In Vitro Inhibition of *Cryptosporidium parvum* Infection by Human Monoclonal Antibodies

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Cryptosporidium parvum infection of the small epithelial intestine causes unremitting diarrhea and malabsorption that can lead to chronic and sometimes fatal illness in patients with AIDS. The illness may be ameliorated by passive oral immunoglobulin therapy. The objective of this study was to produce anti-*Cryptosporidium* human monoclonal antibodies for evaluation as potential therapy. All human monoclonal cell lines that produced *C. parvum* antibodies were originally generated from the peripheral blood lymphocytes of a human immunodeficiency virus-seronegative woman. She had recovered from *C. parvum* infection and had a high specific antibody titer. Hybridization of these lymphocytes with a tumor cell line was accomplished by hypo-osmolar electrofusion. Twelve clones were identified by enzyme-linked immunosorbent assay (ELISA) as secreting anti-*Cryptosporidium* antibodies after the initial hybridization. From the 12 positive clones, two high antibody-secreting clones, 17A and 17B, were maintained in long-term culture. A second hybridization produced two other human monoclonal cell lines, EC5 and BB2. Human monoclonal antibody from the first two cell lines bound to *C. parvum* sporozoites and oocysts by immunofluorescence. The ability of human monoclonal antibodies to inhibit *C. parvum* infection in vitro was assessed by using a human enterocyte cell line, HT29.74. The antibodies of the four different human hybridomas inhibited infection by 35 to 68% ($P < 0.05$) compared to a control irrelevant human monoclonal antibody derived in a similar fashion. Human monoclonal antibodies are candidate molecules for immunotherapy of *C. parvum* infection.

Cryptosporidium parvum is a protozoan parasite that causes enteritis in humans and several animal species. Infection occurs in the gut epithelial mucosa and may cause malabsorption and severe diarrhea. Cryptosporidiosis is a common cause of chronic diarrhea in patients with AIDS, leading to wasting and eventual death (6). No effective therapy for immunodeficient individuals with cryptosporidiosis exists.

More than 40 different drugs have been evaluated, with little or no success (6). Oral immunotherapy with hyperimmune cow colostrum has been used to treat cryptosporidiosis successfully in a very small number of patients, but this therapy is difficult to standardize and is not well tolerated (13, 14). Murine monoclonal antibodies have been successful in treating cryptosporidiosis in a mouse model and have been utilized to detect infection in immunodeficient human patients but have not been evaluated in clinical trials (4, 8, 12). In contrast, human monoclonal antibodies have been evaluated in trials with large numbers of patients for illnesses such as septic shock (HA-1A) and cytomegalovirus (3, 16). Human monoclonal antibodies are a potential alternative immunotherapy against cryptosporidiosis. The present report describes the development of human monoclonal antibodies against *C. parvum* which bind to the parasite and neutralize infectivity in vitro.

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MATERIALS AND METHODS

Human hybridoma development. Peripheral blood lymphocytes (PBLs) were donated by two persons, a human immunodeficiency virus (HIV)-seronegative woman who recovered from cryptosporidiosis and her seropositive husband, who had AIDS and chronic cryptosporidiosis (7).

The HIV-seronegative wife recovered completely from cryptosporidial enteritis 6 months before donating her lymphocytes. She remained asymptomatic, without diarrhea, even after continued exposure to *C. parvum* oocysts from her husband. Anticryptosporidial antibody was present in high titer (optical density [OD]; 2.355 by enzyme-linked immunosorbent assay [ELISA]) in her serum.

PBLs were also donated by her husband, who was in an advanced stage of AIDS, with a CD₄ count of 10 cells/mm³. He continued to shed oocysts after 6 months of infection and eventually died with cryptosporidiosis-associated malabsorption and weight loss.

Human monoclonal antibodies were generated from the donated lymphocytes by using hyperosmolar electrofusion (9). PBLs were isolated by density gradient centrifugation and subsequently stimulated with phytohemagglutinin (Seromed) for 4 days at 37°C in 5% CO₂ in 24-well plates. Phytohemagglutinin-stimulated lymphocytes and H73C11 heteromyeloma cells were washed in L₃75 fusion medium containing 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 0.1% bovine serum albumin (Serva), and sorbital sufficient to adjust the osmolarity to 75 mosm (9). The fusion partners were mixed in L₃75 fusion medium at a ratio of two lymphocytes per heteromyeloma cell to final concentrations of 5×10^6 lymphocytes and 2.5×10^6 heteromyeloma cells per ml. A 200-ml volume of the cell mixture was placed in a standard helical fusion chamber. The cells were aligned by using 250 V/cm at a frequency of 1.7 MHz for 30 s and a single-wave fusion pool of 1,250 to 1,750 V/cm for 15 μ s. After 10 min, the chambers were rinsed with 1 ml of growth medium without phenol red (Greiner) and the cells were plated into four wells of a 24-well plate. The plates were cultured at 37°C in 5% CO₂. After 24 h, 1 ml of hypoxanthine-aminopterin-thymidine medium (Boehringer) was added to each well. After 8 to 10 days in culture, hybridoma clones were detected microscopically.

