Immunoelectron-Microscopic Localization of Antigenic Sites of *Cryptosporidium parvum* and an Assessment of the Role of Monoclonal Antibodies and Hyperimmune Bovine Colostrum in Controlling Cryptosporidiosis

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INTRODUCTION

Cryptosporidium, a protozoan parasite, is an important cause of diarrheal disease in man and several species of animals. Well known to veterinarians in the 1950's and 60's as a cause of diarrhea in animals, the protozoa was first recognized as a cause of human gastroenteritis in 1976 (1, 2). Relatively few cases were diagnosed until cryptosporidiosis was reported to be a life-threatening infection in individuals with the acquired immune deficiency syndrome (AIDS) (3). Medical interest in the epidemiology, diagnosis, and treatment of cryptosporidiosis has increased dramatically since then. Now, in many areas, *Cryptosporidium* is among the top three or four enteric pathogens identified (4). The disease is self-limiting in immunocompetent individuals. For the majority of immunocompromised patients, such as those with AIDS whose illness is a major factor contributing to death, no agent antidiarrheal compound offers clear benefit (5). Infection persisting for more than a month in persons thought to be at risk from AIDS may be considered a diagnostic marker for development of the immunodeficient state (6).

In 1907 a previously unrecognized coccidian protozoa was found in the stomach of the common mouse and named *Cryptosporidium muris* (7). Another species, *C. parvum*, was subsequently identified in the small intestine of the dame host (8). A detailed description of the ultrastructure of various *Cryptosporidium* life cycle stages was published in 1966 (9). Relatively little is known about the antigenic relatedness of the different development stages of *Cryptosporidium*, even though we possess a rather complete knowledge of the fine structural detail of all developmental stages. This paucity of knowledge makes it difficult to completely understand the biology and host-parasite interactions of *Cryptosporidium*.

Treatment of cryptosporidios is, especially in immunodeficient persons, has been unsucessful in most cases. The development of an effective treatment has been limited by the lack of *in vitro* cultivation systems to study the biochemical and metabolic requirements of *Cryptosporidium* sp., and by the lack of a reliable, small-animal model of clinical disease for screening the efficacy of drug compounds (10). Of the numerous antimicrorbial and antiparasitic drugs administered to immunodeficient persons with intestinal cryptosporidiosis, the macrolide antibiotic spiramycin is the only one reported to date to have some efficacy (11). Most patients receiving this treatment, however, continued to shed *Cryptosporidium* oocysts in their feces (11). Suckling mice treated daily with orally administered mixtures of anti-sporozoite monoclonal antibodies showed significantly reduced parasite loads compared with control mice at four days postinfection, even if infections were not completely interrupted (12). These findings suggest that

immunomodulation or passive transfer of antibodies may be of value in controlling cryptosporidial infections. There are currently no vaccines available to prevent cryptosporidiosis in humans, hence the urgent need for an effective and safe anticryptosporidial agent.

This paper outlines research to study two aspects of *Cryptosporidium*. First, specific antigenic determinants were identified and followed through the growth cycle of *C. parvum* to investigate antigenic sharing of molecular epitopes among the different life cycle stages. Secondly, the importance of passive immune protective mechanisms in cryptosporidial infection was assessed by following the course of infection in neonatal mice which have been subjected to treatments using either monoclonal antibodies (mAbs) or hyperimmune bovine colostrum.

METHODS

Production of anti-*Cryptosporidium* monoclonal antibodies

Two month old BALB/C mice were immunized by intramuscularly injecting of a 0.2 m*l* preparation containing merozoites or sporozoites in an equal volume of Freund's complete adjuvant. Two weeks later, a subsequent booster injection was given intraperitoneally (i.p.) using the same antigen in Freud's incomplete adjuvant. Three weeks later, the mouse was boosted by i.p. injection of antigen with no adjuvant for two successive weeks. Mice were eye-bled to one week later to determine the titer of *Cryptosporidium* antibodies. A mouse whose serum titer against *Cryptosporidium* was over 1,000 was selected for fusion. After a rest period of 4 weeks, the antigen containing merozoites or sporzoites, dissolved in 0.4 m*l* saline solution, was injected i.p. On the following day, antigens suspended in 0.1 m*l* saline was injected intravenously. Three days after the intravenous boost, the spleen was removed and its cells for the hybrid fusion.

