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Production of Monoclonal Antibody to the Infective Stage of *Cryptosporidium* Infection in AIDS Patients

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Cryptosporidium parvum has been recognized as a significant cause of life-threatening diarrhea in Acquired ImmunoDeficiency Syndrome (AIDS) patients. Clinical diagnosis of cryptosporidial infections has been primarily based on the detection of infective stage, oocysts, in stools. Anti-*Cryptosporidium* oocyst monoclonal antibody (mAb), IgG2a, recognizing an antigen of 97 kDa was generated to be used for diagnosis of *Cryptosporidium* infection in AIDS patients using an immunofluorecence. It appeared to react with the surface antigens. Transmission electron micrographs of the infective stage of *Cryptosporidium* recognized by this mAb demonstrated sporolulated oocysts, which measure 4~6 μm, and sporozoites excysting from oocysts.

Cryptosporidium sp. is among the top three or four enteric pathogens that have been identified (1). *Cryptosporidium* infection in humans has been described only within the past decade. Cryptosporidiosis is a common opportunistic infection in AIDS patients and is a major factor leading to death (1, 10, 11). The onset of AIDS in the United States brought attention to its association with diarrheal illness when 21 patients with AIDS and cryptosporidiosis were reported to the Center for Disease Control (8, 9). The emergence of AIDS and AIDS-related infections brought human cryptosporidiosis to the forefront of interest in 1982 (10).

Human infection with *Cryptosporidium* has been described on six continents but is most prevalent in developing nation (13). *Cryptosporidium* infection begins with the intake of the infective stage, oocysts. Oocysts obtained from the feces of infected animals are infective for other hosts including humans (1, 13). *Cryptosporidium* oocysts have been considered as a target phase for the diagnosis of cryptosporidiosis in AIDS patients and for developing a vaccine against it (8, 10, 11).

Conventional acid-fast and auramine staining methods have been widely used to detect oocysts in feces of the infected individuals. However, their applications have been limited due to the lack of specificity, i.e., they cross-reacted with other organisms (1, 5, 15). A monoclonal antibody specific for oocysts of *Cryptosporidium parvum* has been developed (14, 15) and been on the market by the Meridian Diagnostics Inc. (Cincinnati, OH, U.S.A.). This product has been imported for diagnosis of *Cryptosporidium* infection from out-patients in Korea (5). Most hospitals, however, have preferred to use conventional staining methods due to the high cost of the Meridian's product so that the necessity of development of a diagnostic kit using a monoclonal antibody has been emphasized in Korea.

This study was performed to develop a monoclonal antibody specific for the infective stage of *C. parvum* which could be used to diagnose *Cryptosporidium* infection in AIDS patients and to observe the ultrastructure of oocysts recognized by the monoclonal antibody.

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MATERIALS AND METHODS

Tissue Fixation and Preparation for Electron Microscopy

Tissue fixation was done as previously described (7). Four day old suckling mice were orally infected with purified oocysts and used four of eight days post infection (PI) for tissue preparation. *Cryptosporidium* oocysts were purified (14, 15) and provided from the Oocyst Production Unit of Dr. Charles R. Sterling's laboratory at the University of Arizona, U.S.A. The anesthetized animal was tied to the operating board with its back down. The abdominal cavity was opened by a midline incision with lateral extensions. The thorax was opened and the right atrium was cut to facilitate flow of solutions. A polyvinyl catheter (No. 8 F feeding tube) was inserted into the left ventricle, and heparinazed 0.025 M phosphate buffer saline (PBS) (1 unit/ml, pH 7.4, osmolarity up to 320 milliosmolar) was allowed to flow. After the capillaries were cleared of blood, the perfusate was switch-

ed to fixative containing 1% glutaraldehyde (Polysciences, Warrington, PA, U.S.A.) in PBS. Perfusion was continued for approximately 15 min. Following perfusion, the terminal ileum was excised and cut into 1 mm cubes. Tissue pieces were immersed in the same fixative used for perfusion for 15 min and then rinsed in buffer. Specimens were stored in buffer at 4°C until used.