Screening of anticryptosporidial antibodies. ELISA was performed to confirm secretion of anti-*Cryptosporidium* antibody after microscopic detection of the hybridomas and subsequently during subcloning. For the ELISA, microtiter wells were plated with 5 μ g of sonicated *C. parvum* oocysts per well. The plate was blocked with milk and then incubated with undiluted hybridoma supernatant, each in triplicate, for 1 h at 37°C. Wells were then treated with a 1:5,000 dilution of alkaline phosphatase-conjugated anti-human immunoglobulin (Sigma) for 1 h at 37°C and exposed to substrate. The reaction was monitored by measuring absorbance at 492 to 450 nm. A commercial pooled-immunoglobulin preparation (Sigma) known to contain a high titer of anti-*Cryptosporidium* antibodies was

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FIG. 1. Hybridoma supernatant binding to *C. parvum* by ELISA. Anti-*Cryptosporidium* antibody reactivity within hybridoma supernatant as measured by ELISA. A 100- μ l volume of undiluted supernatant was incubated with 5 μ g of cryptosporidial antigen. The positive control was a 1:1,000 dilution of a commercial polyclonal antibody with a high anticryptosporidial antibody titer. The negative control was medium. All experiments were done in triplicate and were repeated if the OD values varied by a *P* value of over 0.05. Ig, immunoglobulin.

used as a positive control, and medium (RPMI 1640; Gibco) with 2 mM L-glutamine (Gibco), 100 $\times$ pen/strep (Gibco), 10% fetal calf serum (HyClone), and nonessential amino acids (Sigma) was used as a negative control. All experiments were done in triplicate and were repeated if the OD values varied by over 0.05.

Immunofluorescence assays. An indirect immunofluorescence assay was performed to detect antibody binding to the *Cryptosporidium* sporozoite stage of the parasitic life cycle. Undiluted supernatant was incubated with sporozoites for 1 h at 24°C (2). After washing with phosphate-buffered saline, sporozoites were exposed to a fluorescein-conjugated anti-human immunoglobulin for 1 h at room temperature. Sporozoites were then washed and viewed with a fluorescence microscope. The positive control was a 1:1,000 dilution of convalescent-phase human serum post-*Cryptosporidium* infection with a high antibody titer (7).

In vitro inhibition. In vitro inhibition was tested by using a human enterocyte cell line, HT29.74, as previously described (1, 5, 7, 11). This cell line was chosen for its ability to differentiate into cells that resemble the small intestinal epithelium when grown in medium deprived of glucose. Cells were grown to 70% confluence (24 h) in glucose-free Leibovitz L-15 medium (Gibco) containing 5 mM galactose, 6 mM pyruvate, 1 mM L-glutamine, 20 mM HEPES, antibiotics (as described above), and 10% dialyzed fetal calf serum. Oocysts were then added at $10^6/2\text{-cm}^2$ well. Oocysts were mixed with 150 μ l of unconcentrated supernatant containing monoclonal antibody for 10 min and then added to HT29.74 cells. The oocysts, supernatant, and cells were then incubated at 37°C for 24 h. Cells were fixed for 10 min in methanol and stained with hematoxylin and eosin. The number of cryptosporidial schizonts per 1,000 cells was determined with a light microscope. Human monoclonal antibody binding to schistosome antigens was used as a negative control, and experiments were run in triplicate.

RESULTS

PBLs were obtained from an HIV-seronegative woman who had recovered from cryptosporidiosis and from an HIV-seropositive man with chronic cryptosporidiosis. PBLs were fused with a heteromyloma cell line to produce viable clones. Two separate fusions of PBLs from the HIV-seronegative donor who had recovered from cryptosporidiosis resulted in 90 hybridomas. Of these hybridomas, 12 secreted antibodies to *C. parvum* as detected by ELISA.

