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Ultrastructural Localization of a Common Antigen of Sporozoites and Merozoites of *Cryptosporidium* by Immunogold Labeling Technique Using a Monoclonal Antibody

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Monoclonal Antibody 와 Immunogold 표지법에 의한 *Cryptosporidium* 의 Sporozoites 와 Merozoites 의 공통항원의 구조적 위치 결정

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Relatively little is known about the antigenic relatedness of the different developmental stages of *Cryptosporidium*. A monoclonal antibody (mAb), an IgG3, was produced against the *Cryptosporidium* merozoite stage by immunizing mice with merozoite preparation. This monoclonal was reacted with sporozoite antigens in Western blotting resulting in recognition of an epitope on a 3.5-kDa antigen. An immunoelectron microscopic technique was used to investigate the antigenic relatedness of *Cryptosporidium* sporozoites and merozoites. Mouse intestine was fixed with 1% glutaraldehyde and embedded in LR White. Thin sections were then sequentially treated with murine IgG3 mAb and anti-mouse IgG conjugated to 15-nm diameter colloidal gold. This mAb showed similar (surface/cytoplasmic) immunoelectron microscopic colloidal gold labeling patterns with sporozoites and merozoites, indicating epitope sharing between these two stages. This information might be useful for identifying possible epitopes to which a vaccine could be developed.

Cryptosporidium spp. a protozoa, is an important cause of diarrheal disease in man and several species of animals (1). *Cryptosporidium* infection in humans has been described only within the past decade. Relatively few cases were diagnosed until cryptosporidiosis was reported to be a life-threatening infection in immunocompromised individuals, especially those with the acquired immune deficiency syndrome (AIDS) (2). Medical interest in the treatment of cryptosporidiosis has increased dramatically since then. For the majority of AIDS patients, no agent or antidiarrheal compound offers clear benefit (3). There are currently no vaccines available to prevent cryptosporidiosis in humans.

A detailed description of the ultrastructure of various *Cryptosporidium* life cycle stages was published in 1966 (4). However, even though our knowledge of the fine structural details of all developmental stages is rather complete, little is known about the antigenic relatedness of these life cycle stages. This lack of knowledge makes it difficult to completely understand the antigenic expression of *Cryptosporidium* throughout the life cycle stages.

The present study outlines research to investigate the identification of possible epitopes to which a vaccine could be developed using the post-embedding colloidal gold-labeling technique.

Materials and Methods

Anti-merozoite hybridoma production

A two-month-old BALB/c mouse was immuniz-

Key words: *Cryptosporidium* antigen, immunogold labeling technique, monoclonal antibody

ed intramuscularly with a partially purified merozoite preparation suspended in an equal volume of Freund's complete adjuvant. Two weeks later, a subsequent booster injection was given intraperitonally (i. p.) using the same antigen in Freund's incomplete adjuvant. Three weeks later, the mouse was boosted by an i.p. injection of antigen with no adjuvant weekly for two weeks. After a rest period of five weeks the merozoite antigen, dissolved in a saline solution, was injected i.p. and, no the following day, intravenously with 0.1 m*l* of the preparation. Three days later, the spleen was removed and its cells fused with mouse myeloma cells (P3-X63-Ag8.653) suspended in RPMI-base.

The resulting hybridoma cells were grown in hypoxanthine, aminopterine, thymidine-selective RPMI-1640 supplemented with 15% fetal calf serum in 24-well culture plates (5). Hybridomas that produce antibodies against merozoites were screened by an indirect immunofluorescent assay against *Cryptosporidium* merozoites air-dried onto poly-l-lysine-coated microscope slides. Hybridomas secreting antibodies to merozoite determinants were cloned by limiting dilution and subclassed by indirect immunofluorescent assay using isotype-specific biotinylated anti-mouse immunoglobulin antisera (Zymed Laboratories Inc., South San Francisco, California).

Polyacrylamide gel electrophoresis

Approximately 1×10^9 Percoll-purified sporozoites were suspended in a 400 μl NET buffer (10 mM Tris · HCl, 150 mM NaCl, 50 mM phenylmethylsulfonylfluoride, 50 mM N-a-p-tosyl-L-lysine chloromethyl ketone, 0.5% Nonidet P-40 (vol/vol) (Sigma Chemical Co., St. Louis, Missouri). The suspension was incubated 10 min with agitation at room temperature and then centrifuged at 20,000 × g for 2 min. The supernatant was decanted and stored at 4°C. The sporozoite membrane preparation was diluted with a sample buffer (25 mM phosphate buffer, 1% SDS, 140 mM 2-mercaptoethanol, 0.015% bromophenyl blue, 6.0 M urea, 10% glycerol) and boiled for 4 min before applying to gels (Bio Rad Laboratories, Richmond, California).

