols.

Preparation of parasite oocysts and sporozoites

A mixture of intact oocysts and freshly excysted sporozoites was prepared for use in immunolocalization assays. A purified suspension of C.
parvum oocysts in PBS was allowed to excyst for 90 min (37°C/5% CO₂) in the absence of any further treatment (Bonnin et al., 1991; Robertson et al., 1993). Excystation efficacy was monitored by light microscopy. In some studies, purified oocysts were freeze-thaw permeabilized prior to immunolabeling.

**Preparation of parasite homogenate**

A 5-ml suspension of 10⁶ oocysts/ml in 25 mM PBS was sonicated 5 X 45 seconds (50 mW) on ice to produce the C. parvum homogenate (CPH) used in subsequent ELISA protocols. CPH prepared in this fashion was assayed for total protein concentration (Bicinchonimic Acid Protein Assay, Pierce Scientific, Rockford, Illinois) and typically yielded total protein values in the range of 150-200 µg/ml. CPH was stored at -20°C prior to use.

**ELISA**

A modified ELISA was used to detect AAT interactions with CPH. A 100-µl aliquot of CPH was added to test wells in 96-well flat bottom polystyrene microtiter plates (Corning Glass Works, Corning, New York) in the presence of a carbonate coating buffer, pH 9.4. Immobilization was performed overnight at 4°C. Coated plates were washed with PBS/0.05% Tween 20 (PBST) and blocked with 1% gelatin/PBST for 4 hr at 25°C. The blocked plates were washed with PBST and 100 µl of a 50 µg/ml dilution of purified human AAT (Sigma Chemical Company, St. Louis, Missouri) was added to each test well. The plates were incubated for 60 min at 37°C. The
interaction between AAT and components of the immobilized CPH provided a stabilized antigen for an indirect (2 antibody) ELISA (Crowther, 1995). Subsequent steps were conducted at 37°C and were followed by PBST washing. A 100-μl aliquot of 1:100 rabbit anti-human AAT, IgG fraction, (Sigma) was added to test wells and incubated for 30 min. A 1:2000 dilution of horseradish peroxidase (HRPO)-conjugated goat anti-rabbit IgG (Sigma) was then added to test wells (100 μl), followed by a 30 min incubation period. Plates were washed a final time with PBST and 100 μl of 4% O-phenylenediamine (OPD) dihydrochloride/0.02% hydrogen peroxide was added to each well. The plates were then incubated for 10 min at 25°C and protected from light. Antibody binding and ELISA reactivity was interpreted as an increase in optical density measured at 450 nm (OD₄₅₀) (Microplate Autoreader, Bio-Tek Instruments, Inc., Winooski, Vermont) relative to control wells containing immobilized CPH, but untreated with human AAT. Variation between OD₄₅₀ values was examined for statistical significance utilizing 2-tailed t-test analysis and simple analysis of variance (ANOVA) on a STATVIEW™ data analysis program (Haycock et al., 1992).

Blocking studies were performed using porcine pancreatic elastase (PPE), a cognate target enzyme of AAT, to evaluate the functional specificity of AAT complexes formed with components of CPH. PPE binds covalently to the AAT molecule and occupies its reactive site loop, thereby eliminating the potential for further inhibitor activity by the bound serpin (Joslin et al., 1991,
1993; Perlmutter, 1993). PPE was mixed with AAT in varying molar ratios and the protease-serpin mixture was incubated 60 min at 37°C. The PPE-treated AAT was then added to gelatin-blocked, CPH-coated test wells and the ELISA completed as described. OD$_{450}$ values for PPE-blocked ELISA studies were compared to AAT:CPH reactivity in the absence of PPE treatment for statistically significance differences.

**Immunofluorescence microscopy**

A partially excysted mixture of oocysts and sporozoites was added to each well of a 10-well immunofluorescence glass slide, air-dried at 25°C, and heat-fixed. All subsequent steps were performed at 37°C in a humidity chamber and separated by thoroughly washing with PBST. Each sample was incubated successively in purified human AAT (50 μg/ml X 60 min), rabbit anti-human AAT, IgG fraction, (1:100 X 30 min), and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (1:100 X 30 min). Immunofluorescence assay (IFA) antibodies were obtained from Sigma. Slides were again washed with PBST, covered with a nondrying mounting medium, and examined by epifluorescence microscopy. Related studies utilized freeze-thaw permeabilized oocysts as the initial sample with all subsequent procedural steps identical to those described for the partially excysted samples.
Immunoelectron microscopy

Pre-embedding immunogold labeling: To emphasize surface labeling, a mixture of intact oocysts and free sporozoites was fixed with 0.5% glutaraldehyde in PBS at 4°C for 10 min and labeled as described by Sibley and Sharma (1987) for transmission electron microscopy (TEM). In brief, the lightly fixed parasites were washed in cold PBS and incubated successively in human AAT (50 µg/ml X 60 min), rabbit anti-human AAT, IgG fraction (1:20 X 30 min), and 10 nm colloidal gold-conjugated goat anti-rabbit IgG (1:20 X 30 min) with each step separated by PBST washing at 4°C. Immunogold labeling reagents were obtained from Sigma. Following the immunolabeling procedure, parasites were fixed a second time in 0.5% glutaraldehyde at 4°C for 20 min and dehydrated in an iced ethanol gradient. Samples were impregnated with LR White resin (Electron Microscopy Sciences, Ft. Washington, Pennsylvania) at 25°C and polymerized overnight at 60°C in gelatin capsules. Thin sections of LR White-embedded samples were mounted on nickel grids, carbon-coated, and viewed by TEM (Zeiss 902CEM, Thornwood, New York) after contrast-enhanced staining with saturated uranyl acetate (4 min at 50°C) and lead citrate (2 min at 25°C).

Post-embedding immunogold labeling: Samples of a C. parvum excystation mixture were fixed with 0.5% glutaraldehyde/2% paraformaldehyde in PBS at 4°C for 20 min. Fixed parasites were washed in cold PBS, dehydrated in an iced ethanol gradient, and embedded in LR White
resin. Thin sections were mounted on nickel grids, carbon-coated, and floated for 10 min in PBS/0.5% bovine serum albumin (BSA)/0.02% Tween 20. The samples were washed with PBST and transferred onto human AAT (50 μg/ml) for 60 min at 37°C. Grids were successively floated on rabbit anti-human AAT, IgG fraction (1:50 in blocking buffer) for 30 min and 10 nm colloidal gold-conjugated goat anti-rabbit IgG (1:20 in blocking buffer) for 30 min; each step was followed by PBST washing. After the final wash, grids were rinsed with water and viewed by TEM after contrast-enhanced staining.

RESULTS

Detection of AAT interaction with C. parvum

Figure 2-1 shows the pattern of ELISA reactivity observed for AAT interactions with 3 different protein coatings, i.e., BSA, CPH, and PPE. ELISA reactivity demonstrated a statistically significant increase (P < 0.001) in optical density following incubation of the immobilized CPH with AAT relative to immobilized CPH not exposed to AAT. The pattern of reactivity observed for AAT-treated C. parvum was consistent with the increase in OD₄₅₀ values observed in reaction wells containing immobilized PPE as an initial antigen coating. A concentration-dependent increase in OD₄₅₀ was apparent following AAT incubation with serial dilutions of CPH (Fig. 2-2). Figure 2-3 shows the blocking effect of PPE on serpin interaction with CPH. A loss of
AAT:CPH reactivity (OD$_{450}$) was measured when AAT was incubated with PPE (37°C X 60 min) before adding the serpin to ELISA test wells.

**Immunolocalization of AAT:**

*parasite complexes*

*Indirect immunofluorescence labeling:* A partially excysted mixture of *C. parvum* oocysts and sporozoites, incubated with human AAT and analyzed by an indirect IFA, demonstrated preferential labeling of the sporozoite stage of the parasite (Fig. 2-4). Sporozoites typically exhibited a diffuse, moderately intense fluorescence pattern on their surface membrane.

Intact oocysts did not bind the FITC-conjugated ligand. Figure 2-5 illustrates the immunofluorescence labeling pattern observed in freeze-thaw permeabilized oocysts incubated with AAT. These samples revealed a prominent fluorescence labeling of sporozoites within unexcysted, and otherwise unlabeled, oocysts. Collectively, these observations show that AAT interactions were limited to *C. parvum* sporozoites.

*Immunoelectron microscopy:* AAT interactions at the ultrastructural level were investigated in thin sections of oocysts and sporozoites. Immunogold labeling of *C. parvum* following incubation with AAT demonstrated a prominent labeling of the surface of excysted sporozoites; a smaller number of gold particles were observed in the intracellular matrix of sectioned parasites (Fig. 2-6). As expected, the pattern of surface-associated immunogold labeling was particularly well defined when samples were
labeled prior to resin impregnation and polymerization. Post-embedding labeling of sections through oocysts containing sporozoites showed gold particles in association with the sporozoite stages, but in much smaller numbers than observed for free sporozoites (Fig. 2-7). Surface-associated gold labeling was typically uniform and evenly distributed on free sporozoites; there was no discernible pattern of gold particles that would suggest a differential gradient of AAT complexes on the sporozoite.

*Cryptosporidium parvum* oocysts did not bind the gold-conjugated ligand in either the pre- or post-embedding labeling procedures, suggesting a lack of AAT interaction with the oocyst wall. As seen in Figure 2-7, small clusters of gold particles were occasionally observed within the perimeter wall of unexcysted oocysts, but not in direct association with any structural element of the internal oocyst wall.

**DISCUSSION**

This study is the first to demonstrate a functional interaction between a human serpin (AAT) and components of the sporozoite stage of *C. parvum*. This interaction, presumably the result of complexes formed between AAT and parasite proteases, was detected by ELISA (Figs. 2-1, 2-2) and clearly observed following IFA (Fig. 2-4). The complexes were further visualized by TEM as a developmental stage-specific interaction primarily associated with components of the sporozoite surface membrane (Fig. 2-6). Our findings, in conjunction with Okhuysen et al.'s (1994) demonstration of arginyln
aminopeptidase activity, suggest that *C. parvum* sporozoites express a serine protease-like component that is functionally similar to trypsin (Powers et al., 1993). Classified in terms of substrate specificity, the anionic residues forming the active site “pocket” of trypsin-like enzymes preferentially hydrolyze peptides at cationic amino acid residues, such as arginine or lysine (Craik et al., 1985; Perona et al., 1993; Powers et al., 1993; Perona and Craik, 1995). Whereas the preponderance of scientific evidence points to neutrophil elastase as the primary physiologic target of AAT inhibition (Beatty et al., 1980; Gadek et al., 1981; Huber and Carrell, 1989; Joslin et al., 1993; Döring, 1994), the serpin molecule was originally described for its in vitro inhibitory effect on trypsin (Moll et al., 1958; Bundy and Mehl, 1959).

AAT is a single chain glycoprotein that performs its inhibitory action through the formation of a 1:1 molecular complex with a serine protease (Perlmutter, 1993). A reactive site loop of the AAT molecule interacts with the corresponding active site of a target enzyme and blocks proteolytic activity by the bound enzyme; in effect AAT acts as a substrate for the protease. The serpin:enzyme complex (SEC) formed by the reactive site interactions demonstrates high affinity in its binding association under physiological conditions, primarily as a result of covalent attractions between the serpin and its cognate enzyme. The AAT reactive site loop imparts a degree of functional specificity to the molecule; this portion of the molecule closely mimics the physiochemical properties of an appropriate substrate to achieve serpin:protease recognition. Once formed, the SEC is rapidly cleared from
circulation by specific receptor-mediated endocytosis in the parenchyma of the liver (Perlmutter et al., 1990; Joslin et al., 1991).

The ELISA used in this study was designed to detect AAT:C. parvum interactions by adapting the functional specificity of AAT to probe CPH for protease-like components capable of interacting with the serpin molecule. To achieve this end, we exploited 2 characteristics of the SEC. First, the AAT molecule forms a very stable complex with a target enzyme (Joslin et al., 1993; Stein and Carrell, 1995), stable enough to withstand the manipulations accompanying the selected immunoassays. Second, SEC formation is accomplished while retaining a relatively intact structural configuration of the AAT molecule, thus permitting its detection by immunological techniques directed against epitopes on that portion of the glycoprotein protruding from the SEC (Joslin et al., 1991; Björk et al., 1993). The demonstrated loss of ELISA reactivity following pretreatment of AAT with PPE (Fig. 2-3) suggests that once elastase occupies the AAT reactive site loop, it effectively blocks serpin recognition of analogous structures in the CPH.

The results of IFA and immunogold labeling show a clear distinction in the labeling intensity between free sporozoites and sporozoites within intact oocysts. Figure 2-6 illustrates a qualitatively enhanced recognition of AAT complexes on freshly excysted sporozoites compared to unexcysted sporozoites within otherwise intact oocysts (Fig. 2-7). We postulate that serine protease-like components of C. parvum sporozoites are in an enzymatically inactive form prior to excystation. In the zymogen
(proenzyme) form, protease activity would remain at very low levels until activated by one or more conditions that “trigger” excystation. Okhuysen et al. (1994) described enhanced aminopeptidase activity after prolonged incubation in conditions favorable to excystation; similarly, we demonstrated a qualitatively greater detection of AAT complexes formed with excysted sporozoites (Fig. 2-4) and in permeabilized oocysts following incubation with AAT at 37°C (Fig. 2-5). Because AAT binds to the active site of a target enzyme, SEC formation favors the enzymatically active form of proteases (Perlmutter, 1993; Perona et al., 1993; Perona and Craik, 1995).

The lack of immunolabeling associated with intact oocysts indicates a lack of AAT interaction with the oocyst wall and implies an absence of serine protease-like expression on this stage of parasite development. We did observe some oocyst sections that contained a small number of gold particles within the confines of an intact oocyst wall (Fig. 2-7). This observation may reflect the dissociation of AAT complexes from sporozoites as an artifact of the fixing, sectioning, and labeling process. Conversely, intraoocyst gold conjugate may indicate that metabolically active sporozoites undergo low-level expression and shedding of protease-like components while sequestered within an intact oocyst.