The fusion protocol followed the method of de St Groth and Scheidegger (13). Spleen cells was prepared by teasing pieces of spleen in RPMI-base medium without serum. The cell suspension was allowed to sit for approximately 2 min. The supernatant was removed from the sediment. Splenocytes were washed twice by centrifugation. A fusion mixture consisting of a 10 : 1 ratio of spleen to mouse myeloma cells (P3-X63-Ag8.653) was suspended together in RPMI-base. The cell suspensions was centrifuged and the medium removed as completely as possible. A 50% polyethyleneglycol 4000 solution was slowly added to the cell pellet. The cells were centrifuged at 400g for 5 min. The supernatant was removed and the cells resuspended in medium supplemented with 10% fetal bovine serum. The cell suspension was distributed in equal amounts into successive wells of 24 well plates for selection of hybrids growing with HAT containing medium and placed in CO_2 incubator. Hybridomas that produce antibodies were cloned by limiting dilution in 96 well plates (14). Limiting dilution cloning was done twice to generate a clonal population. Hybridomas that produce antibodies against merozoites or sporozoites were screened by an immunofluorescent procedure.

Polyacrylamide gel electrophoresis and western blotting

Sporozoites were suspended with 1 m*l* of 150 mM NaCl, 5 mM Tris, and 0.02% sodium azide (NET) buffer, pH 7.4, containing 0.5% of the nonionic detergent Nonidet P-40, and 1 mM each of the enzyme inhibitors N-tosyl-L-lysylchloromethyl ketone an phenylmethylsulfonyl fluoride(Sigma). After incubating at room temperature for 10 min the suspension was centrifuged at 20,000g for 2 min. The supernatant-solubilized membrane preparation was decanted and stored at 4C until used.

SDS-PAGE electrophoresis was carried out on slab gels according to the method of Laemmli(15). The gel system consisted of a 10 to 20% gradient resolving gel and a 4% stacking gel. A sporozoite membrane sample was treated with an equal vo-

lume of sample buffer containing 1% SDS, 140 mM mercaptoethanol (Sigma), 0.015% bromophenol blue (Sigma), 100 mM phosphate buffer and 10% glycerol (Fisher) and placed in a boiling water bath for 4 min. Slabs were electrophoresed at 18℃ at a constant 100 mM.

Transfer of reduced and nonreduced sporozoite antigens from a SDS-polyacrylamie slab gel to nitrocellulose sheets was performed according to the electrophoretic blotting procedure of Towbin et al. (16). Transfer was done at 4℃ with a constant 20 volts overnight followed by 30 volts for 2 hours. Following transfer, the strips were probed with anti-*Cryptosporidium* monoclonal antibody for 1 hr, washed with TBS, and incubated for 1 hr with biotinylated goat anti-mouse lgG antibody (Amersham corp.). The nitrocellulose strips were then exposed to an appropriate solution of streptavidin-horseradish peroxidase (Amersham Corp.). Antigen bands with bound antibodies were visualized by a color reaction utilizing a substrate containing hydrogen peroxide and 4-chloro-1-naphtol (Sigma).

Immunoelectron microscopic localization of antigenic sites of *C. parvum* using monoclonal antibodies

Four day old suckling mice were orally infected with oocysts and used four or eight days post infection (PI), for tissue preparation. The whole body of a mouse was perfused with 1% glutaraldehyde (Polysciences) in PBS. Perfusion was continued for 15 min. Following perfusion, the terminal ilium was excised and cut into 1 mm cubes. Tissue pieces were immersed in the same fixative used for perfusion and then rinsed in buffer.

Post-fixation in osmium was avoided in post-embedding systems because of its adverse effect on antigenicity. The specimens were rinsed with distilled water, dehydrated with ethanol. The tissues were then placed in 70% ethanol-LR White resin (1 : 1), and finally into LR White resin. Polymerization was accomplished in vacuum oven at 50℃ for 24 hr. Ultra-thin sections were cut with a diamond knife on an Sorvall MT-2B ultra microtome, mounted on 100-mesh formvar coated nickel grids, and processed for immunohistochemistry.

The grids were rinsed on drops of 0.1% BSA-Tris buffer. The washed grids were then incubated on drops of buffer supplemented with 5% normal goat serum and transferred to appropriate anti *Cryptosporidium* monoclonal antibody solutions. Grids were subsequently rinsed in TBS-BSA and incubated with goat anti-mouse lgG or lgM antibody conjugated with colloidal gold particles (15 or 10 nm). After incubation, the grids were rinsed in TBS-BSA followed by distilled water. The sections were dried and stained with uranyl acetate and lead cirtate and examined by a Hitachi H-500 electron microscope.

