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Production of Monoclonal Antibodies by Hybridomas Sensitized to Sporozoites of *Cryptosporidium parvum*

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Cryptosporidium parvum Sporozoites 에 감작된 Hybridomas 에서의 Monoclonal Antibody 생산

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Hybridoma cell lines, which secrete monoclonal antibodies (mAbs) against the surface antigens of *Cryptosporidium parvum* sporozoites, were produced by fusing spleen cells of *C. parvum* sporozoite-immunized mice with P3-X63-Ag8 myeloma cells. Two cloned antibody-secreting cell lines, Korl and Ea2, were established and produced IgG1 and IgG2a antibodies, respectively. Percoll-purified sporozoites were solubilized and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Western blot assay demonstrates that an antigen of 20-kDa was bound by monoclonals. By indirect immunofluorescence microscopy, mAb exhibited uniform binding to the sporozoite surface.

Cryptosporidium parvum, an intestinal protozoa primarily inhabiting the brush border of enterocytes, has been found to be a significant cause of diarrheal disease in man and several animal species (1). Infected immunocompetent humans usually have a short-term illness accompanied by watery diarrhea, whereas immunocompromised individuals may experience life-threatening diarrheal disease. In humans, protracted diarrheal disease caused by *Cryptosporidium* has been reported in AIDS patients (2), individuals undergoing immunosuppressive treatment (3), and hypogammaglobulinemic patients (4).

As sporozoites are the infective stage of *C. parvum. Cryptosporidium* may increase reproductive potential by cyclic development of sporozoites released from autoinfective oocysts. Sporozoites comprise the extracellular stage and are expected to be major antigens eliciting immune response in infected hosts. Therefore, detailed analysis of *C. parvum* sporozoite antigens must await the purification of sporozoites and the production of a wider spectrum of hybridomas, which produce mAbs that react with the myriad antigens undoubtedly present in the preparations.

The present study was undertaken to produce hybridomas that can be used to generate mAbs against sporozoite antigens in an effort to identify and characterize antigens of sporozoites and other developmental stages that may be important in immune response.

Materials and Methods

Sporozoite purification and solubilization

Percoll gradient-recovered oocysts were washed with PBS at 1,500g (10 min × 3) and resuspended to 220 m*l* PBS at a concentration of 1-2 × 10^8/m*l*. The oocyst suspension was mixed with an equal volume of excysting solution (0.5% trypsin, 1.5% sodium taurocholate in PBS) and incubated 40-60 min in a 37°C water bath (5). The sporozoite mixture was washed with Alserver's solution and resuspended to

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a volume of 4 m*l*. A 1-m*l* aliquot of excysted sporozoites was layered over 9 m*l* of the Percoll solution and centrifuged at 20,000g for 30 min. The centrally located sporozoite band recovered from the Percoll gradients was washed with PBS and counted using a hemacytometer.

Approximately $2\text{-}3 \times 10^9$ sporozoites were pelleted and resuspended 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-alfa-tosyl-L-lysylchloromethyl ketone and phenylmethylsulfonyl fluoride (Sigma, St. Louis, Missouri) (6). 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 4°C until used.

Monoclonal antibody production

Adult BALB/c mice were immunized with approximately 1×10^6 sporozoites (SP). Immunizations were carried out on days 0 (SP + Freund's complete adjuvant delivered intramuscularly), 14 (SP + Freund's incomplete adjuvant delivered intrapertioneally), and 28 (SP delivered intravenously). On day 32 the spleens were removed and fused with P3/X63/Ag8.653 mouse myeloma cells employing polyethylene glycol 4000 (E. Merck, Darmstadt, West Germany). The resulting hybridoma cells were grown in hypoxanthine, aminopterine, thymidine (HAT)-selective RPMI-1640 supplemented with 15% fetal calf serum in 24-well culture plates (7). Supernatants were screened for specific antibodies using an indirect immunofluorescent assay against air-dried *Cryptosporidium* sporozoites.

Hybridomas secreting antibodies to sporozoite determinants were cloned by limiting dilution and sub-classed by indirect immunofluorescent assay using isotype-specific biotinylated antimouse immunoglobulin antisera (Zymed Laboratories Inc., South San Francisco, California). Culture supernatants from serially passaged hybridomas were stored at 4°C until used.