There is substantial evidence of a structural rearrangement of the AAT molecule following complex formation with a protease target that presents a small (pentapeptide) sequence of the AAT protein (Perlmutter et al., 1990; Perlmutter, 1993; Stein and Carrell, 1995). This exposed peptide fragment
includes the carboxyl terminal sequence of the protein and has been attributed a functional role in signaling upregulation of AAT synthesis in cells bearing a specific SEC receptor (Schreiber and Aldred, 1993; Perlmutter, 1993; Potempa et al., 1994). We speculate that a protease-like component expressed by C. parvum sporozoites may help upregulate AAT levels mediated by specific receptor recognition of SEC complexes. Though lacking parasite specificity, this primitive defense mechanism may afford protection against proteolytic activity during the course of early infection and minimize host vulnerability to exogenous proteases while an adaptive immune response is being mounted against the parasite. An interesting association is apparent given the in vitro demonstration of an “acute phase” protein response in extrahepatic sites (Molementi et al., 1993; Perlmutter, 1993). These observations, in particular Molementi’s demonstration of AAT production in human intestinal epithelial cells, suggest the possibility of a self-amplifying serpin response to cryptosporidial proteases sustained by upregulation of AAT synthesis in the immediate vicinity of parasitic infection. While the potential for local (i.e., intestinal epithelium) AAT production may exist as a homeostatic response to exogenous proteases, a recent study reported a lack of correlation between the intensity of cryptosporidial infection and fecal AAT clearance (Goodgame et al., 1995). The influence of extrahepatic AAT production in intestinal parasitic infections is yet to be fully defined.

The role of protease inhibitors in abrogating the effects of exogenous parasite proteases is an area of active investigation. This class of regulatory
proteins has been reported to reduce intraerythrocytic stages of *Plasmodium vinckei* in mice (Rosenthal et al., 1993); affect the maturation and development of *Trypanosoma* spp (Meirelles et al., 1992), *Strongyloides stercoralis* (McKerrow et al., 1990), and *Dirofilaria immitis* (Richer et al., 1993); and reduce the in vitro infectivity of *Eimeria* spp (Adams and Bushell, 1988; Fuller and McDougald, 1990; Cohen et al., 1991). Modha and Doenhoff (1994a) reported the formation of complexes between human AAT and surface membrane components of *Schistosoma* spp cercariae. Their data suggest a putative role for human serpin activity during schistosomal infection. The nature of the reported interaction, AAT binding to parasite proteases, suggests that a nonspecific defense mechanism against exogenous parasite proteases facilitates the homeostatic balance between protease and anti-protease activity. Modha and Doenhoff further suggested (1994b) that schistosome interactions with circulating serpin molecules are an example of immune evasion by antigenic mimicry. The formation of AAT complexes with surface-expressed proteases blocks the conformational availability of these immunogenic parasite proteins to immune effector cells. A foreign organism coated by endogenous host serpin proteins could potentially evade immune recognition.

Bristow et al. (1995) recently demonstrated a cell surface adhesion and fusion mechanism between a viral pathogen and host target cells involving recognition between serine protease-like receptors and corresponding structures analogous to serpin reactive sites. The search for comparable
surface adhesins with regard to *C. parvum* recognition of host epithelial cells and subsequent membrane fusion may elucidate the early invasion sequence of this parasite. It is conceivable that cryptosporidial protease-like components are involved in interactions at the host cell membrane level such as has been demonstrated with related coccidians (Adams and Bushell, 1988; Fuller and McDougald, 1990). Several investigators have reported a putative role for surface-expressed glycoproteins or lectin-associated membrane affinity to aid in the initial attachment of *C. parvum* sporozoites to host cells (Bonnin et al., 1991; Tilley et al., 1991; Petersen et al., 1992; Mitschler et al., 1994; Tilley and Upton, 1994). The initial adherence of sporozoites to host cell membranes may facilitate the spatial orientation of parasite proteases that further enhance cell attachment and, potentially, contribute to the parasite’s unique intramembranous location within infected cells.

This study demonstrated a functional interaction between human AAT and a protease-like component of the initial invasive stage of *C. parvum*, the sporozoite. This interaction was predominately associated with the outer surface of excysted sporozoites; AAT complexes were not detected on either the internal or external surface of the oocyst wall. These findings suggest that the putative role of human AAT in regulating exogenous protease activity should include a protease-like component expressed by *C. parvum*. It would be of particular interest to evaluate the anticryptosporidial
potential of serpin compounds by studying the effects of AAT on C. parvum
infection in permissive cell culture systems and laboratory animal models.

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Figure 2-1. Pattern of ELISA reactivity for alpha-1-antitrypsin interactions with 3 different coating proteins. *Cryptosporidium parvum* homogenate (CPH), porcine pancreatic elastase (PPE), and bovine serum albumin (BSA) were used to coat ELISA test wells prior to incubation with alpha-1-antitrypsin (AAT). ELISA reactivity for wells coated with CPH and PPE was significantly greater ($P < 0.001$) than values determined for untreated wells and for the BSA control. The data reflect 2 experiments containing a total of 4 replicates of BSA and 12 replicates each for wells coated with CPH and elastase. Data are presented as the mean ± SD.
Figure 2-2. Concentration-dependent reactivity of alpha-1-antitrypsin: *Cryptosporidium parvum* homogenate interactions. OD$_{450}$ values for AAT:CPH (–•–) increased proportional to increases in the concentration of CPH [total protein]. Controls consisted of CPH without exposure to AAT, treated with either phosphate buffered saline (–o–) or 2% (w/v) bovine serum albumin (– – ) in place of the serpin. The data represent 2 experiments of 2 replicates each for control values and 4 replicates each for AAT treatment of CPH. Data are presented as the mean ± SD.
Figure 2-3. Effects of porcine pancreatic elastase pretreatment of alpha-1-antitrypsin on ELISA reactivity. Blocking was demonstrated by pretreating AAT with PPE prior to incubation of *C. parvum* homogenate (CPH) (—●—). OD$_{450}$ values for AAT:CPH reactivity decreased in response to an increase in the ratio of [PPE] to [AAT]. Test wells containing bovine serum albumin (BSA) as a coating antigen (—□—) were unaffected by PPE blocking. Substituting phosphate buffered saline (PBS) for PPE in the pretreatment step (—○—) resulted in a slower loss of ELISA reactivity, suggesting a dilution effect on [AAT]. The data reflect 2 experiments of 4 replicates for each test well containing CPH and duplicate values for the BSA control. Data are presented as the mean ± SD.
Figure 2-4. Indirect immunofluorescence microscopy of alpha-1-antitrypsin interactions with Cryptosporidium parvum. Uniform fluorescence labeling was evident on the surface of excysted sporozoites following AAT exposure (arrow). Note the absence of FITC-conjugated ligand on the oocyst wall (arrowhead). Scale bar = 10 μm.
Figure 2-5. Immunolabeling of permeabilized oocysts. Freeze-thaw permeabilized oocysts incubated with AAT were permissive to immunolabeling of serpin complexes formed with sequestered sporozoites (arrow); the oocyst wall remained refractory to AAT interaction. Scale bar = 10 μm.
Figure 2-6. Transmission immunoelectron micrograph of Cryptosporidium parvum sporozoites following incubation with alpha-1-antitrypsin. Immunodetection of AAT interactions with C. parvum at the ultrastructural level is shown by the dense gold labeling on the surface of excysted sporozoites. Scale bar = 350 μm.
Figure 2-7. Transmission electron micrograph of alpha-1-antitrypsin interactions with encysted *Cryptosporidium parvum* sporozoites. An ultrathin section of an intact oocyst containing longitudinal and transverse sections through the sporozoites is observed. A minimal number of gold particles are seen on the sectioned sporozoites following incubation with AAT (arrow). A few small clusters of gold particles are present within the confines of the oocyst wall (arrowhead), suggesting the low-level secretion of a protease-like component by sequestered sporozoites or shedding of the serpin:enzyme complex during labeling. Scale bar = 235 μm.
CHAPTER 3

PROTEASE ACTIVITY ASSOCIATED WITH EXCYSTATION OF

CRYPTOSPORIDIIUM PARVUM OOCYSTS

ABSTRACT: A Cryptosporidium parvum homogenate (CPH), prepared from partially excysted oocysts, was examined for proteolytic activity capable of hydrolyzing azocasein. Protease activity, measured at pH 7.0, was not detected in fresh oocysts, but increased progressively with incubation at 37°C. Activity peaked after 60 min incubation, but progressively decreased with extended incubation intervals. Cryptosporidial protease activity was significantly inhibited (P < 0.01) by the serine protease inhibitors phenylmethaneethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DIFP), aprotinin, alpha-1-antitrypsin (AAT) and the cysteine protease inhibitor L-trans-epoxysuccinyl-leucylamido-(4-guanidino)-butane (E-64). No single inhibitor completely blocked CPH-associated protease activity; however, the combination of PMSF and E-64 inhibited >95% of the azocasein hydrolysis measured in untreated control samples. The same group of inhibitors were further evaluated for their ability to inhibit excystation of C. parvum oocysts. PMSF, DIFP, aprotinin, and AAT significantly inhibited (P < 0.05) oocyst excystation at 15-, 30-, and 60-min incubation intervals; E-64 had no significant inhibitory effect on excystation. The results of this study demonstrate proteolytic activity during peak periods of excystation. Further, we showed that cryptosporidial protease activity was sensitive to both serine and cysteine protease inhibitors, but only serine protease inhibitors

significantly inhibited oocyst excystation. These findings provide preliminary evidence of cryptosporidial proteases of both serine and cysteine protease classes and suggest that serine protease(s) are functionally associated with excystation.

**INTRODUCTION**

*Cryptosporidium parvum* (Apicomplexa: Cryptosporidiidae) is a protozoan parasite that preferentially invades epithelial cells lining the microvilli of the small intestine (Fayer et al., 1990; O'Donoghue, 1995). The most severe consequence of human cryptosporidiosis occurs in the immunodeficient host; *C. parvum* is an opportunistic pathogen of substantial morbidity in the acquired immunodeficiency syndrome (AIDS) patient population. The immunocompromised host develops a profuse, watery diarrheal illness which may progress to a chronic, patent infection of the intestinal epithelium (Gellin and Soave, 1992; Goodgame et al., 1995). The chronicity and severity of cryptosporidiosis are accentuated by the lack of therapeutic agents capable of eradicating the parasite.

The infective stage of *C. parvum* is the oocyst, a thick-walled, double-layered structure containing 4 sporozoites. The intact oocyst is particularly resistant to environmental stress, chemical disinfectants, and many municipal water purification processes (Campbell et al., 1982, 1993; MacKenzie et al., 1994; CDC, 1995). Sporozoites excyst from the oocyst following ingestion and attach to the intestinal microvilli; parasite development proceeds within
an enigmatic residence, an intracellular, but extracytoplasmic, parasitophorous vacuole. A small proportion (approximately 20%) of the oocysts produced in an infected host are ensheathed within a thin, single layer outer wall. This thin-walled oocyst undergoes endogenous sporulation and rapidly excysts in the intestinal lumen, accounting for the autoinfective cycle observed in chronically infected hosts (Current and Garcia, 1991; Chui and Owen, 1994; O’Donoghue, 1995). The characteristics of early cryptosporidial infection, including excystation, are an area of active investigative effort. Studies have identified conditions that favor excystation and reported protocols to optimize in vitro sporozoite production (Robertson et al., 1992, 1993; Upton et al., 1994); however, the biochemical processes that facilitate the emergence of sporozoites from an infective oocyst remain largely undefined.

Recently, investigators have described arginyl aminopeptidase activity (Okhuysen et al., 1994) and a putative cysteine proteinase (Nesterenko et al., 1995), both associated with the outer membrane of sporozoites. Our previous study detected an interaction between the human serine protease inhibitor alpha-1-antitrypsin (AAT) and the surface of excysted sporozoites (Forney et al., 1996b). Additionally, we have demonstrated a significant anticryptosporidial potential for selected protease inhibitors, including human AAT, in a bovine fallopian tube epithelial (BFTE) cell culture system (Forney et al., 1996a). Collectively, emerging evidence suggests that cryptosporidial protease/peptidase activity is requisite for *C. parvum*
excystation and the initial dynamics of host cell infection. Speculation on the role of endogenous proteolytic enzymes during excystation prompted the present study to evaluate *C. parvum* for protease activity associated with peak periods of oocyst excystation. Further, our objectives included a study of the effects of selected inhibitors on parasite protease activity and oocyst excystation.

**MATERIALS AND METHODS**

**Parasite propagation and isolation**

Oocysts were maintained by passage in experimentally infected Holstein calves, purified from feces using discontinuous sucrose and isopycnic Percoll gradients (Arrowood and Sterling, 1987), and stored in 2.5% potassium dichromate (K2Cr2O7) at 4°C. Oocysts were decontaminated in 10% (v/v) bleach for 10 min at 4°C and washed thoroughly with sterile Hanks' balanced salt solution (HBSS) to remove residual bleach and K2Cr2O7. Decontaminated oocysts were harvested following centrifugal concentration and resuspended in sterile RPMI medium (HyClone Laboratories, Inc., Logan, Utah).

**Excystation studies**

Aliquots of purified oocysts were added to sterile plastic microcentrifuge tubes and intact oocysts were counted in a hemacytometer using phase contrast microscopy. Oocysts were then incubated at 37°C in the
absence of any further treatment (Bonnin et al., 1991; Robertson et al., 1993; Upton et al., 1994). Tubes were removed from the incubation environment at fixed time intervals, i.e., 15, 30, 60, 90, 120, and 180 min, and intact oocysts were again enumerated (excluding empty oocyst “ghosts” and partially-excysted oocysts). The difference between the number of oocysts counted prior to incubation and the number of oocysts counted after incubation was expressed as a percent of the original, i.e., pre-incubation, number. This value was termed “percent of excystation” and represented an indirect assessment of excystation.