Tissue Embedding and Sectioning

The specimens were post-fixed in 2% OsO_4. After postfixation, the tissues were washed in the phosphate buffer, dehydrated with a graded ethanol series (30, 50, 70, 95%, 15 min; 100% - 3×, 15 min, with agitation, room temperature), transferred to propylene oxide, and embedded in Epon according to the usual procedure. Ultra-thin sections were cut with a glass or diamond knife on an Sorval MT-2B ultra microtome, mounted on 100-mesh formvar coated nickel grids, and processed for electron microscopy.

Immunization of Mice

The 7 weeks old female BALB/c mice were injected intraperitoneally with oocysts of *C. parvum* and boosted twice at 2 weeks interval. Mice were tail-bled on the 10th day to determine the titer of anti-*Cryptosporidium* oocyst antibodies after the third injection. The final tail injection was given intravenously with *Cryptosporidium* at four weeks after the third boosting. Oocysts in PBS were given for every injection. Hyperimmune mice were sacrificed 3 days after the final booster, and spleen was removed for preparation of mono-splenocytes.

Preparation of Splenocytes

Spleen of hyperimmune mouse was used for B lymphocyte preparation for cell fusion. Spleen was removed by aseptic technique from the mouse's peritoneum and homogenized on the wire mesh screen by use of the plunger of the syringe to produce mono-splenocyte. The mono-splenocytes were washed twice with basal media.

Cell Fusion

P3-X63/Ag 8.653 Myeloma and splenocytes were mixed (10 : 1) into the 50 ml conical tube, filled with basal media to 40 ml, and centrifuged at 400×g for 10 min. The supernatant was removed and 1 ml of polyethylene glycol 4000 was slowly added to the pellet of cells while gently flicking for 1 min and held for 1 min. Then 20 ml of the basal media were slowly added to the cells with gently flicking for 4 min. These procedures were performed at 37°C in a water bath. The cell mixture was centrifuged at 300×g for 5 min and the supernatant was removed. The thymocyte-conditioned media (150 ml) were added to suspend the cell mixture. The fused cells were dispensed into 24 well tissue culture plates and cultured at 37°C in a CO_2 incubator. After 24 h of incubation, 1 ml of HAT media was added to each well.

Screening of Hybridoma by Indirect Immunofluorescence Assay (IFA)

At 10 to 15 days post fusion, colonies of growing hybridoma were visible in 24-well tissue culture plates. Antigen smears of *Cryptosporidium* were prepared by smearing oocysts in PBS on glass slides, then air-dried, and fixed by heating. The smears were incubated for 30 min at 20°C with 100 μl of hybridoma supernatants added on to them and the another smear was incubated with saline as a negative control. After washed three times with PBS, the smears were incubated with 100 μl of fluorescein-isothiocyanate-conjugated goat anti-mouse IgG+IgG+IgA (Cappel, West Chester) diluted to 1/1000 in PBS for 20 min. The oocysts were washed three times and steeped in PBS containing 10% (vol/vol.) glycerol. Immunofluorescence was evaluated with a fluorescence microscope (Olympus BH-2, Japan).

Cloning of Hybridoma

Hybridoma producing monoclonal antibodies to *Cryptosporidium* oocysts were cloned by limiting dilution in 96-well plates to less than one cell per well. For cloning cells, the wells with colonies were rescreened and recloned. The cells were diluted to 1×10^7 cells/ml into complete media after being centrifuged at 400×g for 10 min. One hundred microliters of the cell solution with 22 cells were then dispensed into the first 3 lanes of 96 well-plates containing 100 μl of thymocyte conditioned medium per well. The rest cells were diluted again with 4 ml complete medium so that the cell numbers were 4.4 in 100 μl medium and dispersed into the next 3 lanes of the plates 100 μl per well. Complete medium of 1.5 ml was then added into the last cells and 2.2 cells in 100 μl were added into each well of the last 2 lanes of the plates. All of the cells were cultured in a 5 % CO_2 incubator at 37°C.