Passive transfer of immunity against *Cryptosporidium* infection in neonatal mice using monoclonal antibodies.

Neonatal BAB/c mice were infected *per os* with oocysts at 4 days of age. They received the primary antibody dose or saline one hour prior to oocyst infection and subsequent doses once daily for 4 or 8 days until the mice were sacrificed. Parasite infection were assayed by sacrificing each mouse by cervical dislocation and removing the terminal ileum. The terminal ilium was fixed in formalin, embedded in paraffin, microtome sectioned longitudinally and stained with hematoxylin and eosin. These longitudinal sections were examined for parasite life cycle stages along villus surfaces. The quantitation of parasite density along vilus surfaces was performed by counting the number of parasitic forms per high power field in the tissue sections for each mouse. Three rendomly chosen mice from each litter were examined for the presence of parasites.

Passive transfer of immunity against *Cryptosporidium* infection in neonatal mice using hyperimmune bovine colostrum

Colostrum samples were obtained from the first milking after parturition one cow immunize with adjuvant plus saline (control colostrum) and one cow immunized with adjuvant plus sonicated oocysts or sporozoites (hyperimmune colostrum).

The hyperimmune and control colostrum samples were defatted, and the skim colostrum freeze-dried. Bovine lgG content was determined by single radial immunodiffusion and expressed on a wt/wt basis. The skim colostrum preparations were reconstituted in distilled water and provided a bovine lgG concentration of 5 mg in a 100μl volume for administration *per os*. The lgG dose is high, equivalent to 1.7g lgG/Kg body weight, but was chosen to maximize the potential for efficacy.

Three series of experiments were performed in duplicate with eight neonatal mice per treatment group. All neonatal mice were infected orally with *Cryptosporidium* oocysts in 50μl distilled water at 5-7 days of age. All treatments and dosages of oocysts were given *per os* directly into the stomach through polyethylene tubing whose inside and outside diameters are 0.38 mm and 1.09 mm, respectively, atteched to the needle (25G 5/8) of a 1 cc tuberculine syringe. The control experiments were designed to determine if the daily administration of saline affects the manifestation of crytosporidiosis in neonatal mice. Additionally, sham infected animals were gavaged with control and hyperimmune skim colostra daily for five days to determine adverse effects, if any.

The effect of anti-cryptosporidial hyperimmune colostrum on cryptosporidial infections in neonatal mice was examined. Each mouse was sacrificed by cervical dislocation and the terminal ileum removed. The terminal ileum was embedded in paraffin, sectioned and stained with hematoxylin and eosin after being fixed in 10% buffered formalin for two days. Then, the number of protozoa along villus surfaces was counted per high power field in the tissue sections to quantitate protozoa density.

Data analysis

Statistical analyses of these data were done by goodness of fit tests and a model II nested analysis of variance (17) accommodating unequal sample sizes in an effort to determine the significance of treatments on parasite infections. The null hypothesis of eual therapeutic or prophylactic efficacy was tested by the F-ratio for valence in the ANOVA table. Multiple comparisons among the treatment group means (Tukey's studentized range test) were performed to identify specific treatment or prophylaxis groups with variances significantly dfferent from the control group. The 95% confidence intervals were put around the sample means and the evidence in these intervals were examined to study the magnitudes of the differences among means. Analysis of variance was computed using statistical Analysis System program (SAS) accessed through RVAX (Computer Center, The University of Arizona).

RESULTS AND DISCUSSION

Knowledge of the life history of *Cryptosporidium* within the host and of the antigenic relationship between the various developmental stages facilitates a more thorough understanding of host responsiveness to infection with this parasite. The distribution of defined antigenic sites through the life-cycle stages of *Cryptosporidium parvum* has been mapped by electron microscopic immuno-gold labeling using anti-*C. parvum* monoclonal antibodies. This study represents the first application of this methodology towards defining the antigenic relatedness of *Cryptosporidium* life cycle stages at the ultrastructural level.

Using three anti-*C. parvum* mAbs (Fig. 1) and immunoelectron microscopy, antigenic determinants recognized by these mAbs were mapped during the development of *Cryptosporidium* within its host. The antigens recognized by these mAbs appear to be uniformly distributed in both the cytoplasm and on the surface of the parasite. They also appear on the inner membrane of host epithlial cells.