Preparation of parasite homogenate

Bleach-treated oocysts were incubated at the timed intervals indicated previously and then snap frozen in liquid nitrogen to suspend further excystation. These partially excysted mixtures of oocyst and sporozoite forms were thawed in a 37°C water-bath and sonicated 6 X 45 seconds (50 mW) on ice to produce a C. parvum homogenate (CPH). Samples of the resulting sonicate were examined by light microscopy to ensure that CPH was free of intact oocysts and sporozoites. Peak protease activity was observed in homogenates prepared from oocyst incubated for 60 min; for subsequent inhibitor sensitivity studies, a 3-ml suspension of 10^6 oocysts/ml in RPMI medium was incubated at 37°C for 60 min and sonicated as before. CPH prepared in this fashion was assayed for total protein concentration
(Bicinchoninic Acid Protein Assay, Pierce Scientific, Rockford, Illinois) and the final total protein concentration was adjusted to 200-250 μg/ml.

**Assay for proteolytic activity**

Azocasein (Sigma Chemical Company, St. Louis, Missouri) was used as a substrate in a microassay protocol for proteolytic activity (Plantner, 1991). In brief, 200 μl aliquots of a 2 mg/ml solution of azocasein in 100 mM sodium phosphate buffer, pH 7.0, was prewarmed 15 min at 37°C. Evaluation of protease activity was confined to pH 7.0, consistent with the neutral excystation environment used in this study. A 50-μl aliquot of CPH was added to each tube and the reaction mixture was incubated at 37°C for 10 hr. Following incubation, 40-μl of 50% cold trichloroacetic acid (TCA) was added to each tube to precipitate undigested azocasein and tubes were placed on ice for 60 min. The reaction solution was clarified by centrifugation (7,000 g for 10 min). A 200-μl aliquot of the reaction supernatant was added to individual wells in a 96-well microtiter plate; each well contained 20 μl of 10 N NaOH. The absorbance of the alkalinized solution was read at 450 nm (A_{450}) in an automated microtiter plate reader (Microplate Autoreader, Bio-Tek Instruments, Inc., Winooski, Vermont). Diluted commercial trypsin (Type IV; Sigma) was used as a control for casein hydrolysis.
Protease inhibitors

The protease inhibitors alpha-1-antitrypsin (AAT; Chemicon International, Inc., Temecula, California), aprotinin (ICN Biochemicals, Inc., Aurora, Ohio), L-trans-epoxysuccinyl-leucylamindo-(4-guanidino)-butane (E-64; Sigma), diisopropyl fluorophosphate (DIFP; Sigma), and phenylmethylsulfonyl fluoride (PMSF; Sigma) were evaluated for their effect on CPH-associated protease activity and oocyst excystation. Dilutions of AAT and aprotinin were prepared in 10-μg/ml and 100-μg/ml concentrations in sterile 25 mM phosphate-buffered saline (PBS); DIFP, E-64, and PMSF were first dissolved in dimethyl sulfoxide (DMSO; Sigma) and subsequently diluted to 1-mM and 10-mM concentrations in PBS. Inhibition studies involved the pre-incubation of equal volumes of CPH and protease inhibitors for 30 min at 25°C prior to assay with azocasein. The differences in A450 values for inhibitor-treated CPH samples and untreated control samples (similarly diluted) were compared for statistical significance using ANOVA (Fisher’s PLSD).

To evaluate the effect of protease inhibitors on excystation, purified oocysts were mixed with equal volumes of protease inhibitor solutions and subjected to the excystation conditions previously described. The number of oocysts that excysted in inhibitor-treated groups was expressed as a percent of the number of oocysts excysted in untreated control samples. Inhibitors
requiring preparatory dissolution in DMSO were matched with excystation controls containing equal concentrations of DMSO.

RESULTS

Excystation studies

Under standard conditions (37°C, RPMI medium) excystation increased rapidly during the initial 90 min (Fig. 3-1). The percent of excystation at 120 (74.5 ± 1.41) and 180 (76.7 ± 2.12) min was increased over shorter incubation times, but did not significantly differ from the percent of excystation at 90 min (68.5 ± 1.06). In effect, 89.3% of the total excystation observed occurred within the first 90 minutes of incubation, with 78.2% occurring within the first 60 min.

Table 3-1 shows the effects of selected protease inhibitors on the excystation dynamics of C. parvum oocysts. The serine protease inhibitors PMSF, DIFP, aprotinin, and AAT significantly inhibited excystation (P < 0.05) in the sampling intervals from 15 to 60 min, but the percent of excystation obtained at 90-180 min was not significantly reduced below control levels by any of the protease inhibitors we evaluated. PMSF, at a concentration of 10 mM, was the most potent excystation inhibitor evaluated. The cysteine protease inhibitor E-64 had no significant inhibitory effect on excystation.
**Protease activity**

Protease activity was detected in CPH prepared from oocysts incubated at 37°C. Figure 3-1 shows the relationship between azocasein hydrolysis ($A_{450}$) and the length of time oocysts were incubated prior to homogenization. Protease activity was absent in unincubated oocysts; azocasein hydrolysis increased with oocyst incubation time, and peak activity was measured in CPH prepared from oocysts that were incubated for 60 min. Azocasein hydrolysis progressively declined in CPH prepared from oocysts incubated for 90- to 180-min intervals.

Inhibitor sensitivity studies were conducted on CPH prepared from oocysts that had been incubated for 60 min to coincide with the period of highest hydrolytic activity. Cryptosporidial protease activity was significantly inhibited ($P < 0.05$) by the serine protease inhibitors PMSF, DIFP, and AAT at each of the concentrations evaluated (Table 3-2). Aprotinin significantly inhibited azocasein hydrolysis ($P < 0.05$) when tested at a concentration of 100 μg/ml; a 10-μg/ml concentration of aprotinin had no significant effect on protease activity. The cysteine protease inhibitor E-64 significantly inhibited ($P < 0.05$) azocasein hydrolysis at concentrations of both 1 mM and 10 mM. The combination of 10 mM PMSF and 10 mM E-64 inhibited 95.8% of the hydrolytic activity measured in unchallenged control samples.
DISCUSSION

This study reports the detection of proteolytic activity in homogenates prepared from C. parvum oocysts collected during peak periods of excystation. CPH-associated protease activity paralleled the increase in excystation observed with incubation of oocysts at 37°C (Fig. 3-1). Our findings showed that protease activity peaked at the 60-min incubation interval and steadily declined when oocysts were incubated for longer periods. Further, this study suggests that CPH contained proteases sensitive to both serine and cysteine protease inhibitors.

The pattern of oocyst excystation observed in this study is consistent with previous reports of excystation dynamics. Upton et al. (1994) reported a high rate of excystation during the initial 90 min of incubation at 37°C in RPMI medium. Whereas our study did not attain as high a percent of excystation as reported by those authors, the rapid rate of excystation we observed in the 15- to 90-min intervals is in substantial agreement. Robertson et al. (1993) recommended a protocol for in vitro excystation that limited oocyst incubation to 30 min at 37°C to maximize sporozoite viability. In vitro studies strongly suggest that sporozoite viability, and subsequent infectivity, is optimally associated with the peak excystation period occurring during the initial 30-90 min of incubation at 37°C.

As seen in Table 3-1, the pattern of excystation inhibition (inhibition by PMSF, DIFP, aprotinin, and AAT; lack of inhibition by E-64) strongly suggests
that a *C. parvum* serine protease(s) is functionally associated with excystation. Michalski et al. (1994) reported similar findings in a homogenate prepared from sporulated *Eimeria tenella* oocysts. Their study detected a mixed inhibitor sensitivity pattern indicative of the presence of both serine and cysteine proteases in *E. tenella* oocysts.

The findings of this study suggest that *C. parvum* oocysts may also contain multiple proteases with inhibitor sensitivities characteristic of both the serine and cysteine protease classes. Nesterenko et al. (1995) reported a cysteine protease detected in solubilized sporozoite membranes. Because the homogenate used in the present study contained components of both oocysts and sporozoites, the E-64-sensitive component of CPH may represent the cysteine protease previously described. The potential expression of multiple proteases in a crude homogenate of *C. parvum* is certainly not an unexpected finding in protozoans. Multiple proteases have been reported in a number of parasitic genera, including *Entameoba*, *Plasmodium*, *Trichomonas*, *Trypanasoma*, and *Leishmania* (McKerrow et al., 1993) Alternately, there is evidence of a malarial protease that exhibits an inhibitor sensitivity pattern suggesting serine and cysteine protease properties in a single enzyme (Deguercy et al., 1990).

Our previous study reported an interaction between human AAT and *C. parvum* (Forney, 1996b). The localization of AAT complexes on the external surface of excysted sporozoites suggested the expression of a protease-like component on this early developmental stage of the parasite. The
The present study supports this hypothesis by demonstrating proteolytic activity and showing the antagonistic properties of serine protease inhibitors on excystation. The mechanism by which serine protease inhibitors block excystation is not fully understood. It has been shown that bleach treatment increases the permeability of the oocyst wall and enhances excystation (Reduker and Speer, 1985; Robertson et al., 1993). Our use of bleach to decontaminate oocysts may have provided a means for inhibitors to penetrate the oocyst wall and inhibit intra-oocyst proteases. We have not evaluated the effect of serine protease inhibitors on excystation of unbleached oocysts.

The observation that inhibitor-treated oocysts eventually approached control levels of excystation after 90-180 min incubation suggests that excystation is only partially blocked by the inhibitors and concentrations evaluated in this study. The half-life of some inhibitors, such as PMSF and DIFP, is relatively short and loss of inhibitory effect may be attributable to the short half-life of these compounds. An alternative postulate is the potential for compensatory mechanisms in the parasite life-cycle that may facilitate excystation in an inhibitory environment. For example, it has been shown that cysteine proteases can proteolytically degrade the AAT molecule and effectively eliminate its inhibitory potential for serine proteases (Johnson et al., 1986; Potempa et al., 1986; Wright and Scarsdale, 1995). The presumptive indication of cysteine protease activity in CPH suggests a mechanism to potentially mediate the inhibitory effects of AAT on oocyst excystation.
In summary, the findings of this study suggest that multiple proteolytic components, one sensitive to serine protease inhibitors and the other to the cysteine protease inhibitor E-64, are expressed by *C. parvum* during excystation. These findings further suggest that cryptosporidial serine proteases are functionally associated with excystation. It is of particular interest to note that PMSF, AAT, and aprotinin, in addition to inhibiting excystation, have previously been shown to significantly reduce *C. parvum* infection in a permissive BFTE cell culture system (Forney et al., 1996a; Yang et al., 1996). Thus, protease inhibitors may reduce parasite infectivity in vitro by antagonizing excystation. Altering the dynamics of excystation may potentiate an effective anticryptosporidial strategy.

**LITERATURE CITED**


Table 3-1. Effect of protease inhibitors on excystation of *Cryptosporidium parvum* oocysts.

<table>
<thead>
<tr>
<th>Protease Inhibitor</th>
<th>Concentration</th>
<th>% Inhibition*</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>15†</td>
</tr>
<tr>
<td>phenylmethlysulfonyl fluoride (PMSF)</td>
<td>10 mM</td>
<td>87.9‡ ± 12.6</td>
</tr>
<tr>
<td>diisopropyl fluorophosphate (DIFP)</td>
<td>10 mM</td>
<td>66.7‡ ± 10.8</td>
</tr>
<tr>
<td>aprotinin</td>
<td>100 µg/ml</td>
<td>48.5‡ ± 12.7</td>
</tr>
<tr>
<td>alpha-1-antitrypsin (AAT)</td>
<td>100 µg/ml</td>
<td>82.8‡ ± 11.8</td>
</tr>
<tr>
<td>L-trans-epoxysuccinyl-leucylamino-(4-guanidino)-butane (E-64)</td>
<td>10 mM</td>
<td>14.9 ± 8.1</td>
</tr>
</tbody>
</table>

*Percent of control excystation inhibited by protease inhibitor treatment as described in Materials and Methods. Data derived from duplicate samples from 3 separate experiments. Values represent mean ± SD.
†Time (min) of incubation at 37°C.
‡Significantly different (P < 0.05) from control group.
Table 3-2. Effect of selected inhibitors on excystation-associated protease activity.

<table>
<thead>
<tr>
<th>Protease Inhibitor</th>
<th>Concentration</th>
<th>% Inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMSF</td>
<td>10 mM</td>
<td>88.4 ± 4.2†</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>67.0 ± 6.6†</td>
</tr>
<tr>
<td>DIFP</td>
<td>10 mM</td>
<td>80.8 ± 5.8†</td>
</tr>
<tr>
<td></td>
<td>1 mM</td>
<td>72.6 ± 4.1†</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>100 µg/ml</td>
<td>65.7 ± 4.9†</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml</td>
<td>9.8 ± 2.6</td>
</tr>
<tr>
<td>AAT</td>
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<td>60.7 ± 4.4†</td>
</tr>
<tr>
<td></td>
<td>10 µg/ml</td>
<td>30.8 ± 2.3‡</td>
</tr>
<tr>
<td>E-64</td>
<td>10 mM</td>
<td>37.7 ± 8.8†</td>
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<td></td>
<td>1 mM</td>
<td>22.6 ± 3.5‡</td>
</tr>
<tr>
<td>PMSF + E-64</td>
<td>10 mM (each)</td>
<td>95.8 ± 6.7†</td>
</tr>
</tbody>
</table>

*Percent of protease activity in CPH controls inhibited following treatment with protease inhibitors as described in Materials and Methods. Data derived from duplicate samples from 3 separate experiments. Values represent mean ± SD.

