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Production of a Monoclonal Antibody and Ultrastructure of the Sporozoite of *Cryptosporidium parvum*

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Cryptosporidium parvum **causes a life-threatening diarrhea in acquired immunodeficiency syndrome (AIDS) patients. The sporozoite stage of *C. parvum* has been known to be a target in treating cryptosporidiosis in AIDS patients as it is an extracellular stage. A sporozoite was ultrastructurally observed. It has a crescent shape with a rounded posterior end and a tapering body. The compact nucleus was located at the posterior end. A monoclonal antibody was produced, which recognized a 43 kDa of sporozoite antigens in a western blot analysis and showed the surface labeling in immunofluorescence.**

Key words: ***Cryptosporidium*****, AIDS, sporozoite, ultrastructure, monoclonal antibody**

Cryptosporidiosis is a common opportunistic infection in AIDS patients and may be a major factor leading to death. Characteristically, the diarrhea is profuse and watery, with as many as 71 stools/day and up to 17 liters/day reported (7,8,9). *Cryptosporidium* was first described by Tyzzer (16) from laboratory mice. Cryptosporidiosis was considered to be a rare infection in animals, and in human it was thought to be the result of a little-known opportunistic pathogen of immune-deficient individuals outside its normal host range (2, 11). Cryptosporidiosis was first noted as a human infection through a similar occurrence of overwhelming watery diarrhea in an immunosuppressed patient, and now there is a growing awareness of this opprtunistic parasitic protozoan as a potential pathogen (6,9,14). In many areas, *Cryptosporidium* sp. is among the top three or four enteric pathogens identified (3,10).

Infection follows the ingestion of oocyst, smallest among the coccidia. Oocysts obtained from the feces of infected animals are immediately infective for other hosts including humans (1,5). In the lumen of the gut the oocyst wall ruptures in response to bile salts to release four motile sporozoites (4,11). Sporozoites are extracellular stages of *Cryptosporidium parvum* (4). As they are extracellular, sporozoites are likely to become susceptible to the immune response against (7,10).

Sporozoites of *C. parvum* has been considered to be a target stage in treating cryptosporidiosis in AIDS patients and developing a vaccine against it (8,10,11). This study was performed to investigate the ultrastructure and antigenic analysis of a sporozoite. In this paper we describe the ultrastructure of a sporozoite of *Cryptosporidium parvum* and the production of a monoclonal antibody.

Materials and Methods

Tissue fixation and preparation for electron microscopy

Four day old suckling mice were orally infected with percoll-cleaned oocysts and used four or eight days post infection (PI) for tissue preparation. Heparinaized 0.025 M phosphate buffer saline (PBS) (1 unit/ml, pH 7.4, osmolarity up to 320 milliosmolar) and fixative were placed 130 cm above the worktable for tissue perfusion. The animal anesthetized 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 heparinaized PBS was allowed to flow. After the capillaries were cleared of blood, the perfusate was switched to fixative containing 1% glutaraldehyde (Polysciences, Warrington, PA, U.S.A.) in PBS. Perfusion was continued for apporximately 15 min. Following perfusion, the terminal il-

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ium 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℃ until used.

Tissue embedding and sectioning

The specimens were post-fixed in 2% OsO_4. After post-fixation, 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. Ultrathin 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 6 weeks old female BALB/c mice were used. Mice were injected intra-peritoneally with sporozoites of *C. parvum* and boosted at 3 weeks later. Mice were tailbled on the 22nd day to determine the titer of anti-Cryptosporidum antibodies. The final tail injection was given intravenously with *Cryptosporidium* at three weeks after the second boosting. Sporozoites in PBS were given for every injection. Hyperimmune mice were sacrificed 3 days after the final booster, and spleen cells were removed to be fused with myeloma cells.

Preparation of splenocytes

Spleen of the mouse was used as B lymphocyte for the cell fusion. When the cell fusion was performed, spleen was removed by aseptic technique from mouse's peritoneum and it was transformed to homogeneity on the wire mesh screen by plunger of syringe (5 cc), and washed with basal medium (20 ml) and centrifuged at 400×g for 10 min. They were washed with basal media twice.

Cell fusion

Splenocytes and myeloma were mixed (10:1) into the 50 ml conical tube, and 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 stayed for 1 min. The 20 ml of the basal media were slowly added to the cells with gently flicking for 4 min. These procedures were performed at 37℃ in the water bath. The cell mixture was centrifuged at 300×g for 5 min and then the supernatant was removed. The thymocyte conditioned media (150 ml) were added and suspended to the cell mixture. The fused cells were dispensed into 24 well tissue culture plates and cultured at 37℃ in a CO_2 incubator. After 24 hours of cell fusion, 1 ml of HAT media was added to the cell.