Polyacrylamide gel electrophoresis (PAGE)

Gradient gels of 10-20% polyacrylamide or standard 10% polyacrylamide gels were used with a discontinuous buffer (8). A 250- μl sporozoite membrane sample (approximately 1.05 mg/m*l*) was diluted with an equal volume of sample buffer (100 mM phosphate buffer, 1% sodium dodecyl sulfate (SDS), 140 mM 2-mercaptoethanol. 0.015% bromophenol blue, 6.0 M urea, and 10% glycerol) and boiled for 4min. Electrophoresis was performed at a constant 100 mA at 18°C. Prestained high and low molecular weight markers (Bethesda Research Laboratories, Gaithersburg, Maryland) were incorporated into each electrophoretic run. Gels not used for western blotting were fixed and silver stained using the Gel Code stain kit (Pierce Chemicals, Rockford, Illinnois).

Western blotting

Following SDS-PAGE, proteins were transferred to nitrocellulose (9) using a Bio-Rad Trans-Blot cell (Bio-Rad Laboratories, Richmond, California). Electrophoretic transfer at 4°C was achieved be employing a constant 30 volts overnight followed by 60 volts for 2 hr. The efficiency of protein transfer was determined by silver staining the polyacrylamide gel after transfer as well as by staining nitrocellulose strips with amido black. Following transfer, nitrocellulose strips were blocked for 30 min in a solution of 1.0% powdered goat milk dissolved in 0.05 M Tris-buffered saline (TBS), pH 7.5 (10). The blocked strips were incubated in hybridoma supernatant for 1 hr, washed with TBS, and incubated for 1 hr with biotinylated anti-mouse IgG (Vector Laboratories Inc., Burlingame, California) diluted 1:400 in TBS. The strips were then washed with TBS and reacted for 1 hr with streptavidin-horseradish peroxidase (Bethesda Research Laboratories) diluted 1:400 in TBS. After a final TBS wash, color development was accomplished using 0.05% 4-chloro-1-naphthol and 0.015% H_2O_2 (Kirkegaarde and Perry Laboratories, Gaithersburg, Maryland). Transfer conditions and reagent dilutions were identical in all blotting experiments.

Indirect immunofluorescent assay

Sporozoites of *C. parvum* were obtained as previously described. Sporozoite-specific mAbs were applied directly to the air-dried slide for 15 min. After a 0.01 M phosphate buffered saline (PBS, pH 7.2) wash, flourescein-conjugated, goat anti-mouse immunoglobulins were added and the mixture

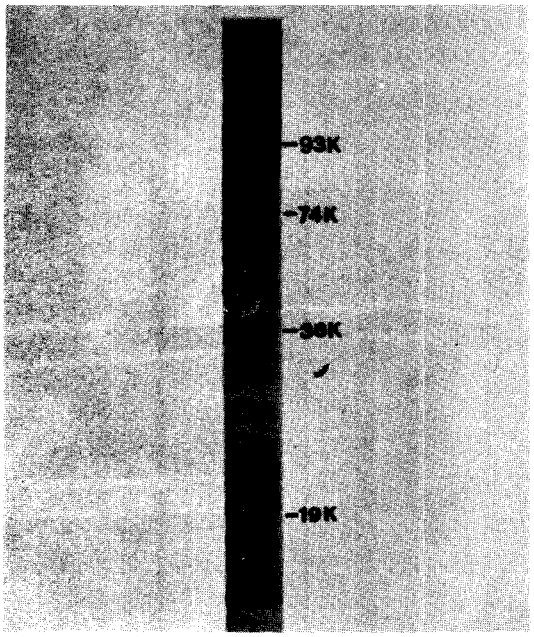

Fig. 1. Silver stained NET-solubilized sporozoite proteins separated by 10-20% gradient gel SDS-PAGE.

was incubated for 15 min. After a final PBS wash, the antigens were mounted n 10% glycerol in PBS and viewed using a Zeiss UV epifluor microscope.

Results

Sporozoite excystation and purification

Optimal sporozoite release (80% excystation) from percollcleaned oocysts occurred when trypsin and taurocholate were combined in excysting fluids. Quantitative recoveries of 63% or greater were routinely obtained using the Isopycnic Percoll gradient technique with approximately 2.2% contamination by intact oocyst.

Anti-*C. parvum* sporozoite monoclonal antibody

Hybridoma cell lines, which secrete antibodies directed against *Cryptosporidium* sporozoites, were produced. Two cloned antibody-secreting cell lines, Kor1, and Ea2, were established. These clones produced IgG1 and IgG2a antibodies, respectively (Fig. 2).