†Significantly different (P < 0.01) from untreated control group.
‡Significantly different (P < 0.05) from untreated control group.
Figure 3-1. Effect of oocyst incubation time at 37°C on excystation and protease activity. Percent excystation (primary Y-axis) observed at fixed incubation intervals (--O--). $A_{450}$ values (secondary Y-axis) for azocasein hydrolysis in C. parvum homogenates (CPH) prepared from oocysts incubated for designated intervals (--●--). Data points represent the mean value of duplicate samples from a typical experiment; replicate experiments produced similar results.
CHAPTER 4

ANTAGONISTIC EFFECT OF HUMAN ALPHA-1-ANTITRYSIN ON EXCYSTATION OF CRYPTOSPORIDIUM PARVUM OOCYSTS

ABSTRACT: This study evaluated the effects of the human serine protease inhibitor alpha-1-antitrypsin (AAT) on in vitro excystation and infectivity of Cryptosporidium parvum. Oocysts used in excystation studies were subjected to bleach treatment, either directly from potassium dichromate storage or following cesium chloride (CsCl) purification. Unbleached CsCl-purified oocysts were used in control samples. Excystation was monitored at 37°C in RPMI medium in the presence of 0, 100, 500, or 1000 μg/ml AAT. Samples were collected at timed intervals and the mean percent of excystation in AAT-treatment groups was compared to untreated control sample means. AAT significantly inhibited (P<0.05) excystation in a concentration-dependent manner at incubation intervals from 15-90 min. AAT did not significantly alter the excystation dynamics of unbleached oocysts. The effects of AAT were further evaluated in cultured bovine fallopian tube epithelial (BFTE) cells. Bleached oocysts, suspended in RPMI medium containing 0, 1, 10, 100, 500, or 1000 μg/ml AAT, were used to inoculate confluent BFTE cell monolayers. Inoculated cells were maintained at 37°C until collected at 24, 48, 72, or 96 hr post-inoculation. Alternately,

sporozoites, excysted at 37°C and collected by filtration, were used to inoculate BFTE cells under the same conditions. The mean number of parasites counted in AAT-treated, oocyst-inoculated cells was significantly less ($P<0.01$) than control mean values at 24 and 48 hr post-inoculation; longer post-inoculation intervals (72-96 hr) exhibited a decreased inhibitory effect. AAT did not significantly inhibit parasite infection when cultures were inoculated with C. parvum sporozoites. These data suggest that the anticryptosporidial potential of AAT is primarily associated with an antagonistic effect on oocyst excystation.

INTRODUCTION

*Cryptosporidium parvum* (Apicomplexa: Cryptosporidiidae) is an enteropathogenic parasite that causes a diarrheal illness in humans. This protozoan preferentially invades the apical surface of epithelial cells lining the small intestine and exhibits the potential to colonize the intestinal and respiratory epithelia of immunocompromised hosts (Current et al., 1983; O'Donoghue, 1995). The severity of human cryptosporidiosis is appreciable in the AIDS patient population and infection is frequently accompanied by chronic, protracted diarrhea (Petersen, 1992; Goodgame et al., 1993, 1995). A number of potential anticryptosporidial compounds have been evaluated, yet there remains no definitive treatment for cryptosporidiosis that eradicates the parasite or reduces the chronicity of infection in susceptible hosts.
Alpha-1-antitrypsin (AAT) is a single chain glycoprotein composed of 394 amino acid residues with a molecular weight of approximately 56 kDa (Travis and Salvesen, 1983). AAT is found in peripheral circulation and provides homeostatic regulation of proteolytic activity from endogenous proteases (Baumstark, 1967; Perlmutter, 1993). In humans, AAT helps mediate the systemic effects of inflammation and is particularly effective in inhibiting its cognate target enzyme, neutrophil elastase. At present, pharmaceutical preparations of AAT are therapeutically indicated to supplement inadequate AAT levels in patients with congenital deficiencies; its use as a potential anticoccidian reflects a novel application of the compound.

Previous investigators have proposed the induction of parasite-derived mechanisms within the oocyst as the probable biochemical initiators of C. parvum excystation (Fayer and Leek, 1984; Reduker and Speer, 1985; Reduker et al., 1985; Woodmansee, 1987). Enzyme activity, specifically endogenous proteolysis, has been shown to facilitate excystation in the protists Giardia muris and Eimeria tenella (Feeley et al., 1991; North, 1992; McKerrow et al., 1993; Michalski et al., 1994). Cryptosporidial oocyst can be induced to excyst by incubating at elevated temperatures and, although the level of excystation is quite low, water or buffered saline can serve as an excystation medium at 37°C (Fayer and Leek, 1984; Current, 1990). Exposing oocysts to protocols that putatively enhanced the permeability of the oocyst wall significantly increases the rate of excystation at 37°C. Accordingly, bleach treatment, acidification,
deoxycholic acid, taurocholic acid, and trypsin have been reported to optimize excystation and subsequent in vitro infection (Reduker et al., 1985; Robertson et al., 1993; Upton et al., 1994).

Although a role for proteolytic activity in excystation has been postulated for several years, only recently has proteolytic enzyme activity been reported in *C. parvum*. Okhuysen et al. (1994) reported arginyl aminopeptidase activity in excysted *C. parvum* sporozoites and suggested a putative role for aminopeptidase activity in excystation. A subsequent report by Nesterenko et al. (1995) demonstrated cysteine protease activity in solubilized membrane fractions of excysted sporozoites. Our previous studies have detected proteolytic activity (azocasein hydrolysis) in a parasite homogenate prepared from oocysts and sporozoites during peak periods of excystation (Forney et al., 1996a). Further, we have reported an interaction between AAT and *C. parvum* that suggests the formation of serpin:enzyme complexes immunolocalized on the surface of freshly excysted sporozoites (Forney et al., 1996b).

In the present study we have pursued a thorough evaluation of the antagonistic effect of human AAT on the excystation of *C. parvum* oocysts. In particular, experiments were designed to investigate oocyst wall permeability, enhanced by bleach treatment, as a factor in AAT-mediated inhibition of excystation. We further evaluated the anticryptosporidial activity of AAT on both oocyst- and sporozoite-inoculated cultures and monitored serpin effects on in vitro infectivity over extended post-inoculation incubation intervals.
MATERIALS AND METHODS

Parasite isolation and preparation

Oocysts (Iowa strain) used in this study were propagated in experimentally infected Holstein calves, isolated from feces using sucrose and isopycnic Percoll gradients (Arrowood and Sterling, 1987), and stored in potassium dichromate (K₂Cr₂O₇) at 4°C. In preparation for analysis, oocysts were packed by centrifugation and the bulk of the K₂Cr₂O₇ was removed. The oocyst pellet was decontaminated in a 20% bleach (v/v) solution (Chlorox®, 5.25 % sodium hypochlorite in stock concentration) for 10 min at 4°C and washed thoroughly with sterile Hanks' balanced buffered saline (HBSS, HyClone Laboratories, Logan, Utah) to remove residual bleach and K₂Cr₂O₇. Oocysts were harvested by centrifugation and resuspended in sterile RPMI medium (HyClone). Alternately, the oocyst pellet was purified by CsCl flotation (ref) and used either directly following CsCl-purification or treated with bleach as previously described. CsCl-purified oocysts were washed in HBSS and resuspended in RPMI medium.

Bleached-decontaminated oocysts, prepared directly from K₂Cr₂O₇ storage, were used in BFTE cell infectivity studies. Sporozoites for cell culture inoculation were prepared from bleach-decontaminated oocysts suspended in RPMI medium at a concentration of 1 X 10⁷ oocysts/ml. The oocyst suspension was aspirated into sterile, prewarmed syringes, and incubated at 37°C for 1 hr. The resulting mixture of oocysts and sporozoites was passed
through a sterile 5-μm filter (Millipore Corporation, Bedford, Massachusetts) under gentle pressure and resuspended in RPMI medium to a final concentration of $2 \times 10^6$/ml sporozoites. Samples of the filtered sporozoite inoculum were examined by brightfield microscopy and found to be negative for intact oocysts.

**Excystation studies**

AAT (Chemicon International, Temecula, California) was initially reconstituted as a 2000-μg/ml stock solution in sterile RPMI medium and further diluted in RPMI medium as needed. Oocyst samples (i.e., bleach-treated before or after CsCl-purification) were mixed with AAT at concentrations of 100, 500, and 1000 μg/ml. Intact oocysts were counted in each sample using a hemocytometer and phase contrast microscopy to establish a baseline oocyst number prior to incubation. Oocysts were then incubated at 37°C, removed from the incubator at fixed time intervals (15, 30, 60, 90, 120, and 180 min), placed on ice to suppress further excystation, and intact oocysts were again counted. Unbleached, CsCl-purified oocysts were evaluated as control samples, both with and without AAT in the excystation medium. Because of the low rate of excystation observed for unbleached oocysts, incubation intervals were extended to 1-hr increments for a total of 6 hr when assessing AAT effects. Empty "ghost" oocysts and partially excysted oocysts were not included in the enumeration process. The difference in the number of oocysts counted before and after incubation was expressed as a
percent of the original, pre-incubation number. This value was termed the “percent of excystation” and used for comparative analysis of excystation between treated and control samples.

**In vitro infectivity**

Infectivity studies involved the inoculation of BFTE cells with $10^5$ bleach-decontaminated oocysts/ml in RPMI medium containing AAT at concentrations of 1, 10, 100, 500, and 1000 μg/ml. The stock serpin solution was passed through a 0.8-μm/0.2-μm syringe filter (Acrodisc® PF, Gelman Sciences, Ann Arbor, Michigan) prior to use in cell culture and further diluted in RPMI medium as required. A 1.0-ml aliquot of the inhibitor:oocyst mixture was used to inoculate BFTE cell monolayers grown to confluence on glass coverslips placed in individual wells of a 24-well culture plate (Corning Glass Works, Corning, New York). Monolayers inoculated with *C. parvum* oocysts in the absence of serpin treatment served as an infection control group. Inoculated cells were maintained in a candlejar at 37°C for 24 hr (Luechtfeld et al., 1982). After 24 hr incubation, the residual inoculum was rinsed off with sterile RPMI medium and cell monolayers were retreated with RPMI medium containing the same concentration of AAT as in the inoculum; coverslips representing the 24-hr post-inoculation treatment group were collected at this time. The remaining treatment groups were further incubated at 37°C/5% CO₂ and collected at 48-, 72-, and 96-hr post-inoculation. A second experiment involved the addition of AAT to BFTE cell
monolayers that had been inoculated with $10^5$ C. parvum oocysts and cultured for 24 hr prior to treatment. Coverslips were collected 24 hr after AAT treatment was applied to the established infections. A third treatment protocol involved the use of C. parvum sporozoites as a source of culture inoculum. Sporozoites were mixed with the same AAT levels noted previously to achieve a final concentration of sporozoites at $1 \times 10^6$/ml. A 1.0-ml aliquot of the AAT:sporozoite mixture was applied to BFTE cell monolayers and inoculated cells were maintained as described. Coverslips were collected at 24 and 48 hr post-inoculation. A final investigation paralleled the initial experiment, but was limited to a single AAT concentration (1000 µg/ml) in an oocyst inoculum. Treatment included the reapplication of AAT each 24-hr interval following the initial inoculation to evaluate the effect of daily retreatment; this manipulation helped ensure sufficient bioavailability of the serpin compound. Coverslips were collected at 24, 48, 72, and 96 hr post-inoculation. In all experiments, cell monolayers were removed at designated intervals, washed with RPMI medium, fixed in absolute methanol (10 min/25°C), rinsed with 25 mM PBS, and stained with Giemsa. Coverslips were mounted (inverted) on glass slides with a permanent mounting medium and examined by brightfield microscopy.

**Parasite enumeration**

Parasite enumeration in infected BFTE cells was performed by the method of Yang et al. (1996). In brief, parasites were counted in a single scan
(1,000X, oil immersion) across the diameter of a mounted coverslip (approx.
65 high power fields). Intact oocysts observed in BFTE cell monolayers at the
tend of the designated post-inoculation periods were considered to be residual
inoculum and were not counted. The mean number of parasites counted in
each of the AAT treatment groups was expressed as a percent of the mean
number of parasites counted in the infection control group. Differences in
infection numbers were compared for statistical significance by simple
ANOVA (Fisher's PLSD).

RESULTS

Excystation

The observed excystation dynamic of bleach-treated oocysts was
consistent with previous reports (Fayer and Leek, 1984; Woodmansee, 1987;
Upton et al., 1994). The percent of excystation increased rapidly during the
initial 90 min of incubation at 37°C with a progressive decline in the rate of
excystation after 180 min incubation (Fig. 4-1). Unbleached CsCl-purified
oocysts excysted at low levels over extended incubation periods. These
oocysts required 37°C incubation for 6 hr before reaching mean excystation
levels of ≥30%; comparable excystation yields were obtained within 30 min
with bleach-treated samples.

AAT significantly inhibited (P<0.05) excystation of bleach-treated
oocysts in a concentration dependent manner (Fig. 4-2). The observed
antagonistic effect was particularly evident during the early incubation
intervals from 15-90 min, but decreased progressively with extended incubation times. Further, both the duration of excystation inhibition and the degree of recovery were influenced by serpin concentration. Variation in bleach treatment (i.e., bleached directly from K$_2$Cr$_2$O$_7$ storage or following CsCl-purification) had no significant effect on AAT-mediated inhibition. As shown in Figure 4-3, excystation of CsCl-purified oocysts was not significantly inhibited by 1000 μg/ml AAT; similar results were obtained for lower concentrations of the serpin (data not shown).