Electrophoreses of sporozoite antigen preparations essentially followed the standard Laemmli system (6). The continuous gradient gel of 10-20% acrylamide gel with 4% stacking gels was employed for molecular weight determination. Electrophoresis was carried out at 18°C at a constant 100 mA (Protein Gel Electrophoresis Unit, Bio Rad Laboratories). Gels used for silver staining were fixed and stained using the Gel Code silver stain system (Pierce Chemicals, Rockford, Illinois).

Western blotting

Following electrophoresis, the gels were transferred to a Bio Rad Trans-Blot apparatus for transfer of proteins to nitrocellulose paper. Transfer was carried out overnight at 4°C and 30 volts (constant) followed by 2 hours at 60 volts. Gels were stained to verify protein transfer. Nitrocellulose strips were blocked with 1.0% powdered goat milk dissolved in TBS for 30 min. The blocking solution was replaced with undilute hybridoma supernatants and incubated for 1 hour. The nitrocellulose strips were washed with TBS and incubated with TBS-diluted (1:1000) biotinylated goat antimouse Ig (Bethesda Research Laboratories Inc., Gaithersburg, Maryland) for 1 hour, washed with TBS, incubated with TBS-diluted (1:1000) streptavidin-horseradish peroxidase (Bethesda Research Laboratories Inc., Gaithersburg, Maryland) for 1 hour, washed with TBS, and then developed with 0.05% 4-chloro-1-naphthol and 0.015% H_2O_2 (Kirkegaarde and Perry Laboratories, Gaithersburg, Maryland).

Immunoelectron microscopy

Four-day-old suckling mice were orally infected with 10^7 percoll-cleaned oocysts and perfused with fixative containing 1% glutaraldehyde (Polysciences, Warrington, Pennsylvania) in PBS four days post infection. Following perfusion, the terminal ilium 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 the buffer.

The specimens were rinsed with distilled water and dehydrated with 30%, 50% and 70% ethanol for 1 min each. The tissues were then placed in 70% ethyl alcohol-LR White resin (1:1) and finally into LR White resin. The following morning the tissue was embedded in LR White acrylic resin (Ernest Fullam, Latham, New York) in gelatin capsules. Polymerization was accomplished anaerobically in a vacuum oven at 50°C for 24 hours.

Ultra-thin sections were cut with a diamond knife

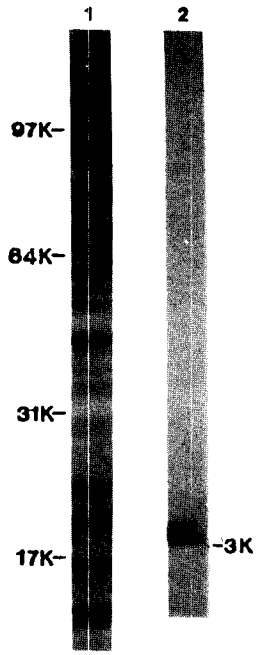

Fig. 1. Silver-stained 10-20% gradient gel SDS-PAGE of a sporozoite membrane preparation (lane 1) and Western blot of NET-solubilized sporozoite material using anti-merozoite monoclonal antibody, showing strong reactivity to an epitope on a 3.5-kDa antigen (lane 2).

on a Sorvall MT-2B ultra microtome and mounted on 100-mesh formvar nickel grids. The grids were rinsed on drops of 0.1% BSA-Tris buffer composed of 20 mM buffered saline, supplemented with 1 mg/ml BSA and 20 mM NaN_3. The washed grids were incubated on drops of buffer supplemented with 5% normal goat serum (15 min) and transferred to appropriate anti-*Cryptosporidium* antibody solutions for 1-hour incubation. Grids were washed on the buffer, incubated with goat anti-mouse IgG antibody conjugated with colloidal gold (GAM G15, Janssen Pharmaceutica, Beerse, Belgium), washed and dried. Sections were stained with uranyl acetate and lead citrate and examined with a Hitachi H-500 electron microscope.