Each of the mAbs tested appeared to be localized in all developmental stages of *C. parvum* (18) : trophozoites, merozoites in type I (Fig. 2) and II meronts, microgametocytes, macrogamonts, unsporulated oocysts, sporozoites in oocysts and free sporozoites. Cross reactions involving these anti-

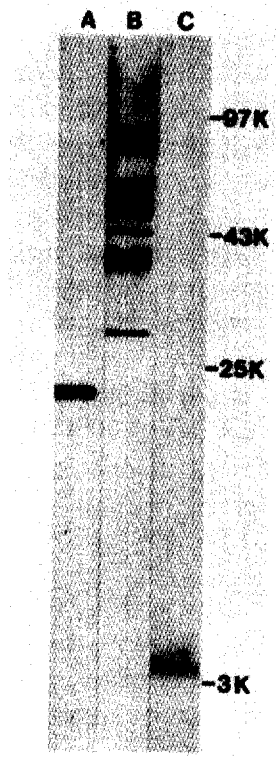

Fig. 1. Western blot analysis of NET-solubilized sporozoite antigens reacted with monoclonal C6B 6 (A) and C4A1 (B) and Cmg3 (C).

Fig. 2. Immunoelectron micrograph of a type I meront of *C. parvum* reacted with mAb C4A1 demonstrating labeling of the parasite's surface and cytoplasm as well as the inner membrane of the infected epithlial cell. X21,300.

gens may be explained by the persistence of antigenic determinants throughout the life cycle stages. Similar antigenic sharing has not been demonstrated for most other coccidia.

In another coccidian parasite *Eimeria,* which has similar life cycle stages, there appear to be antigenic differences between the various developmental stages (19). In another coccidium, *Plasmodium,* the antigens of the parasite are highly stage-specific ; i. e., each is expressed in only a single developmental stage. Each developmental stage of most coccidian parasites has its characteristic shape and distinctive set of functions ; it inhabits a particular microenvironment and interacts with a specific target tissue, while all life-cycle stages of *Cryptosporidium* are confined to the same intracellular but extracytoplasmic habitat. This implies that although all the stages of *Plasmodium* have the same complement of genes, a different part of the genome is being expressed in each development stage. For *Cryptosporidium,* the same part of the genome might be expressed in each developmental stage.

Epitope sharing of *C. parvum* may be important not only for understanding the biology and host-parasite interactions of this parasite but also for developing a vaccine or immunotherapeutic strategy against *Cryptosporidium.* Because there is antigenic cross-reactivity between the different developmental stages of *C. parvum.* antibodies against a common antigen may attack and neutralize several life-cycle stages, potentially making the development of a vaccine or immunotherapeutic modality of treatment easier.

The presence of parasite antigens in the host membrane cell suggests that some antigens of *Cryptosporidium* may be inserted into the host cell membrane by invading merozoites and sporozoites. It is also possible that certain of these antigens may modify the host cell to allow for parasite growth by serving as receptors or transport molecules for essential exogenously supplied nutrients and enzymes needed by the parasite. The number of antigens which may be inserted in this manner

into the host cell membrane remains to be determined.

Control of enteric cryptorsporidiosis by the host immune system is indicated by the following observations. (i) The disease is self-limiting in immunocompetent hosts and stimulates the production of antibodies to *C. parvum* (20), but is persistent in immunodeficient hosts and in athymic (nude) mice (10) ; (ii) recovered immunocompetent calves and humans are resistent to reinfection (21). For these reasons, investigations on immunologic approaches to control this disease were initiated.

Anti-*Cryptosporidium* monoclonal antibodies Cmg3, C6B6 and C4A1 used for immunoelectron microscopy were examined for their ability to modulate experimental crytosporidial infection in BALB/c neonatal mice for two different periods, i.e., 4 and 8 days. The fact that Cmg3, C6B6, and C4Al bind to asexual and sexual stages of *C. parvum* suggested that oral administration of ascitic fluids containing mAbs to oocyst inoculated suckling mice may modulate cryptosporidial infections.

To determine whether parasite loads in treated mice differed significantly, parasite numbers along villus surfaces were counted and the results subjected to repeated measures analysis of variance. Since difference was significant at $P<0.05$, the null hypothesis was rejected that there was no significant difference in the variance among treatments.

A multiple comparison of treatment group means was performed (using Tukey's studentized range test) in an attempt to identify treatment groups differing significatly from the control group because the ANOVA demonstrated a significant difference between treatments at the 0.05 level. Suckling mice treated daily with orally administered mixtures of Cmg3, C6B6, and C4A1(Kor) or only with Cmg3 (Ea) showed significantly reduced parasite loads compared to control mice at four days post infection (Table 1). However, when neonatal mice were treated with two different treatments for eight days, only the mouse group treated with mixtures of three mAbs showed significant differences.