**In vitro infectivity**

AAT, at concentrations ≥10 μg/ml, significantly inhibited ($P<0.01$) parasite infection in the 24- and 48-hr post-inoculation groups in cells inoculated with bleach-treated oocysts (Table 4-1). The inhibitory effect of AAT exposure decreased with increased culture incubation intervals; significant inhibitory activity ($P<0.01$) was limited to serpin levels ≥500 μg/ml in the 72-hr post-inoculation group and ≥1000 μg/ml at 96 hr after inoculation. BFTE cell monolayers with established *C. parvum* infection were refractory to AAT and no significant inhibition was observed at any of the time periods evaluated (Table 4-1).

AAT had no significant inhibitory effect on parasite numbers when sporozoites were used to inoculate BFTE cell cultures (Fig. 4-4). Similarly, the reapplication of human serpin at 24-hr intervals for the duration of post-inoculation incubation periods did not significantly enhance the inhibitory
effect of the compound on in vitro infection relative to a single reapplication of AAT (Fig. 4-5).

The observed results led us to question whether AAT had an adverse effect on BFTE cells such that parasite numbers were reduced independent of any potential anticoccicial activity. To help clarify this issue, BFTE cell monolayers were incubated with each of the respective concentrations of AAT for 24 hr prior to inoculation. Cells were then rinsed 4 times with RPMI medium to remove residual serpin, inoculated with either bleach-treated oocysts or sporozoites, and reincubated at 37°C/5% CO₂ for 24 hr. Infection rates obtained following AAT preincubation were not statistically different from the mean number of parasites counted in control samples preincubated with RPMI medium in lieu of AAT (data not shown). No cytotoxic effects were observed in culture cells incubated for up to 96 hr with AAT at the concentrations noted.

**DISCUSSION**

The results of the present study provide substantial evidence to link the in vitro anticryptosporidial activity of AAT to an antagonistic effect on oocyst excystation. This association is apparent from the differential inhibitory capacity of AAT on oocyst-inoculated cell cultures and the absence of significant inhibition on established in vitro infections. The lack of anticryptosporidial efficacy in this later group suggests that AAT exerts minimal effect on the asexual propagation of intermediate stages (merogony).
Further, AAT did not demonstrate a significant inhibitory effect on BFTE cells inoculated with freshly excysted sporozoites, suggesting that serpin activity does not alter parasite attachment and invasion. Neither was the diminution of anticryptosporidial activity over extended post-inoculation intervals attributable to a lack of biological availability of the serpin compound; retreatment of infected BFTE cells at daily (24-hr) intervals did not preclude the loss of inhibitory efficacy.

Our data indicated that while AAT reduced the rate of excystation for bleached-treated oocysts, the serpin did not inhibit the excystation of unbleached oocysts. These findings suggest that parasite excystation is dependent upon endogenous mechanisms that are protected from serpin activity by the oocyst wall. It is further apparent that permeability of the oocyst wall is requisite for AAT interactions with oocysts and/or sporozoites sufficient to alter excystation prior to the emergence of viable sporozoites. The oocyst wall is an effective barrier to AAT as long as the integrity of the wall remains intact.

Infection control samples (i.e., lacking AAT treatment) inoculated with sporozoites had considerably lower infection rates and exhibited greater variability than observed in cells inoculated with oocysts. While microscopic examination of aliquots taken from sporozoite suspensions prior to inoculation did not reveal the presence of intact oocysts, oocysts were occasionally encountered on sporozoite-inoculated coverslips. Filtration methods to produce *C. parvum* sporozoites minimize handling and reduce
the time interval from excystation to culture inoculation, but do not completely eliminate the inadvertent inclusion of oocysts in the final inoculation. Some of the variation noted in AAT treatment of sporozoite-inoculated cells may be influenced by serpin effects on spurious oocysts in filtered samples and may not be solely representative of AAT effects on the early stages of in vitro infection. These groups lacked the concentration- and time-dependent relationships between AAT treatment and parasite numbers observed in oocyst-inoculated cultures.

The antagonistic effect of AAT on excystation appears to be a transient process; continued incubation produced excystation yields levels approaching approximately 65-90% of the control group mean values. Despite the relative increase in excystation with continued incubation, it is noteworthy that oocyst inoculum containing AAT had significantly lower infection levels in the early post-inoculation treatment groups. The viability of emerging sporozoites appears to be directly related to the time lapse between the induction of excystation-related activity and final escape from the oocyst wall. Specifically, the time lag between the initial activation of excystation and the availability of susceptible host cells is a critical factor in *C. parvum* infectivity. The viability of excysted sporozoites is significantly diminished by excessive handling or delays in inoculating cultures (Robertson et al., 1993). Sporozoite viability is relatively intolerant of delayed emergence and a loss of infectivity is evident in AAT-treated oocysts. It is reasonable to speculate that a significant portion of the finite energy stores within the sporozoite may be
consumed in the excystation process; failure to find an obligatory host cell shortly after emergence is detrimental to parasite infectivity. A similar relationship between sporozoite energy expenditure and diminished viability has been reported in the related coccidian *Eimeria tenella* (Yashwant et al., 1993).

The findings presented support previous assertions that parasite-derived biochemical mechanisms induced by elevated temperature and, possibly, other conditions in the microenvironment of excysting oocysts trigger the activation and emergence of sporozoites (Fayer and Leek, 1984; Woodmansee, 1987). Of particular interest is the association between antagonized excystation during initial incubation intervals and a subsequent reduction in BFTE cell infection demonstrated in this study. The implications of these findings suggest that not only is excystation induced by an activation of enzymatic processes, but once activated, bioenergetic demand may be the most important limitation of sporozoite viability.

On the basis of this study, we conclude that AAT antagonizes *C. parvum* excystation when it can gain entry into the oocyst; enhanced permeability of the oocyst wall is necessary for the serpin to inhibit excystation. Further, these data suggest that sporozoite activation within the intact oocyst wall includes, at least in part, the expression of proteolytic enzyme activity. It is the association of protease activity with excystation, and the functional specificity of the AAT molecule for the active site of serine proteases, that provides the most likely mechanism for serpin-mediated
inhibition. The delayed excystation dynamic attributed to AAT accounts for its anticryptosporidial activity against in vitro infection, putatively by altering endogenous proteolytic activity within the intra-oocyst milieu.

LITERATURE CITED


Table 4-1. In vitro anticryptosporidial activity of alpha-1-antitrypsin in BFTE cell culture.

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<th>[AAT]*</th>
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</table>

*Concentration in µg/ml.
†Duration of AAT exposure (hr). A positive (+) sign indicates treatment was delayed until after inoculation; all other groups were treated at the time of culture inoculation with C. parvum oocysts.
‡Significantly different from control group (P<0.01); data represent mean ± SD for duplicate samples in 2 separate experiments.
Figure 4-1. Pattern of excystation for bleach-treated *Cryptosporidium parvum* oocysts. Excystation yield from oocysts harvested directly from K₂Cr₂O₇ storage (–o–) or following CsCl-purification (–●–) compared to unbleached CsCl-purified oocysts (–■–). Values represent mean ± SD for 3 replicates from each of 3 separate experiments.
Figure 4-2. Antagonistic effect of alpha-1-antitrypsin on *Cryptosporidium parvum* excystation. CsCl-purified, bleach-treated oocysts incubated at 37°C in the presence of 100 (■), 500 (●), or 1000 (○) μg/ml purified human AAT. Data for bleach decontaminated oocysts from K₂Cr₂O₇ storage was similar. Values represents mean ± SD for duplicate samples from 3 separate experiments.
Figure 4-3. Effect of alpha-1-antitrypsin on excystation of unbleached *Cryptosporidium parvum* oocysts. CsCl-purified oocysts incubated at 37°C in RPMI containing 1000 μg/ml human AAT (—○—) relative to untreated control samples (—●—). Values represents mean percent excystation for each incubation interval ± SD for duplicate samples from 2 separate experiments.
Figure 4-4. Effect of alpha-1-antitrypsin on infection of cultured epithelial cells inoculated with Cryptosporidium parvum sporozoites. Filter-isolated sporozoites were cultured in RPMI medium containing AAT for 24-hr (---) or 48-hr (-O-) post-inoculation intervals. Values represents mean ± SD for duplicate samples from 2 separate experiments.
Figure 4-5. Effect of alpha-1-antitrypsin retreatment on parasite numbers in cultured epithelial cells inoculated with Cryptosporidium parvum oocysts. Data represents the mean number of parasites counted in BFTE cell monolayers, expressed as a percent of untreated control sample means, containing 1000 μg/ml AAT and cultured for 24 (white bar) or 48 (black bar) hr post-inoculation. Values indicate mean ± SD for duplicate samples from 2 separate experiments.
CHAPTER 5
IN VITRO ANTICRYPTOSPORIDIAL POTENTIAL OF SERINE PROTEASE INHIBITORS

INTRODUCTION

This chapter comprises 3 separate manuscripts originally prepared in the abbreviated format of a Research Note consistent with the requirements of the publishing journal. Though prepared as individual manuscripts, these notes provide a composite evaluation of the anticryptosporidial potential of select protease inhibitors with a particular emphasis on AAT. The ultimate contribution of this dissertation may reside in the applicability of these studies to the needs of the medical community; the increasing incidence of cryptosporidiosis in immunodeficient and immunocompromised individuals has substantially increased the morbidity and mortality of HIV infection. Overwhelming bouts of diarrheal illness, in excess of 70 liters of watery diarrhea a day, severely degrade the quality of life for AIDS patients. The lack of a curative agent to combat chronic cryptosporidial infection is a very real problem and efforts to identify candidate compounds for therapeutic trial are a worthy endeavor.

The reader should recognize the progressive evolution and refinement of the studies upon which this chapter is based. The initial evaluation of AAT as a potential anticryptosporidial compound revealed a potent effect for the inhibitor in concentrations $\geq 100 \mu g/ml$. Subsequent studies showed that
the inhibitory potential of AAT was noted at much lower levels and peaked at 24- to 48-hr post-inoculation; we later noted that efficacy waned when post-inoculation intervals were extended to \(\geq 72\) hr. It became increasingly apparent that AAT, and possibly other compounds in the general class of serine protease inhibitors, were potent antagonists of excystation of \(C.\ parvum\) oocysts. As an antiexcystation agent, it is reasonable to suggest that the reduction in initial excystation rates observed in the presence of AAT contributed to the decreased infectivity pattern noted in cell cultures inoculated with \(C.\ parvum\) oocysts.

The amplification cycle of type I meronts appears to be refractory to serpin activity and the observed loss of in vitro efficacy for AAT during mergony likely reflects the propagation of intermediate stages of \(C.\ parvum\). This hypothesis is strongly supported by results indicating a decreased inhibitory potential for AAT during prolonged post-inoculation intervals and a lack of significant inhibition of established infections. Further, AAT had no significant inhibitory effect on infection when excystation was accomplished prior to inoculation; sporozoite inocula containing AAT produced in vitro infection rates statistically indistinguishable from untreated control groups.

The potent inhibition of excystation and early infection by AAT, followed by a progressive loss of anticryptosporidial activity, led to the consideration of a multi-compound evaluation. A strategy was employed to exploit the anticryptosporidial characteristics of AAT in combination with a second anticryptosporidial agent that preferentially targeted developmental
intermediates of the parasite. The aminoglycoside paromomycin was selected as a second treatment compound and this combination (i.e., AAT and paromomycin) demonstrated a potent synergistic activity against *C. parvum* infection in vitro. Thus, a rational approach to multi-compound evaluation in an in vitro system proved to be a highly successful strategy in preventing cryptosporidial infection.

The manuscripts presented in this chapter have been modified from their original style to conform to format consistency and facilitate the reader’s review of their contents. The abstract prefacing each section has been summarized in this introduction and a single, comprehensive literature cited section completes the chapter.

**EFFICACY OF SERINE PROTEASE INHIBITORS AGAINST *CRYPTOSPORIDIUM PARVUM***

The dynamic of host cell infection by *Cryptosporidium parvum* (Apicomplexa: Cryptosporidiidae) and the parasite’s enigmatic residence within an intramembranous vacuole are poorly characterized events in the life-cycle of this organism. Other apicomplexan species reportedly undergo a series of events during which the apical complex of the organism attaches, orients, and, ultimately, facilitates penetration of the host cell membrane to

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assess an intracellular location for growth and development (Aikawa et al., 1978). The biochemical properties of sporozoans appear to include 1 or more protease-mediated steps in the host cell adhesion and invasion process. Hadley et al. (1983) demonstrated a protease-dependent mechanism involved in parasite attachment and penetration of erythrocytes by *Plasmodium knowlesi* merozoites. Their study reported a significant reduction in infectivity following the exposure of merozoites to selected protease inhibitors. Other investigators have reported analogous findings in *Plasmodium falciparum* (Dluzewski et al., 1986), *Eimeria tenella* (Fuller and McDougald, 1990), and *Eimeria vermiformis* (Adams and Bushell, 1988), in which protease inhibitors reduced the in vitro infection of permissive host cells.