Fusion with PBLs from the HIV-seropositive donor with chronic cryptosporidiosis and AIDS resulted in 74 hybridomas, none of which secreted anti-*Cryptosporidium* antibodies. These clones were not evaluated further.

By ELISA, the OD of the supernatant from these clones from the seronegative donor ranged from 0.170 to 1.354. 17BD4, 17A1D, EC5, and BB2 were among the clones that continued to show the highest antibody titer and were fur-

FIG. 2. Indirect immunofluorescence assay to detect antibody binding to *C. parvum* sporozoites. Immunofluorescent binding of anticryptosporidial antibodies 17A1D (a) and 17BD4 (b) to *C. parvum* sporozoites was compared with a positive control of a 1:1,000 dilution of convalescent-phase post-*Cryptosporidium* infection serum with a high antibody titer (c). An oocyst is present in panel a along with sporozoites. No binding was detected when an irrelevant hybridoma supernatant was used as a negative control.

ther characterized (Fig. 1). By affinity chromatography, all human monoclonal antibodies were determined to be immunoglobulin G1 (15).

Immunofluorescence studies utilizing supernatant from clones 17BD4 and 17A1D confirmed antibody binding to the *C. parvum* sporozoites (Fig. 2). Antibody from the EC5 hybridoma bound to sporozoites weakly, in a diffuse pattern. BB2 did not bind by immunofluorescence (data not shown).

There was significant inhibition of infection in vitro, ranging from 35% with clone 17BD4 to 68% with BB2 (Fig. 3). This is in comparison to an irrelevant human monoclonal immunoglobulin G1 antibody to *Schistosoma japonicum* (15).

DISCUSSION

C. parvum infection continues to be a significant problem in persons with AIDS. As is evident from the recent outbreak of *C. parvum* in Milwaukee due to contamination of the water

FIG. 3. In vitro inhibition of *C. parvum* infection. A 100- μ l volume of hybridoma supernatant was incubated with 10^6 oocysts at 32°C for 10 min and then added to HT29.74 cells. *C. parvum* schizonts were counted after 24 h in culture. Inhibition ranged from 35% by 17BD4 to 68% by BB2. All experiments were done in triplicate. *P* values relate inhibition compared to the negative control. Numbering within bars represents percent inhibition. Standard deviation is shown by error bars.

systems, this pathogen is ubiquitous and a significant cause of morbidity and mortality in immunosuppressed individuals (10). Therefore, the need for an effective and tolerable treatment becomes increasingly important.

C. parvum is a mucosal pathogen which does not invade beyond the epithelial cells. Immunotherapy with orally administered antibody could theoretically prevent infection of susceptible cells by sporozoites. Immunotherapy, in the form of hyperimmune cow colostrum, has been shown to be effective against cryptosporidial infection in patients with AIDS (13, 14). The limited success of bovine colostrum has led to the exploration of monoclonal antibodies and other forms of immunotherapy as a viable treatment. Much of the research has focused on the production of mouse monoclonal antibodies against cryptosporidiosis. Perryman et al. demonstrated that mice treated with mouse monoclonal antibodies had significantly reduced *C. parvum* oocyst shedding and that the antibodies specifically protected the ileum, cecum, and colon (12). Bjorneby et al. reduced cryptosporidial infection in the intestinal lining by using a neutralizing mouse monoclonal antibody (4).

Human monoclonal antibodies can be developed in large quantities and have been shown to be well tolerated in clinical trials. Ziegler et al. demonstrated that human monoclonal antibodies are safe and effective in the treatment of gram-negative bacteremia and septic shock (16). Human monoclonal antibodies, which are derived from individuals who have cleared an infection, may bind to more relevant protective antigens. For this reason, human monoclonal antibodies may be better suited for immunotherapy.

We have demonstrated that PBLs from an immune individual who has recovered from cryptosporidiosis can be utilized to develop human monoclonal antibodies that bind to *C. parvum* by ELISA and immunofluorescence assay and inhibit infection in vitro. Fusion of PBLs from a person who has AIDS and chronic cryptosporidiosis did not successfully result in hybridomas secreting cryptosporidial antibodies. This may be due to the severe immunodeficiency, with associated lymphocyte dysfunction, or to the lack of specific protective responses to *C. parvum*.

These initial studies have shown that stable human monoclonal antibodies can be developed which bind to the sporozoite stage of the parasitic life cycle and inhibit infection in vitro. Further studies are needed to develop additional human monoclonal antibodies with a range of inhibitory activity for testing in animal and, possibly, human trials for immunotherapy of *C. parvum* infection.

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