Screening of hybridoma by indirect immunofluorescece assay (IFA)

At 10 to 14 days post fusion, colonies of growing hybridoma clones were visible in 24-well tissue culture plates. Smears on glass slides were prepared from *Cryptosporidium* sporozoites in PBS, air-dried, and fixed by heating. The smears were incubated for 30 min at 20℃ with 100 ul of hybridoma supernatants, washed with PBS three times, incubated with 100 ul of fluorescein-isothiocyanate-conjugated goat anti-mouse IgG+IgG+IgA (Cappel, West Chester) diluted to 1/1000 in PBS for 20 min. The cells were washed three times and dropped in PBS containing 10% (vol/vol.) glycerol. Immunofluorescence was evaluated with a fluorescence microscope (Olympus BH-2, Japan, ×1000)

Cloning of hybridoma

Hybridoma producing monoclonal antibodies to *Cryptosporidium* were cloned by limiting dilution in 96-well plates to less than one cell per well. For cloning cells, the wells with colony 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. The cells were then dispensed into first 3 lanes of 96 well plates, which were previously added with 100 μl of thymocyte conditioned media, so that each will contain 22 cells per well with its volume of 100 ul. 4 ml of complete media was added into rest cells, which were dispensed into next 3 lanes with 100 μl of 4.4 cells per well. 1.5 ml of complete media was added into last cells and they were dispensed into last cells and they were dispensed into the last 2 lanes by 100 ul of 2.2 cells per well. All of the cells were cultured in 5% CO_2 incubator at 37℃.

SDS-PAGE analysis of *Cryptosporidum* sporozoite antigens

Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS-PAGE) was carried out in 10~20% gradient separating gel and 5% stacking gel, using a micro slab gell electrophoresis system (SE 250-Might 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℃ for 5 min. After electrophoresis, the gels were stained with Coomassie blue R-250 (Sigma, St., Louis, U. S.A.) for 20 min, or used for Western blot analysis without staining. Following molecules were used as the standard molecular markers (Bio-Rad, U.S.A.): myosin

(200 kDa), galactosidase (116 kDa), rabbit muscle phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), hen egg white lysozyme (14.4 kDa) and bovine pancreases apotinin (6.5 kDa).

Western blot analysis of sporozoite antigens using monoclonal antibodies

After electrophoresis, proteins were transferred to nitrocellulose sheets using an electro blotter (Hoefer, Sanfrancisco, CA., U.S.A.). Using buffer containing 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 applied 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 nitrocellulose was blocked with 3% BSA in Tris buffered saline (TBS; 10 mM Trizma base, 150 mM NaCl, pH 7.5) for 2 hours at 37°C and washed three times with TBS. The nitrocellulose was incubated with hybridoma supernatant for 1 hour 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 hour at 37°C. Following each step, unbound reagents were removed with TBS by three washes for 30 min. The blots were finally treated for 10 to 20 min at room temperature with a substrate solution consisting of 30 mg/ml chloronaphtol, 10 ml methanol, and 30 ul H_2O_2 (30%) in 50 ml TBS. The developed blots were washed with distilled water and photographed.

Results and Discussion

Transmission electron microscopy of sporozoites

Sporozoites were observed excysting from oocysts in mucosal scrapings of the ileum and large intestine. The sporozoite has a crescent shape with a rounded posterior end and a tapering body (Fig. 1). The compact nucleus was located in the posterior end of the parasite. These invasive forms escaped through an opening in the oocyst wall that apparently resulted from dissolution of a single sutural junction and partial inward collapse of the wall covering the anterior of the four sporozoites (7,9). Once the opening was formed, sporozoites exited quickly by rapid forward gliding movements, leaving an oocyst wall surrounding a large refractile globule (4). Living sporozoites were long and slender and had a rounded posterior end that tapered to a pointed anterior end. The anterior third of the sporozoite was curved sharply. Sporozoites exhibited rapid gliding movements, usually in the direction of curvature. Gliding was often accompanied by side-to-side flexing of the anterior end. Many sporozoites are observed probing the microvillous border and subsequent invasion by the sporozoite into this region of the host cell (11). During the invasive process, sporozoite become shorter and thicker, eventually assuming the form of spherical uninucleate meronts (15).

Fig. 1. Transmission electron micrograph of the sporozoite of *Cryptosporidium parvum*. The sporozoite are crescent shaped with a rounded posterior end and a tapering body. The compact nucleus is located at the posterior end. This invasive form escapes through an opening in the oocyst wall that apparently results from dissolution of a single sutural junction and partial inward collapse of the wall covering the anterior of the four sporozoites. ×15,000.