Table 1. Multiple comparisons-differences between monoclonal antibody passive transfer treatment group means.

Mean*	6.745	2.870	3.150
Treatment	CL	Kor	Ea
Kor	3.875***		
Ea	3.585***	0.280	

Table values are the differences between treatment means
Confidence=0.95, DF=48, MSE=4.757
Critical Value of Tukey's Studentized Range(HSD) =3.420
* =mean of square root transformed data
*** =significant at $p<.05$

The passive transfer of immunity using mAbs has shown to limit the number of parasites which complete the life cycle (22). Monoclonal antibodies transferred passively into the gut lumen appear to be ideally suited for interaction with the extracellular invasive forms (sporozoites, merozoites, and microgametes) of the parasite for several reasons. First, antibodies may act directly on extracellular stages to damage them, or by interacting with components of the complement system. Secondly, antibodies may neutralize sporozoites and merozoites directly by blocking their attachments to new host cells. Finally, antibodies may enhance phagocytosis of extracellular forms mediated by Fc receptors on macrophages.

The partial reduction in the numbers of sporozoites of *Eimeria* found with in the epithelium of immune animals was due to the inhibitory effects of antibodies on invasion, as demonstrated *in vitro* (23). It has been suggested that the hindering of peretration may prolong the exposure of the parasites to lytic factors normally present in the intestinal contents which can damage surface antigens and thereby inhibit the growth of those that do succeed in entering cells (24). This may explain why in immunocompetent hosts crytosporidial infection is transient (25).

In an effort to evaluate further the role of pas-

sive immunity for cryptosporidiosis, hyperimmune bovine colostrum was produced and colostral whey was separated from milk solids including cellular constituents and fat. Skim colostrum was produced by lyophylizing colostral whey and its efficacy evaluated in neonatal mice. Because cryptosporidiosis is entirely limited to the gastrointestinal tract, a trial of oral administration of hyperimmune bovine colostrum to neonatal mice was undertaken. Because there is no evidence for host specificity for this organism (25) and bovine gammaglobulin is known to pass through the entire gastrointestinal tract unaltered (26), it should come in contact with parasites present.

Significantly fewer ($p<.05$) stages of *C. parvum* were found in mice treated with hyperimmune skim colostrum (HSC) or original hyperimmune colostrum (HC) than in mice received control skim colostrum (CSC) or original control colostrum (CC) in therapeutic or prophylactic experimental groups (Table 2). No significant difference existed between HSC and HC treatment regimens as well as between CSC and CC treatments at the 95% confidence intervals. Both HSC and HC demonstrated the same therapeutic and prophylactic effecacy against *C. parvum* infection in mice as reported for calves fed hyperimmune colostrum (27). HSC and HC showed a protective effect on challenge with *C. parvum* oocysts, suggesting that neutralizing activity was present.

Colostrum is known to contain antibodies, T and B lymphocytes, polymorphonuclear leukocytes, and macrophages and their associated cytokines, as well as other biologically active factors such as vitamins, prostaglandins, lactoferrin, and complement (28). All are capable of modulating an immune response. It is not known, however, whether the effector mechanisms of protective immunity against cryptosporidiosis might be mediated by antibody, cells, or other components. The results of this study indicate there are factors present in hyperimmune skim clostrum capable of significantly reducing cryptosporidiosis and that the activity or concentration of these factors is significantly greater in HSC than in CSC. Whether such factors are immunoglobulins or other biologically active substances such as cytokines is presently not known. The fact that HSC showed similar passive protective effects to HC indicates that immune cells are not required. Antibodies, cytokines, or both appear to play an active role in immunity of cryptosporidiosis (29).

Table 2. Multiple comparisons-differences between colostrum passive transfer treatment group means.

Mean*	10.076	9.324	9.108	4.703	4.091
Treatment	saline	CC	CSC	HC	HSC
CC	0.752				
CSC	0.968	0.216			
HC	5.373***	4.621***	4.405***		
HSC	5.985***	5.233***	5.017***	0.612	

Table values are the differences between treatment means
Confidence=0.95, DF=48, MSE=4.757
Critical Value of Tukey's Studentized Range(HSD) =3.420
* =mean of square root transformed data
*** = significant at $p<.05$

Alhough it is not presently known how the hyperimmune colostrum might have exerted its beneficial effects, neutralizing antibodies might specifcally bind to parasites and prevent cell invasion. Conversely, the nonspecific interaction of colostral components with parasites and host effector cells might reduce sporozoite and merozoite infectivity (27).

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