The present study evaluated the anticryptosporidial potential of the protease inhibitors alpha-1-antitrypsin (AAT), antipain, aprotinin, leupeptin, methoxysuccinyl-ala-ala-pro-valine chloromethylketone (MAAPVCK), soybean trypsin inhibitor (SBTI), and phenylmethylsulfonyl fluoride (PMSF) in a bovine fallopian tube epithelial (BFTE) cell culture system. Our previous study (Forney et al., 1996b) detected a functional interaction between human AAT and *C. parvum*, primarily associated with the invasive form (sporozoite) of the parasite. The functional specificity of AAT for the active site of serine proteases influenced the selection of inhibitors used in the present study. Our objective here was to evaluate the efficacy of the selected protease inhibitors in reducing the in vitro infectivity of *C. parvum*. 
The BFTE cell culture system used in this study supports the complete development, i.e., asexual and sexual reproductive cycles, of *C. parvum* (Yang et al., 1996) and has been validated as an analytical tool for screening potential anticryptosporidial compounds in previous studies with Immuno-C™ (Healey et al., 1996) and paromomycin (S. Yang, per. comm.). Protease inhibitors AAT (Chemicon International, Inc., Temecula, California), antipain (ICN Biochemicals, Inc., Aurora, Ohio), aprotinin (ICN), leupeptin (Calbiochem-Novabiochem Corp., La Jolle, California), and SBTI (Calbiochem) were initially prepared in stock solutions of 10, 20, 100, 200, and 1000 µg/ml in RPMI medium (Hyclone Laboratories, Logan, Utah). MAAPVCK (Sigma Chemical Co., St. Louis, Missouri) and PMSF (Sigma) were first dissolved in dimethyl sulfoxide (DMSO; Sigma) and subsequently diluted in RPMI medium to prepare the same concentrations (MAAPVCK) or 2, 4, and 6 mM concentrations (PMSF). Oocysts used in this study were successively propagated in Holstein calves. Isolated oocysts (Arrowood and Sterling, 1987) were decontaminated in 10% bleach for 20 min at 4°C, washed thoroughly with sterile Hanks' balanced buffered saline (HBSS), and resuspended in sterile RPMI medium. Immediately prior to inoculation, purified *C. parvum* oocysts were added to the previously prepared protease inhibitor dilutions to obtain $10^5$ oocysts/ml at the final inhibitor concentrations tested, i.e., 5, 10, 50, 100, and 500 µg/ml or 1, 2, and 3 mM for PMSF. A 1.0-ml aliquot of the inhibitor:oocyst mixture was used to inoculate BFTE cell monolayers grown to confluence on glass coverslips placed in
individual wells of a 24-well culture plate. Each protease inhibitor concentration was tested in either 2 (antipain, leupeptin, SBTI, PMSF, MAAPVCK) or 4 (AAT, aprotinin) well replicates in duplicate experiments. At least 2 wells per plate were inoculated with C. parvum oocysts in the absence of inhibitor treatment and served as an infection control group. Inoculated cells were maintained in a candlejar environment at 37°C. Coverslips were removed at 24 hr postinoculation and washed with sterile RPMI medium. Cell monolayers were fixed 10 min in 100% methanol, rinsed twice with 0.025 M PBS, stained with Giemsa for 2 hr, and rinsed with distilled water. Coverslips were mounted (inverted) on glass slides with a permanent mounting medium and examined by brightfield microscopy (oil immersion, 1000X).

Parasite enumeration was accomplished as previously described (Yang et al., 1996). Oocysts observed in the BFTE cell monolayers at the end of the incubation period were considered to be residual inoculum and not included in the enumeration process. The mean number of parasites counted in each of the protease inhibitor treatment groups was expressed as a percentage of the mean number of parasites counted in the infection control group. The difference between mean values for treatment and control groups was compared for statistical significance by ANOVA (Fisher's PLSD) using a StatView® statistical analysis application.
BFTE cells were permissive to *C. parvum* infection. Typically, at 24 hr post-inoculation, the infection control group exhibited a predominance of the type I and II meront developmental stages with few, if any, residual oocysts remaining from the inoculum. Parasite numbers were significantly reduced (*P* < 0.01) following treatment with leupeptin and SBTI at concentrations of 10 and 50 µg/ml, respectively (Table 5-1). The protease inhibitors AAT, antipain, and aprotinin significantly reduced (*P* < 0.001) parasite numbers at concentrations as low as 5-10 µg/ml. MAAPVCK did not significantly reduce *C. parvum* infection of BFTE cells at any of the concentrations tested. PMSF significantly reduced (*P* < 0.001) parasite numbers to 40% of control values at a concentration of 3 mM (Table 5-2).

AAT, antipain, and aprotinin were the most potent inhibitors of *C. parvum* infection in the BFTE cell culture system. When concentrations of these protease inhibitors were increased to 1,000 µg/ml in the inoculum, parasite numbers were reduced to 1.76 ± 0.4% (AAT), 6.64 ± 1.44% (antipain), and 9.44 ± 1.81% (aprotinin) of the infection control mean (Fig. 5-1). During the microscopic enumeration of parasites in BFTE cell monolayers treated with these 3 protease inhibitors, intact (unexcysted) oocysts were frequently observed. This observation was predominantly associated with protease inhibitor concentrations exceeding 100 µg/ml and was confined to these 3 inhibitors. There was no evidence of cytotoxicity for BFTE cells at the protease inhibitor concentrations evaluated in this study.
Anticryptosporidial potential attributable to protease inhibition strongly suggests that the dynamic influencing initial host cell:parasite interaction is facilitated by parasite protease activity. The present study demonstrated a significant in vitro inhibition of *C. parvum* infection by AAT, antipain, and aprotinin and significant, but quantitatively less, inhibition by leupeptin, SBTI, and PMSF. These findings are generally consistent with studies on related coccidians (Hadley et al., 1983; Adams and Bushell, 1988; Fuller and McDougald, 1990). PMSF was the most potent inhibitor of *Eimeria* spp when evaluated at a concentration of 5 mM. Conversely, Hadley et al. (1983) reported no significant inhibitory effect by either PMSF or antipain on erythrocyte invasion by *P. knowlesi* merozoites. In the present study, a lack of significant inhibition by MAAPVCK, in light of the effects of AAT, antipain, and aprotinin, is a presumptive indication that protease-like activity expressed by *C. parvum* may more closely resemble a trypsin-like serine protease, in terms of inhibitor sensitivity, rather than an elastolytic enzyme (Stein and Trainor, 1986; Potempa et al., 1994).

The results of this study add to the growing body of literature addressing the essential metabolic and physiologic roles of proteases in parasitic infection (North, 1992; Roose and Van Noorden, 1995). Parasite protease activity has been associated with host cell attachment (Arroyo and Aldrete, 1989), degradation of host proteins for parasite nutrition (Chappell and Dresden, 1986), tissue migration and penetration (McKerrow et al., 1990),
cytotoxic effects (Lushbaugh et al., 1981), and parasite maturation and development (Richer et al., 1993).

This study is the first to report a significant reduction in C. parvum infection of cultured cells in the presence of protease inhibitors. These findings support a hypothesis that protease activity, putatively a serine protease enzyme, is an important biochemical constituent of the early infection dynamics of C. parvum. A recent report of arginyl aminopeptidase activity associated with C. parvum (Okhuysen et al., 1994) assessed a potential role for aminopeptidase activity in the excystation of oocysts. Our observation of intact residual oocysts in cell monolayers treated with serine protease inhibitors strengthens the association between proteolytic activity and excystation. These findings further suggest that the anticryptosporidial potential of protease inhibitors may reduce host cell vulnerability to parasite protease activity and may be a prefatory indication of a chemotherapeutic strategy to abrogate cryptosporidial infection.

**ANTICRYPTOSPORIDIAL POTENTIAL OF ALPHA-1-ANTITRYPsin**

*Cryptosporidium parvum* is an intracellular protozoan parasite that preferentially infects epithelial cells lining the microvilli of the small intestine. Human cryptosporidiosis is particularly severe in the

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immunocompromised host and is recognized as a significant cause of morbidity in the AIDS patient population. The present lack of efficacious therapy is an impetus for investigative efforts to identify potential anticryptosporidial agents. Alpha-1-antitrypsin (AAT) is the major serine protease inhibitor (serpin) in human circulation and has a substantial homeostatic role in regulating excess proteolytic activity. Previous studies have detected an interaction between human AAT and components of C. parvum sporozoites (Forney et al., 1996b). To evaluate the functional significance of serpin interactions, we investigated the anticryptosporidial potential of purified human AAT on 2 aspects of the early infection dynamics C. parvum. In this present study, we report the effects of AAT on in vitro excystation and cryptosporidial infection in a bovine fallopian tube epithelial (BFTE) cell culture system.

Oocysts (Iowa strain) were amplified in Holstein calves. Isolated oocysts (Arrowood and Sterling, 1987) were decontaminated in 10% bleach for 10 min at 4°C, washed with sterile HBSS, and resuspended in sterile RPMI medium (HyClone Laboratories). Purified human AAT (Chemicon International, Inc.) was initially prepared in stock solutions of 20, 200, 1000, and 2000 μg/ml in RPMI medium. For excystation studies, oocysts were mixed with equal volumes of the appropriate AAT stock solutions to obtain final serpin concentrations of 10, 100, and 500 μg/ml. Intact oocysts were counted in a hemacytometer using phase contrast microscopy. Oocysts were then incubated at 37°C in the absence of any further treatment (Robertson et
al., 1993; Upton et al., 1994). Tubes were removed from the incubation environment at fixed time intervals, i.e., 15, 30, 60, 90, 120, and 180 min, and intact oocysts were again enumerated (excluding empty oocyst "ghosts" and partially excysted oocysts). The difference between the number of oocysts counted prior to incubation and the number of oocysts counted after incubation was expressed as a percent of the original (preincubation) number. This value was termed "percent excystation" and represented an indirect assessment of excystation. To evaluate serpin effects on infectivity, C. parvum oocysts were added to the previously prepared AAT stock solutions to obtain $10^5$ oocysts/ml at inhibitor concentrations of 10, 100, 500, and 1000 μg/ml. A 1.0-ml aliquot of each AAT:oocyst mixture was used to inoculate separate BFTE cell monolayers grown to confluence on glass coverslips placed in individual wells of a 24-well culture plate. Inoculated cells were maintained in a candlejar environment at 37°C. Monolayers were removed at 24 hr post-inoculation, washed with RPMI medium, fixed with absolute methanol, and stained with Giemsa. Coverslips were mounted (inverted) on glass slides with a permanent mounting medium and examined by brightfield microscopy (oil immersion, 1000X). Parasite enumeration was accomplished as previously described (Yang et al., 1996). Intact oocysts remaining after 24 hr incubation were considered residual inoculum and not included in the enumeration process. The mean number of parasites counted in each of the protease inhibitor treatment groups was expressed as a
percentage of the mean number of parasites counted in an infection control group.

Excystation studies demonstrated that AAT significantly \((P<0.05)\) inhibited oocyst excystation in concentrations \(\geq 10 \mu g/ml\) (Table 5-3). The inhibitory effect was greatest in the early incubation intervals from 0-60 min. Excystation yields typically attained 70-75% of untreated control samples when incubation was continued to 180 min with all but the highest levels of AAT evaluated (Table 5-3). The functional specificity of AAT for the active site of serine protease enzymes suggests that protease activity, putatively a serine protease, facilitates oocyst excystation.

Infectivity of \(C.\ parvum\) oocysts in BFTE cells was significantly inhibited \((P<0.01)\) at each of the AAT concentrations evaluated (Table 5-4). Inhibition of in vitro infection exceeded 50% at AAT levels as low as 10 \(\mu g/ml\) and reached 95.4% at a concentration of 1000 \(\mu g/ml\). The frequent observation of intact (unexcysted) oocysts in BFTE monolayers inoculated with oocysts in the presence of high concentrations of AAT further suggests an association between serpin activity and impaired excystation.

These data describe a potent in vitro anticryptosporidial potential for human AAT and, collectively, strengthen the association between early excystation intervals and subsequent in vitro infection of host cells (Robertson et al., 1993; Upton et al., 1994). The findings of this study add to emerging evidence suggesting a requisite role for proteolytic enzyme activity in the infectivity of \(C.\ parvum\) (Forney et al., 1996c; Nesterenko et al., 1995;
Okhuysen et al., 1994) and, further, suggest the potential use of serine protease inhibitors in a therapeutic strategy to combat cryptosporidiosis.

**SYNERGISTIC ANTICRYPTOSPORIDIAL POTENTIAL OF THE COMBINATION ALPHA-1-ANTITRYPSIN AND PAROMOMYCIN**¹

*Cryptosporidium parvum* (Apicomplexa: Cryptosporidiidae) is an enteropathogenic protozoan that invades the intestinal epithelium in a number of mammalian species (O'Donoghue, 1995). Cryptosporidiosis is particularly severe in the immunocompromised human host and infection is frequently characterized by a profuse, watery (termed "cholera-like") diarrheal illness. Growing concern about the potential for human infection, particularly as acquired through municipal water supplies, and the sequela of chronic infection in susceptible hosts constitutes a significant emerging infectious disease threat (CDC, 1994; MacKenzie et al., 1994). The formidability of chronic infection is accentuated by the present lack of efficacious therapy. The search for anticryptosporidial agents has included the evaluation of numerous compounds in various in vitro and in vivo models, as well as in human clinical trials. This pursuit has been plagued by inconsistent therapeutic responses, recrudescence of infection following

cessation of treatment, and the apparent autoinfective life-cycle of *C. parvum* in chronic infection states.

Reports of proteolytic enzyme activity in the early developmental stages of *C. parvum* have prompted consideration of serine protease, cysteine protease, and arginyl aminopeptidase inhibitors for anticryptosporidial properties. Okhuysen et al. (1994) first reported cryptosporidial aminopeptidase activity and further demonstrated the ability of alpha-aminoboronic acid derivatives to inhibit *C. parvum* peptidase activity and oocyst excystation (Okhuysen et al., 1995). Other investigators have reported cysteine protease activity associated with *C. parvum* sporozoite membrane fractions (Nesterenko et al., 1995) and a reduction in parasite infectivity following exposure of sporozoites to the cysteine protease inhibitor L-trans-epoxysuccinyl-leucylamindo-(4-guanidino)-butane (E-64) in MDCK cell culture (Huang and Petersen, 1996). Additionally, we have demonstrated a potent anticryptosporidial potential for select serine protease inhibitors, including human alpha-1-antitrypsin (AAT), in a bovine fallopian tube epithelial (BFTE) cell culture system (Forney et al., 1996b). These collective observations strongly suggest that protease/peptidase activity is requisite for the early infection dynamics of *C. parvum* and further suggest a potential chemotherapeutic role for protease inhibitors in controlling cryptosporidiosis.