Results

Anti-merozoite monoclonal antibody production

Three hybridoma cell lines, which secrete antibodies directed against *Cryptosporidium* merozoites,

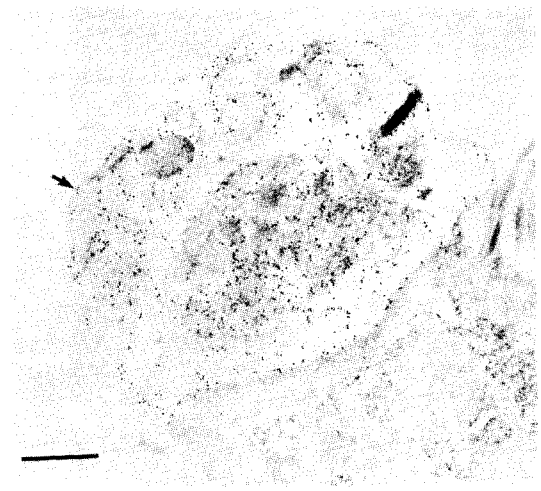

Fig. 2. Transmission electron micrograph of merozoites in a meront stage of *Cryptosporidium* fixed with 1% glutaraldehyde and embedded in LR White. The section was treated sequentially with anti-merozoite monoclonal antibody Jo3 and colloidal gold anti-mouse IgG. Gold particles were localized specifically on the surface and in the cytoplasm of merozoites. Arrow denotes colloidal gold. Bar = 1 μm.

were produced by fusing spleen cells of crude merozoite-immunized mice with murine myeloma cells. One cloned antibody-secreting cell line was established. This clone produced an IgG3 antibody (Fig. 1).

Sprorozoite antigens were obtained from sonicated oocysts and subsequently electrophoresed on SDS-polyacrylamide gel. Silver staining of SDS-PAGE gels has detected a total of about 46 bands ranging in molecular weight from approximately 3 kDa to 300 kDa (Fig. 1, lane 1). These proteins were transferred to nitrocellulose paper and reacted with an anti-merozoite mAb, Jo3, using Western-blot analysis, which revealed that the antibody recognizes an epitope on a 3.5-kDa antigen (Fig. 1, lane 2).

Immunogold labeling

Anti-merozoite monoclonal Jo3 reacted to purified sporozoites in immunofluorescent assays, indicating cross-reaction between these two stages of *Cryptosporidium* (data not shown). Monoclonal Jo3 was used to further examine potential antigen sharing between merozoites and sporozoites at the ultrastructural level using the post-embedding immunogold labeling technique.

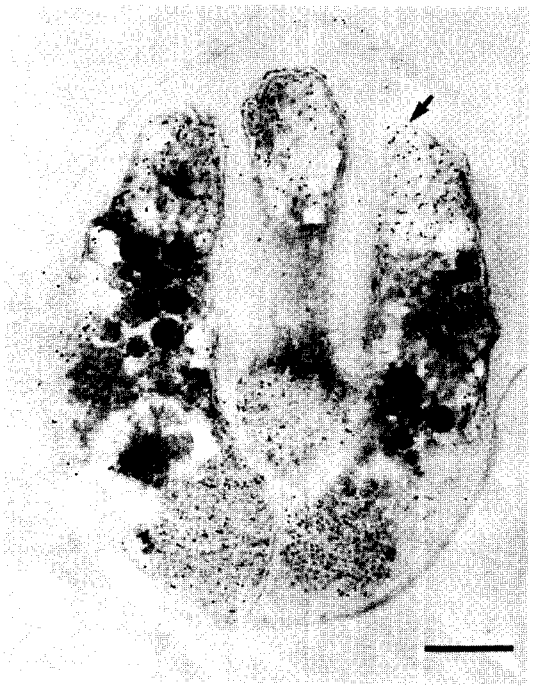

Fig. 3. Transmission electron micrograph of sporozoites in oocyst of *Cryptosporidium* fixed with 1% glutaraldehyde and embedded in LR White. The section was treated sequentially with anti-merozoite monoclonal antibody Jo3 and colloidal gold anti-mouse IgG. Gold particles were present mainly on the surface and in the cytoplasm of sporozoites. Arrow denotes colloidal gold. Bar = 1 μm.