Isotyping of Monoclonal Antibody

The subisotyping kit from American Qualex (San Clemente, CA, U.S.A.) was used for the detection and identification of anti-*Cryptosporidium* oocyst monoclonal antibody subclass. 100 μl of the goat anti-mouse immunoglobulins was added to the 10 ml plate coating solution. 100 μl of the plate coating mixture was added to each well of a 96-well EIA plate. The plate was sealed, incubated for 18~24 h at 4°C, washed with PBS-surfactant and pat dried onto clean, dry towel. 200 μl of diluted blocking serum (1 : 4 in 1×PBS) was added to each well. The plate was incubated at room temperature for one hour, washed with PBS-surfactant and pat dried. 50 μl of each hybridoma supernatants was added to each of the wells of a 8-well column. 50 μl of diluted normal mouse serum (1 : 500) to a 8-well column to serve as a positive control. The plate was incubated at room temperature for one hour, washed with PBS, pat dried and repeated this washing step twice. Two drops of the different typing antisera were added to each row on the plate. 100 μl of PBS-surfactant was added to any wells that do not contain supernatant. This served as a negative

control. The plate was incubated at room temperature for one hour. The contents of the plate was shaken and washed with PBS and pat dried. 100 μl of diluted conjugate was added to each well, incubated at room temperature for one hour, washed with PBS and pat dried three times. 100 μl of the TMB substrate reagent was added to each well. The plate was read at 655 nm in a microtiter plate reader.

Western Blot Analysis of *Cryptosporidium* Oocyst Antigens Using Monoclonal Antibodies

Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis was carried out in 10~20% gradient separating gel and 5% stacking gel, using a microslab gel electrophoresis system (SE 250-mighy Small II, Hoefer, U.S.A.). Antigens (20~40 μg) of cell lysates were dissolved in 60 mM-Tris/HCl buffer (pH 6.8) containing 2% (w/v) SDS, 25% (v/v) glycerol, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue by heating at 100°C for 5 min. After electrophoresis, oocyst antigens were transferred to nitrocellulose membranes using an electroblotter (Hoefer, San Francisco, CA, U.S.A.). The transfer buffer contained 15.6 mM Tris (Sigma, St. Louis, U.S.A.) and 120 mM glycine (Sigma, St. Louis, U.S.A., pH 8.3) at a constant current of 30 mA for 90 min at 4°C. The gel was stained with Amido black (Sigma, St. Louis, U.S.A.) to confirm complete transfer of the proteins. The membranes were blocked with 3% BSA in Tris buffered saline (TBS) 10 mM Trizma base, 150 mM NaCl, pH 7.5) for 2 h at 37°C and washed three times with TBS. The nitrocellulose was incubated with hybridoma supernatant for 1 h at 37°C. Then, horse radish peroxidase conjugated to goat anti-mouse IgG+IgM (Jackson Immuno-Research Lab, Inc., West Grove, PA, U.S.A.) was diluted to 1 : 1000 in 0.5% (w/v) BSA/TBS and incubated with the blots for 1 h at 37°C. Following each step, unbound reagents were removed with TBS by three washes for 30 min. The blots were finally incubated with a substrate solution consisting of 30 mg/ml chloronaphtol, 10 ml methanol, and 30 μl H_2O_2 (30%) in 50 ml TBS for 10 to 20 min at room temperature. The developed blots were washed with distilled water and photographed.