Marshall and Flanigan (1992) reported a significant reduction in *C. parvum* infection in a differentiated human enterocyte cell line, HT29.74, using the aminoglycoside paromomycin. Further studies have reported a
significant anticryptosporidial effect for paromomycin in immunosuppressed mice (Healey et al., 1995), immunosuppressed rats (Rehg, 1994; Verdon et al., 1994), calves (Fayer and Ellis, 1993b), SCID mice (Tzipori et al., 1994), and in human clinical trials (Clezy et al., 1991; Fichtenbaum et al., 1993; Bissuel et al., 1994; Flanigan et al., 1996). A report of paromomycin treatment followed by the administration of letrazuril noted an improved therapeutic response in treating AIDS-related cholangitis (Hamour et al., 1993). Fayer and Ellis (1993a) evaluated the anticryptosporidial efficacy of select aminoglycosides, to include paromomycin, in single and combined therapy trials with tetracycline, in a neonatal mouse model. Their findings showed a significant reduction in cryptosporidial infection following administration of paromomycin, but did not reveal a synergistic effect in combination with tetracycline derivatives.

In the present study we evaluated the combined effects of the serine protease inhibitor (serpin) AAT and paromomycin in BFTE cell culture. A putative role for serpin activity in antagonizing oocyst excystation (Forney et al., 1996a) and the anticryptosporidial efficacy of paromomycin, particularly against intermediate stages of *C. parvum*, suggests that these compounds target different stages in the developmental life-cycle of the parasite. Our specific intent was to exploit the potential for enhanced efficacy from these 2 compounds when used simultaneously in an in vitro assay.

Oocysts used in this study were propagated in experimentally infected Holstein calves, isolated from feces using discontinuous sucrose gradients (Arrowood and Sterling, 1987), and stored in 2.5% potassium dichromate
(K₂Cr₂O₇) at 4°C. Prior to cell culture inoculation, oocysts were decontaminated with a 10% (v/v) bleach solution for 10 min at 4°C and washed with HBSS to remove residual K₂Cr₂O₇ and bleach. Bleach-decontaminated oocysts were resuspended in RPMI 1640 base medium (RPMI; HyClone Laboratories, Logan, Utah) at a concentration of 2 X 10⁵ oocysts/ml.

Primary BFTE cell cultures were prepared by the method of Yang et al. (1996) and used to evaluate anticryptosporidial activity. In brief, epithelial cells were collected from the mucosal surface of bovine fallopian tubes (E. A. Miller & Sons Packing Company, Hyrum, Utah) by flushing the tubes multiple times with sterile HBSS. Cells were thoroughly washed in HBSS, packed by centrifugation (200 X g for 10 min), introduced into culture flasks containing RPMI supplemented with 10% fetal bovine serum (FBS; HyClone), and cultured at 37°C/5% CO₂. Cells were trypsinized and seeded onto glass coverslips in individual wells of 24-well culture plates (Corning Glass Works, Corning, New York) using unsupplemented RPMI. BFTE cells were maintained at 37°C/5% CO₂ until monolayers reached confluence.

Stock solutions of human AAT (Chemicon International, Inc., Temucula, California) and paromomycin (Warner Lambert/Parke Davis, Ann Arbor, Michigan) were prepared in RPMI at concentrations of 1000 μg/ml (AAT) or 2400 μg/ml (paromomycin) and passed through a 0.8-μm/0.2-μm syringe filter (Acrodisc® PF, Gelman Sciences, Ann Arbor, Michigan). Stock concentrations were further diluted in RPMI as needed.
To evaluate the differential efficacy of each compound, decontaminated *C. parvum* oocysts were added to RPMI containing AAT at concentrations of 1, 10, 100, and 500 µg/ml or paromomycin at levels of 150, 300, 600, and 1200 µg/ml. A 1.0-ml aliquot of each of the resulting mixtures (10⁵ oocysts) was used to inoculate BFTE cell monolayers. To evaluate the combined effects of AAT and paromomycin, each concentration of AAT was combined with each level of paromomycin for a total of 16 combined treatment groups. A mixture of *C. parvum* oocysts in RPMI alone was used to inoculate infection control samples. The same concentrations of compounds, single and combined, were added to BFTE cell monolayers to assess the effect of the compounds on BFTE cells. Inoculated cells were maintained in a candlejar environment at 37°C for the initial 24-hr post-inoculation period. At 24 hr post-inoculation, cell monolayers were rinsed with RPMI to remove residual inoculum and either collected or retreated with the same concentration of compound(s) present in the initial inoculum and further incubated at 37°C/5% CO₂.

BFTE cell monolayers were collected at 24, 48, 72, and 96 hr post-inoculation and fixed 10 min in absolute methanol at room temperature. Fixed cells were rinsed with 25-mM PBS, stained with Giemsa, and rinsed with distilled water. Coverslips were mounted (inverted) on glass slides with a permanent mounting medium and examined by brightfield microscopy (oil immersion, 1000X). Parasite enumeration was accomplished as described by
Yang et al. (1996). In brief, parasites were counted in a single scan across the diameter of each coverslip (approx 65 high-power fields) and the mean number of parasites counted in each of the treatment groups was expressed as a percentage of the mean number of parasites counted in an infection control group.

Synergistic potential was evaluated by calculating fractional inhibitory concentration (FIC) indices as described by Berenbaum (1978) and interpreted according to Allen et al. (1982). This method is based on the proportional relationship between IC$_{50}$ values for single and combined treatment groups and was calculated in this study using the following formula:

\[
FIC = \frac{IC_{50} \text{ of } \text{AAT in combination}}{IC_{50} \text{ of AAT alone}} + \frac{IC_{50} \text{ of paromomycin in combination}}{IC_{50} \text{ of paromomycin alone}}
\]

FIC values were interpreted as follows:

- \( FIC < 0.5 \) = Significant synergism
- \( FIC 0.5 - 0.9 \) = Suggestive of synergism
- \( FIC 1.0 \) = Additive effect
- \( FIC 1.1 - 1.9 \) = Indifference or partial antagonism
- \( FIC \geq 2.0 \) = Antagonism

The difference between the mean number of parasites counted in treatment and control groups was compared for statistical significance by ANOVA (Fisher’s PLSD). Similarly, the mean number of parasites counted
in combined treatment groups was compared to both single compound
treatment groups and corresponding infection control groups for statistically
significant differences. The 50% inhibitory concentration (IC$_{50}$) values for
AAT and paromomycin treatment alone, and for combined treatment effects,
were calculated using curve-fitting algorithms in the StatView™ data
analysis program.

The anticryptosporidial effect of AAT on in vitro oocyst infectivity was
concentration dependent and exhibited an inhibitory pattern similar to that
previously reported (Forney et al., 1996b). AAT significantly reduced ($P < 0.01$)
$C.\ parvum$ infection in BFTE cells at concentrations $\geq 1$ µg/ml at 24 hr post-
inoculation relative to untreated control samples. Serpin activity was
progressively less inhibitory to $C.\ parvum$ infection with increased post-
inoculation intervals; $\geq 100$ µg/ml was required to produce a significant
inhibitory effect ($P < 0.01$) at 96 hr post-inoculation (Fig. 5-2a). The pattern of
decreasing efficacy is further evident in the IC$_{50}$ values determined for AAT at
each of the post-inoculation sampling periods (Table 5-5).

Paromomycin significantly inhibited ($P < 0.01$) in vitro cryptosporidial
infection at concentrations $\geq 150$ µg/ml at each of the post-inoculation
sampling intervals (Fig. 5-2b). The inhibitory effect of paromomycin was
concentration dependent and progressively increased with continued
incubation of inoculated cultures. The most potent anticryptosporidial effect
for paromomycin was observed in the 48- to 96-hr post-inoculation intervals
and, at 1200 µg/ml, paromomycin consistently reduced parasite numbers
>90% relative to infection control samples. As seen in Table 5-5, the IC<sub>50</sub> values determined for paromomycin decreased with extended post-inoculation sampling intervals.

The combined application of AAT and paromomycin in an oocyst inoculum significantly inhibited (P <0.01) C. parvum infection at each of the combinations and post-inoculation sampling intervals monitored in this study (Fig. 5-3). The inhibitory effect of combined treatment was concentration-dependent at the 24- to 48-hr post-inoculation sampling intervals. The contributory effect of AAT concentration was less evident in the latter sampling periods, consistent with the diminished anticryptosporidial potency noted for the serpin in single compound treatment groups at 72 and 96 hr post-inoculation.

FIC indices were calculated for combined treatment groups to evaluate the potential synergistic response of the 2 compounds relative to single treatment efficacy. The mean FIC value for combined treatment groups at 24-hr post-inoculation was 0.1835, indicating a significant synergistic interaction for the combination of AAT and paromomycin. Mean FIC values increased with longer incubation intervals, but continued to fall in a range indicative of synergistic activity despite the relative decrease in the anticryptosporidial contribution of AAT (Table 5-1).

The present study is the first to demonstrate a synergistic anticryptosporidial effect for the combination of a serine protease inhibitor and an aminoglycoside. While both AAT and paromomycin have previously
been reported to reduce in vitro *C. parvum* infection, the findings presented here show an enhanced inhibitory effect was achieved when oocysts were simultaneously exposed to these 2 compounds at the time of inoculation. Further, these data illustrate the potential for a chemotherapeutic strategy to control cryptosporidiosis using multiple compounds targeting different developmental stages of the parasite.

The results of this study are in substantial agreement with previous studies of the in vitro efficacy of AAT and paromomycin. Human AAT has been reported to reduce parasite infection in BFTE cells in a concentration and time-dependent manner (Forney et al., 1996c). Similarly, a progressive decrease in the anticryptosporidial activity of AAT was observed with increased incubation periods. Further, AAT has been shown to exert an antagonistic effect on *C. parvum* excystation, putatively altering excystation dynamics with a subsequent decrease in sporozoite viability.

Consistent with the findings of Marshall and Flanigan (1992), paromomycin demonstrated a significant inhibitory effect on *C. parvum* infection at 24 hr post-inoculation. The mode of action for paromomycin in reducing *C. parvum* infection is unknown. In general, aminoglycosides inhibit protein synthesis at the ribosome level and similar activity has been proposed for the inhibitory effect of paromomycin on in vitro *Giardia lamblia* infection (Edlind, 1989). Rehg (1994) evaluated 6 different aminoglycoside compounds for anticryptosporidial activity in an immunosuppressed rat model and found that only paromomycin exhibited a significant inhibitory
effect on *C. parvum* infection. The observation that paromomycin is particularly effective against intermediate stages of *C. parvum* suggests that the compound may preferentially target protein synthesis associated with proliferative asexual development.

The use of multi-compound protocols is a clinically relevant strategy for treating microbial infections. Combined drug therapy has the advantage of reducing toxicity by obtaining an effective response at lower concentrations and may delay the development of drug resistance. In the present study, the combination of 2 compounds putatively active against different developmental stages of *C. parvum* demonstrated the potential to exploit individual anticryptosporidial activities to facilitate an enhanced inhibitory response.

In summary, we have demonstrated that AAT and paromomycin can be used in combination to significantly reduce in vitro cryptosporidial infection. Using the criteria of FIC indices, we have shown a synergistic effect for this multi-compound combination in BFTE cell culture. It is readily acknowledged that in vitro activity is not a universal indicator of analogous in vivo success and caution is warranted in the extrapolation of results based solely on in vitro assays. Further studies of the efficacy, toxicity, and systemic effects of multi-compound treatments in an appropriate experimental in vivo model are needed to better elucidate their potential therapeutic utility. The findings presented here demonstrate the capability to achieve a marked reduction in *C. parvum* infection and may constitute an alternative approach
to conventional single-agent treatment protocols. The present study supports the continued evaluation of multi-agent combinations for enhanced anticryptosporidial activity and suggests exciting possibilities for future investigative efforts.

LITERATURE CITED


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facilitates penetration of the skin by the infective larvae. Experimental Parasitology 70: 134-143.


Table 5-1. Effect of selected protease inhibitors on *Cryptosporidium parvum* infection of bovine fallopian tube epithelial cells.

% of Control

<table>
<thead>
<tr>
<th>[PI]†</th>
<th>AAT</th>
<th>Aprotinin</th>
<th>Leupeptin</th>
<th>SBTI</th>
<th>MAAPVCK</th>
<th>Antipain</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>5</td>
<td>49.1±3.78$§$</td>
<td>83.9±6.56$‡$</td>
<td>89.1±5.41</td>
<td>100.3±4.25</td>
<td>95.2±3.68</td>
<td>67.74±7.15$§$</td>
</tr>
<tr>
<td>10</td>
<td>43.9±4.21$§$</td>
<td>54.2±5.99$§$</td>
<td>64.8±11.98$‡$</td>
<td>95.9±7.34</td>
<td>91.8±5.23</td>
<td>53.11±5.78$§$</td>
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<tr>
<td>50</td>
<td>42.7±1.62$§$</td>
<td>36.1±4.72$§$</td>
<td>58.7±11.21$‡$</td>
<td>70.2±4.25$‡$</td>
<td>79.2±13.65</td>
<td>42.13±6.14$§$</td>
</tr>
<tr>
<td>100</td>
<td>30.4±1.87$§$</td>
<td>16.8±5.13$§$</td>
<td>51.9±4.64$§$</td>
<td>68.3±11.59$‡$</td>
<td>80.2±19.09</td>
<td>20.94±2.16$§$</td>
</tr>
<tr>
<td>500</td>
<td>14.7±6.31$§$</td>
<td>10.9±4.71$§$</td>
<td>43.4±6.57$§$</td>
<td>50.5±2.71$§$</td>
<td>77.5±9.69</td>
<td>15.58±1.44$§$</td>
</tr>
</tbody>
</table>

*Mean number of parasites counted per treatment group expressed as a percent ± SD of the mean number of parasites counted in the infection control group; alpha-1-antitrypsin (AAT), soybean trypsin inhibitor (SBTI), methoxysuccinyl-ala-ala-pro-valine chloromethylketone (MAAPVCK).