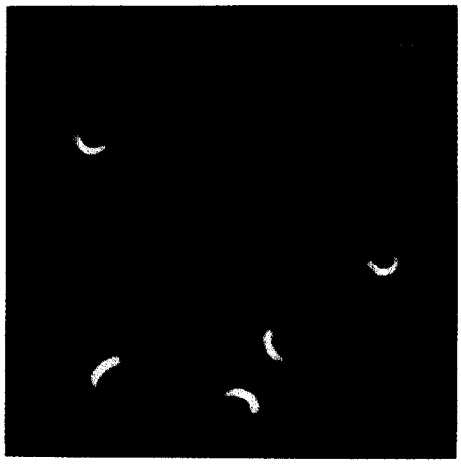

Fig. 2. Immunofluorescent staining patterns of monoclonal antibody KKU reacted with air-dried sporozoites showing typical surface labeling. Sporozoite specific monoclonal was applied directly to the air-dried slide for 15 min. After PBS wash, fluorescein-conjugated, goat anti-mouse immunoglobulins were added and the mixture was incubated for 15 min. After a final wash, the antigens were mounted on 10% glycerol in PBS and viewed using a epifluor microscope. Bar, 5 μm.

Anti-sporozoite monoclonal antibody and immuno-

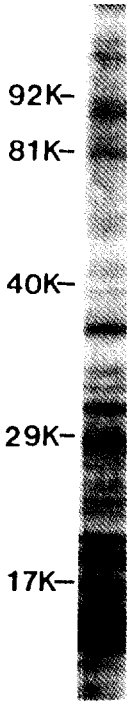

Fig. 3. Silver-stained 10~20% gradient gel SDS-PAGE of NET-solubulized sporozoite antigens. Antigens (20~40 μg) of cell lysates were dissolved in 60 mM-Tris/HCl buffer (pH 6.8) containing 2% (w/v) SDS, 25% (v/v) glycerol, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue by heating at 100℃ for 5 min. After electrophoresis, the gels were stained with Coomassie blue R-250 for 20 min.

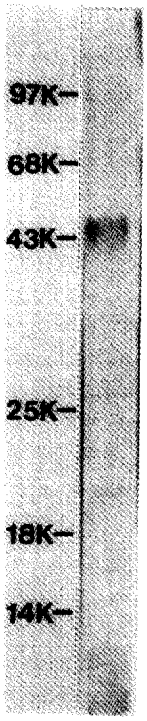

Fig. 4. Western blot analysis of NET-solubulized sporozoite material using monoclonal KKU, showing strong reactivity to the 43-kDa antigen. The nitrocellulose was incubated hybridoma supernatants for 1 hour at 37℃. Then, horse radish peroxidase conjugated to goat anti-mouse IgG+IgM was incubated with the blots for 1 hour at 37℃. The blots were finally treated with a substrate solution consisting of 30 mg/ml chloronaphtol, 10 ml methanol, and 30 μl H_2O_2 (30%) in 50 ml TBS.

fluorescence

After completing the cell fusion, screening, and cloning , one monoclonal antobody-secreting hybridoma KKU against *Cryptosporidium* parvum was obtained and established. This clone produced IgG2b antibody. Monoclonal reacted with what appeared to be the surface of air-dried sporozoites in an immunofluorescence and reacted with that appeared to be the surface of air-dried sporozoites (Fig. 2). Polyacrylamide gel electrophoresis of sporozoite antigens Sporozoite antigens were obtained from sonicated oocysts and subsequently electrophoresed on SDS-polyacrylamide gel. SDS-PAGE of C. parvum sporozoite antigens revealed a profile of proteins ranging in molecular weight from 5 kDa to 150 kDa (Fig. 3). A total of 40 bands was detected. One-dimensional gel electrophoretic analysis was performed on sporozoite protein preparations in which a total of 46 bands ranging in molecular weight from apporzimately 300 kDa to 3 kDa were detected by silver staining of SDS-PAGE gels (12).

Western blot analysis using monoclonal antibody

These proteins were transferred to nitrocellulose paper and reacted with an anti-sporozoite KKU, using western-blot analysis, which revealed that the antibody recognizes an antigen with an apparent molecular mass of 43 kDa (Fig. 4). Immunofluorescent labeling suggests that this antigen is located on the sporozoite surface (Fig. 2). However, one can not exclude the possibility that this antigen is present also inside the sporozoite. Western blots against sporozoite antigens employing immune human, equine, and bovine sera demonstrated distinct reactivity to a 20 kDa antigen (12,13). It is not surprising that the host mounts a humoral response to the sporozoite stage as *Cryptosporidium* parvum differs from other coccidian parasites in that it recycles sporozoites through the generation of thin-walled oocysts (9,14,15).

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