RESULT AND DISCUSSION

Transmission Electron Microscopy of Oocysts

Oocysts we observed contained sporozoites which had a crescent shape with a rounded posterior end and a tapering body. Sporolulated oocysts, which measure 4~6 μm, contained four naked C-shaped sporozoites, which are surrounded by a pellicle and structurally similar to merozoites (7, 9). Sporozoites were observed excysting from oocysts in large intestine (Fig. 1A), The compact nucleus was located in the posterior end of the parasite. Sporozoites contain numerous micronemes, electron-dense bodies, electron-pale vacuoles and highly condensed ribosomes in their cytoplasm.

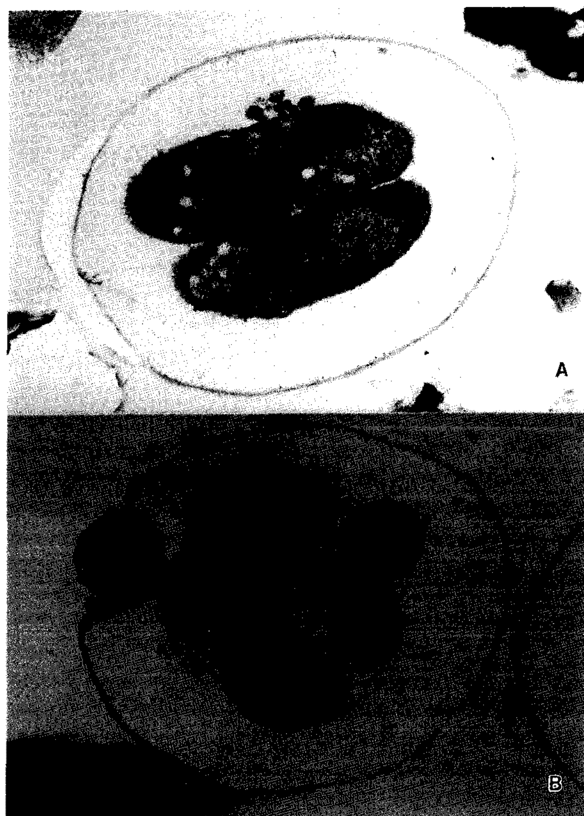

Fig. 1. Transmission electron micrographs of oocysts of *Cryptosporidium parvum*.
Sporolulated oocysts, which measure 4~6 μm, contained sporozoites which have a crescent shape with a rounded posterior end and a tapering body (A). The compact nucleus is located in the posterior end of the parasite. Sporozoites contain numerous micronemes, electron-dense bodies, electron-pale vacuoles and highly condensed ribosomes in their cytoplasm. Sporozoites were observed excysting from oocysts (B). Excystation releases four naked, non-flagellated sporozoites. Once the opening is formed, sporozoites exited quickly by rapid forward gliding movements, leaving an oocyst wall surrounding a large refractile globule. ×15,000.

Oocysts undergo excystation which, *in vitro* at least, requires the combined action of trypsin and bile salts (1). Excystation releases four naked, non-flagellated sporozoites. These invasive forms escape from the oocysts through an opening in the oocyst wall, which apparently results from dissolution of a single natural junction and partial inward collapse of the wall covering the anterior of the four sporozoites (Fig. 1B). Once the opening is formed, sporozoites exit quickly by rapid forward gliding movements (4).

Formation of the oocyst take place often, but not always, in the parasitophorous vacuole (10). Oocysts are

formed from the fertilized macrogamete which undergoes successive changes before and after fertilization (1). Oocysts with both thin and thick walls have been identified. Both types had sporulated when discharged in the feces. It has been suggested that the thin-walled oocysts, which excysted mainly within the same host, are responsible for autoinfection (8, 11, 13).

Anti-oocyst Monoclonal Antibody and Immunofluorescence

One monoclonal antibody-secreting hybridoma STF-U against *Cryptosporidium* oocyst was obtained. This clone produced IgG2a antibody. Monoclonal antibody was applied to air-dried oocysts of *Cryptosporidium* in immunofluorescence and appeared to react with the surface antigens (Fig. 2). However, we can not exclude the possibility that this antigen is also present inside the oocyst. *Cryptosporidium* oocysts, showing apple-green fluorescence against a dark background free of nonspecific fluorescence under epifluorescence microscopy (Fig. 2), were round and easily visible. The negative control showed a dark background without any fluorescence.