† [PI] = Protease inhibitor concentration (μg/ml) in RPMI medium used in the BFTE cell inoculum.

‡ Significantly different from control group mean at *P* < 0.01.

§ Significantly different from control group mean at *P* < 0.001.
Table 5-2. Effect of phenylmethylsulfonyl fluoride on *Cryptosporidium parvum* infection of bovine fallopian tube epithelial cells.

<table>
<thead>
<tr>
<th>[PMSF]*</th>
<th>% of Control†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>100</td>
</tr>
<tr>
<td>1 mM</td>
<td>68.9± 5.23‡</td>
</tr>
<tr>
<td>2 mM</td>
<td>55.9± 4.67‡</td>
</tr>
<tr>
<td>3 mM</td>
<td>40.0± 8.34§</td>
</tr>
</tbody>
</table>

* [PMSF] = Phenylmethylsulfonyl fluoride concentration (mM) in RPMI medium used in the BFTE cell inoculum.

† Mean number of parasites counted per treatment group expressed as a percent± SD of the mean number of parasites counted in the infection control group.

‡ Significantly different from control group mean at *P* < 0.01.

§ Significantly different from control group mean at *P* < 0.001.
Table 5-3. Effect of alpha-1-antitrypsin on *Cryptosporidium parvum* oocyst excystation at 37°C.

<table>
<thead>
<tr>
<th>[AAT] (μg/ml)</th>
<th>15†</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16.5±2.4</td>
<td>27.5±4.1</td>
<td>54.5±8.9</td>
<td>68.5±11.2</td>
<td>74.5±9.8</td>
<td>77.1±10.6</td>
</tr>
<tr>
<td>10</td>
<td>5.2±1.1†</td>
<td>18.5±4.4†</td>
<td>45.5±10.0</td>
<td>52.2±12.7</td>
<td>69.9±14.6</td>
<td>72.1±14.2</td>
</tr>
<tr>
<td>100</td>
<td>3.2±1.8†</td>
<td>9.5±2.1†</td>
<td>39.7±11.8†</td>
<td>57.5±14.4†</td>
<td>64.5±9.8†</td>
<td>69.6±12.7</td>
</tr>
<tr>
<td>500</td>
<td>1.8±0.7†</td>
<td>8.2±1.2†</td>
<td>19.8±6.2†</td>
<td>32.3±8.7†</td>
<td>40.8±10.2†</td>
<td>52.5±13.2†</td>
</tr>
</tbody>
</table>

*Concentration in μg/ml
†Time (min) of incubation
‡Significant at (P<0.05), mean ± SD
Table 5-4. Effect of alpha-1-antitrypsin on *Cryptosporidium parvum* infection in BFTE cells.

<table>
<thead>
<tr>
<th>[AAT]*</th>
<th>% of Infection Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>48.1 ± 6.82†</td>
</tr>
<tr>
<td>100</td>
<td>36.8 ± 3.58†</td>
</tr>
<tr>
<td>500</td>
<td>18.3 ± 7.18†</td>
</tr>
<tr>
<td>1000</td>
<td>4.6 ± 2.21†</td>
</tr>
</tbody>
</table>

*Concentration in µg/ml
†Significant at (P<0.01), mean ± SD
Table 5-5. In vitro anticryptosporidial activity of alpha-1-antitrypsin and paromomycin in single and combined treatment groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24$^\dagger$</th>
<th>48</th>
<th>72</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAT$^\ddagger$</td>
<td>10.2 ± 2.8</td>
<td>26.3 ± 9.8</td>
<td>466.8 ± 52.6</td>
<td>494.6 ± 75.9</td>
</tr>
<tr>
<td>Paromomycin$^\ddagger$</td>
<td>262.7 ± 33.4</td>
<td>128.2 ± 9.1</td>
<td>80.6 ± 12.4</td>
<td>73.4 ± 9.5</td>
</tr>
<tr>
<td>AAT + Paromomycin$^§$</td>
<td>0.1835</td>
<td>0.3781</td>
<td>0.7895</td>
<td>0.8571</td>
</tr>
</tbody>
</table>

$^\dagger$ Treatment applied at the time of cell culture inoculation.
$^\dagger$ Denotes time (hr) post-inoculation.
$^\ddagger$ Values represent mean concentration (μg/ml) ± SD (duplicate samples) capable of reducing parasite infection to 50% of mean control value (IC$_{50}$).
$^§$ Values represent mean fractional inhibitory concentration (FIC) indices.
Figure 5-1. Effect of protease inhibitors on infection of bovine fallopian tube epithelial cell monolayers by *Cryptosporidium parvum* oocysts. Data reflect the mean number of parasites counted at 24 hr postinoculation, expressed as a percent of the infection control mean, following treatment with alpha-1-antitrypsin (–□–), antipain (–▲–), and aprotinin (–●–). Error bars represent 1 SD about group means.
Figure 5-2. Single compound treatment effect on *Cryptosporidium parvum* infection in BFTE cells using alpha-1-antitrypsin or paromomycin. Values represent infection mean ± SD for duplicate samples in 2 separate experiments using (a) AAT or (b) paromomycin. Sampling intervals were conducted at 24 hr (■), 48 hr (○), 72 hr (□), and 96 hr (●).
Figure 5-3. Effects of alpha-1-antitrypsin and paromomycin concentrations in combined treatment groups. Infection values for (a) 24, (b) 48, (c) 72, and (d) 96 hr post-inoculation sampling intervals. Data reflects mean values for treatment groups expressed as a percent of untreated control samples (duplicate samples) from a typical experiment. Replicate experiments produced similar effects.
The studies presented herein provide unequivocal evidence of the recognition and functional interaction of AAT with a component of *C. parvum*, putatively expressing serine protease-like activity during excystation. The biological significance of the observed serpin:parasite interaction is a potent antagonistic effect on oocyst excystation with a concomitant reduction in cryptosporidial infectivity in a permissive cell culture system. It is readily apparent from these studies that the alteration of the early infection dynamics of *C. parvum*, in particular the interval encompassing excystation and initial host cell attachment, has a substantial inhibitory effect on parasite infection.

The antagonistic role of AAT in inhibiting excystation is attributed to the functional specificity of the serpin molecule for the active site of serine protease enzymes. Immunolocalization of SEC interactions on the membrane of excysted *C. parvum* sporozoites suggests the expression of a cognate target enzyme on this early developmental stage of the parasite. Further, the ability to block AAT recognition of *C. parvum* with porcine pancreatic elastase substantiates the specificity of the interaction and demonstrates a functional capacity for serpin activity in mediating the biological activity of the invasive sporozoite.
Select serine protease inhibitors (i.e., PMSF, SBTI, AAT, and aprotinin) and serine/cysteine inhibitors (i.e., leupeptin and antipain) showed potent anticryptosporidial potential in preventing infection in cultured epithelial cells. Further characterization of the anticryptosporidial activity of AAT showed a significant reduction in *C. parvum* infection in BFTE cells at 24-48 hr post-inoculation, but the inhibitory effect of this serpin diminished with continue incubation intervals ranging from 72 to 96 hr post-inoculation. Similarly, AAT had very little effect on established in vitro infections, suggesting a minimal impact on developmental intermediates of the parasite.

The differential antagonistic capability of AAT on oocyst excystation was restricted to oocysts that had been subjected to treatments known to increase the permeability of the oocyst wall (i.e., bleach decontamination, incubation in an acidic medium, exposure to deoxycholic acid); entry of AAT into the intraoocyst milieu required at least partial degradation of the oocyst wall. The excystation of CsCl-purified oocysts not otherwise subjected to any permeability-enhancing chemical pretreatments was not significantly inhibited by AAT. Rather than consigning the role of AAT in antagonizing excystation as "limited" in terms of its applicability to permeabilized oocysts, it is more accurate to view the association as physiologically relevant in light of the progressive passage of oocysts through the digestive system following ingestion. The biochemical analogy to gut passage and excystation in the lumen of the small intestine (i.e., acidification, neutralization, exposure to bile extracts and emulsifying agents) facilitates oocyst excystation and,
ultimately, enhances parasite vulnerability to serpin-mediated reduction in excystation efficiency. The effect of serine protease inhibitors on the excystation dynamics of thin-walled oocysts has yet to be evaluated; the assumed role of this type of oocyst in perpetuating chronic infection in susceptible hosts suggests a particularly interesting study for future consideration.

A curious characteristic of AAT's antagonistic effect on excystation was the progressive loss of inhibitory potential with increased incubation intervals. The half-life of AAT in human circulation is in the range of 3-5 days in the noninflammatory state; degradative capacity secondary to a reduced half-life does not seem to be a plausible explanation for the decreased inhibitory effect AAT had on excystation at 2- to 3-hr incubation intervals. One possible explanation for the time-dependent loss of efficacy is the consumptive nature of the serpin interaction. The serpin molecule binds covalently to a cognate target enzyme and remains bound, under physiologic conditions of the human system, until cleared from circulation. In effect, AAT is a "suicidal" inhibitor. Alternatively, *C. parvum* may possess mechanisms to counter serpin activity and, therefore, reduce the inhibitory capacity of the serpin. The reported ability of cysteine proteases to cleave the reactive site loop of the AAT molecule suggests a mechanism to rapidly degrade the molecule's ability to recognize and complex with proteases. This latter mechanism has been reported to enhance the pathogenicity of bacteria, and the putative identification of cysteine protease activity in *C. parvum*
suggests that studies evaluating degradation of AAT may be of interest in characterizing parasite defense mechanisms to acute phase proteins such as AAT.

The transient nature of AAT effects on excystation was a consistent feature of the studies reported here; continued incubation of AAT-treated oocysts at 37°C typically produced excystation yields approaching approximately 65-80% of control group mean values. Despite the relative increase in excystation with continued incubation, it is noteworthy that oocyst inoculum containing AAT had significantly lower infection levels in the early post-inoculation treatment groups. The viability of emerging sporozoites appears to be directly related to the time interval between the induction of excystation-related activity and final escape from the oocyst wall. Specifically, the time lapse between the initial activation of excystation and the availability of susceptible host cells is a critical factor in C. parvum infectivity. Sporozoite viability appears to be relatively intolerant of delayed excystation and a significant loss in infectivity is evident in AAT-treated oocysts. It is likely that the invasion process (i.e., from excystation to formation of the parasitophorous vacuole) is dependent upon preexisting energy stores within the sporozoite; failure to find an obligatory host cell shortly after emergence is detrimental to infectivity. Though speculative at present, the implications of the results presented herein suggest that not only is excystation induced by an activation of enzymatic processes, but once
activated, bioenergetic demand may be the most critical limitation of sporozoite viability.

On the basis of the results present here, it is concluded that AAT antagonizes *C. parvum* excystation by gaining direct access to sequestered sporozoites; some degree of enhanced oocyst wall permeability is necessary for AAT to inhibit excystation. Further, it is apparent that sporozoite activation within the intact oocyst wall includes, at least in part, the expression of proteolytic enzyme activity. It is the association of proteolytic enzyme activity with excystation, and the functional specificity of the AAT molecule for the active site of serine proteases, that provides the most likely mechanism for serpin-mediated inhibition. The alteration in excystation dynamics caused by AAT accounts for its anticryptosporidial activity against in vitro infection, putatively by altering endogenous proteolytic activity within the otherwise intact oocyst.

In an attempt to compensate for the waning efficacy of AAT with continued post-inoculation intervals, a multiple compound treatment strategy was evaluated. A study was designed to exploit the anticryptosporidial efficacy of 2 compounds that theoretically targeted different stages in the parasite's life cycle. AAT, an apparent excystation antagonist, and paromomycin, reportedly effective against asexual intermediates (i.e., type I and II meronts), were applied both individually and in combination in an in vitro test system. The combined serpin-aminoglycoside activities exhibited a potent anticryptosporidial effect in the
BFTE cell culture system. Further, using the criteria of FIC indices, the study demonstrated that a significant synergistic response was achieved.

The therapeutic application of protease inhibitors in the treatment and prevention of human cryptosporidiosis is premature; considerable research is necessary to better understand the systemic effects of exogenous inhibitors. The results presented in this dissertation demonstrate a potent antiparasitic effect for select protease inhibitors in the absence of observed cytotoxicity. The exact nature of excystation antagonism remains to be fully described; however, it is likely related to serpin activity on one or more cryptosporidial serine proteases requisite to proteolytically enhanced modification of the oocyst wall that facilitates full emergence of the infective sporozoite. Isolation and characterization of the protease(s) associated with excystation will provide a definitive answer to the hypothetical significance of enzyme activity in excystation and invasion of epithelial cells.

While proteases certainly provide opportune targets for antimicrobial intervention, full therapeutic utility of protease inhibitors in the treatment of cryptosporidiosis will likely require the development of synthetic inhibitors with a substantial degree of parasite specificity. This approach is best illustrated by the recent inclusion of synthetic protease inhibitors in antiviral protocols for the treatment of HIV infection in humans. Clinical application of protease inhibitors and substrates as anticryptosporidial agents is dependent upon demonstrating the relevance of targeted proteases to infection and the vulnerability of these targets to chemotherapeutic agents. It
is, therefore, essential that cryptosporidial proteases be isolated and characterized to include a thorough description of their function in the parasite's life-cycle.

The collective contribution of these assembled manuscripts is a solid foundation for future efforts to characterize the structural detail of cryptosporidial proteases related to excystation and early infection dynamics. Protein sequence data and homology comparisons to previously described proteases, both protozoan and mammalian, will provide researchers with a defined target and enhance the potential for effective clinical intervention. Inhibition of protease activity is a scientifically and clinically proven method for preventing infection and ameliorating the pathology associated with infectious disease processes. The further application of this strategy directed against parasitic infection is a logical extension of existing scientific and medical achievement and potentiates the development of a potent arsenal of novel anticryptosporidial compounds.
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