Anti-merozoite IgG3 mAb showed similar (surface/cytoplasmic) immunoelectron microscopic colloidal gold labeling patterns with merozoites (Fig. 2) and sporozoites (Fig. 3), indicating epitope sharing between these two developmental stages of *Cryptosporidium*. Colloidal golds were localized on the innermembrane of host epithelial cells as well as on the parasites.

Discussion

The antigenic relatedness of *Cryptosporidium* sporozoites and merozoites is not known. The vague understanding of antigenic relatedness may result from the lack of immunocytochemical observations of antigenic sites at the ultrastructural level. The Present study was prompted by the hope that a precise mapping of antigenic determinants may contribute to a better understanding of the stage-specific antigenic relatedness of *Cryptosporidium* sporozoites and merozoites. An electron microscopic immunogold labeling technique employing monoclonal antibodies has been applied to locate and follow a specific antigenic determinant through two extracellular stages of *Cryptosporidium*.

Immunoelectron microscopic studies have shown that an antimerozoite IgG3 mAb (Jo3) recognizing a 3.5-kDa antigen (Fig. 1) cross-reacts with sporozoites of *Cryptosporidium* (Fig. 3). Cross-reactions involving this antigen may be explained by the persistence of an antigenic determinant in extracellular merozoite (Fig. 2) and sporozoite (Fig. 3) stages. However, in the case of most coccidian parasites, especially *Plasmodium* that causes malaria, the protein expressions of the cell surface are highly stage-specific; that is, each is expressed in only a single developmental stage, which makes the development of a vaccine difficult. Therefore, epitope sharing o *Cryptosporidum* sporozoites and merozoites may have imporatant implications for the development of a vaccine to control cryptosporidial infection because an antibody against merozoites could attack sporozoites as well.

Cryptosporidium spp. undergoes the complex life cycle stages in the intestine in susceptible hosts: sporozoites, trophozoites, meronts (containing merozoites), microgametes, macrogametes, zygotes, and oocysts (containing sporozoite). Although all developmental stages of *Cryptosporidium* are confined to an intracellular location of epithelial cells, sporozoites and merozoites become extracellular stages by being released from oocyst and meront stages, respectively (1). The presence of sporozoites released from autoinfective oocysts and merozoites undergoing cyclic development may explain why a small inoculum can produce persistent, life-threatening infections in AIDS patients (7). Sporozoites and merozoites could be appropriate target stages for the immune system to attack this protozoa due to their extracellular locations.

Antibodies are ideally suited for interaction with the extracellular invasive forms of *Cryptosporidium* for several reasons. First, an antibody can act directly on merozoites and sporozoites to damage them, either by itself or by interacting with the complement system. Second, an antibody can neutralize sporozoites and merozoites directly by blocking their attachments to new host cells. Finally, an antibody

can enhance phagocytosis of extracellular forms mediated by Fc receptors on macrophages. However, antibodies are ineffective once the protozoa has entered its host cell, that is, become the intracellular stage.

In view of the impending increase in the incidence of AIDS and the lack of effective anti-cryptosporidial drugs, epitope sharing of *Cryptosporidium* merozoites and sporozoites may be useful information for identifying possible epitopes to which a vaccine could be developed.

요 약

*Cryptosporidium*이 인체 내에서 복잡한 life cycle를 거치면서 각 stage마다 만들어지는 단백질 항원의 관계성에 관한 보고가 없어 Cryptosporidiosis에 대한 치료제 개발에 어려움이 있었다.

본 연구는 단일군 항체와 Immunogold labeling 기술을 이용하여 주요 extracellular stages인 sporozoites와 merozoites의 antigenic relatedness를 살펴보았다.

BALB/c 쥐로부터 merozoites에 대한 단일군 항체(Jo3)를 분리하였으며 IgG3형이었다. 정제된 sporozoites를, SDS-PAGE로 분리한 후 Western blot을 이용하여 Jo3를 반응시킨 결과 3,500 Daltons 크기의 sporozoites 항원을 인식하였다.

Jo3를 *Cryptosporidium*에 감염된 tissue section에 반응시킨 후 immunoelectron microscopy를 이용하여 3.5-kDa 항원의 위치와 sporozoites와 merozoites가 똑같은 단백질 항원을 만드는가를 추적해 본 결과 3.5-kDa 단백질 항원이 두 stages에서 공동으로 합성되는 것으로 나타났으며 이 항원은 표면과 세포질 내에 위치하고 있었다.

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