Western Blot Analysis of Oocyst Antigens Using Monoclonal Antibody

Oocyst proteins were transferred to nitrocellulose membrane and detected with the monoclonal antibody STF-U, using western-blot analysis. The results revealed that the antibody recognized an antigen with an apparent molecular size of 97 kDa (Fig. 3). Immunofluorescent labeling suggested that this antigen was located on the oocyst surface (Fig. 2). Arrowood and Sterling developed anti-oocyst monoclonal antibodies OW3 and C1B3. These antibodies showed reactivities to a wide band of 70 to 200 kDa and to a band of 200 kDa, respectively, in western blots (3). Amido black-stained nitrocellulose replicas showed no distinct bands or smears of 70 to 200 kDa, perhaps indicating that there were undetectable proteins or no proteins and these monoclonals recognized an oocyst wall surface determinant in immunofluorescence assays (14).

Cryptosporidium antigens have been investigated. In one study, serum from 93% of patients with cryptosporidiosis reacted primarily with an 23 kDa *Cryptosporidium* oocyst antigen (15). In another, immune sera of humans, calves, and horses reacted to a 20 kDa membrane antigen of sporozoites separated by polyacrylamide gel electrophoresis (12, 13). It is not surprising that the host presented a humoral response to the sporozoites because *Cryptosporidium parvum* sporozoites could repeatedly invade the host epithelial cells

Fig. 2. Immunofluorescent staining patterns of a monoclonal antibody STF-U reacted with air-dried *Cryptosporidium* oocysts showing typical surface labeling.

Smears on glass slides were prepared from *Cryptosporidium* oocysts in PBS, air-dried, and fixed by heating. The smears were incubated with hybridoma supernatants, washed with PBS, incubated with fluorescein-isothiocyanate-conjugated goat anti-mouse IgG+IgM+IgA.

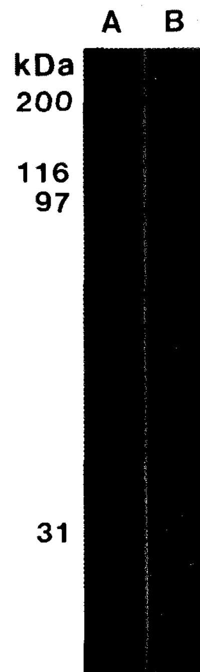

Fig. 3. Western immunoblot analysis of oocyst antigens of *Cryptosporidium* reacted with a monoclonal antibody showing reactivity to the 97 kDa antigen.

After electrophoresis, proteins were transferred to nitrocellulose membranes using an electroblotter and incubated with hybridoma supernatant for 1 hour at 37°C. The blots were finally treated for 10 to 20 mins. with a substrate solution. Lane A: standard markers.

and recycled themselves (9, 14, 15).

Immune response studies on *Cryptosporidium* infection have largely been confined to immunodeficient hosts, especially individuals with AIDS (17). These individuals are predisposed to a variety of opportunistic illnesses, including cryptosporidiosis. Circulating antibodies in response to *Cryptosporidium* infection have been detected in immunocompetent and immunocompromised individuals using immunofluorescence and ELISA (15). Immunologically healthy patients show early fluctuations of IgM followed by elevation of IgG within six weeks. Some AIDS patients produced IgM whereas all of them produced IgG (15).

A monoclonal antibody recognizing an 97 kDa antigen of the infective stage, oocysts, of *Cryptosporidium* was generated to diagnose *Cryptosporidium* infection in AIDS patients and the ultrastructure of oocysts recognized by this mAb was demonstrated.

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