Polypeptides of *Cryptosporidium* sporozoites

Silver staining of SDS-PAGE gels (Fig. 1) revealed a total of about 46 major bands ranging in molecular weight from approximately 300 kDa to 3 kDa. The majority of these bands were transferred to nitrocellulose as demonstrated by amido black staining (results not shown).

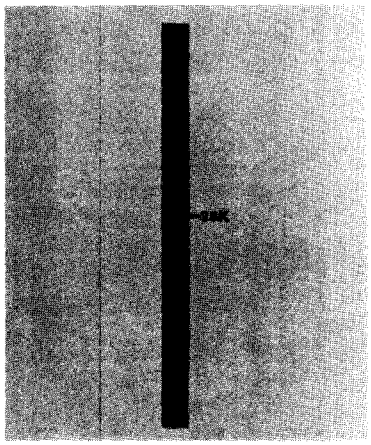

Fig. 2. Western blot analysis of NET-solubilized sporozoite material using monoclonal Kor1, showing strong reactivity to the 20-kDa antigen.

Fig. 3. Indirect immunofluorescent appearance of *Cryptosporidium parvum* air-dried sporozoites labeled by monoclonal Kor1 showing typical surface labeling (bar = 1 μm).

Western blots and immunofluorescence using monoclonal antibody

The western blot of monoclonal antibodies Kor1 or Ea2 reacted against sporozoite antigens is shown in Fig.2. Both of monoclonals showed recognition of a 20-kDa antigen band, which appears to be a major recognition of a 20-kDa antigen band, which appears to be a major sporozoite surface determinant

recognized by human, equine, and bovine immune sera (data not shown). The typical indirect immunofluorescent surface labeling of sporozoites using Kor1 is shown in Fig. 3. Monoclonal Ea2 showed a similar labeling pattern.

Discussion

Immunity to *Cryptosporidium parvum* is poorly understood. Humoral responses to *Cryptosporidium* have been demonstrated by indirect immunofluorescence assay (11) and ELISA (12), but the stage-specific antigens that elicit humoral response to the infective sporozoite stage has not yet been identified. In this regard, the present study was engaged to study three aspects of *Cryptosporidium* sporozoites: purification of sporozoites, separation of sporozoite antigens, and production of sporozoite-specific monoclonal antibody.

The excystation of percoll-cleande oocysts containing sporozoites can be induced independently by trypsin and taurocholate. In this study, optimal sporozoite release (80% excystation) occurred when trypsin and taurocholate were combined in excysting fluids. The apparent synergistic effect of combining trypsin with taurocholate suggests a role for proteases in excystation.

Isopycnic Percoll gradient isolated sporozoites were found to be free of oocyst walls with few contaminating intact oocysts. Quantitative recoveries of 63% or greater were routinely obtained using the Percoll technique, with approximately 2.2% contamination by intact oocyst. The degree of contamination was dependent on the efficiency of excystation. The recovered sporozoites were suitable for antigen analysis by SDS-PAGE, western blotting, nucleic acid analyses in our laboratory.

Cellulose column chromatographic purification of *Cryptosporidium* sporozoites, based on the method of Schmatz *et al.* (13) for *Eimeria* sporozoite purification, was found to be inefficient and produced sporozoite preparations contaminated with intact oocysts and bacteria. Purified sporozoites were essentially free of oocyst walls, however, but the technique was tedious and demonstrated poor sporozoite yield. Riggs and Perryman (14) recently reported the use of Whatman DE-52 column chromatography for sporozoite purification. Their methods varied somewhat from the methods described above. While their initial results were similar to those generated in our laboratory, the optimal recovery conditions were not adequately described, making their results difficult to evaluate.

One-dimensional gel electrophoretic analysis was performed on sporozoite protein preparations. A total of 46 about bands ranging in molecular weight from approximately 300 kDa to 3 kDa were detected by silver staining of SDS-PAGE gels (Fig. 1). Comparisons among sonicated sample preparations, trypsin/taurocholate-treated preparations, and preparations employing enzyme inhibitors showed no apparent protein degradation in the enzyme-exposed preparations (data not shown). Sporozoite surface protein tolerance of enzymes and bile salts is not surprising as they remain viable and infectious to intestinal cells in an environment containing digestive enzymes and bile.

Five anti-sporozoite hybridomas were derived from immunized BALB/c mice. Only those monoclonal antibodies demonstrating sporozoite surface antigen reactivity were examined in detail. Two monoclonal antibodies, Kor1 and Ea2, reacted with a 20-kDa sporozoite antigen in western blots (Fig. 2). Immunofluorescent labeling suggests that this antigen is located on the sporozoite surface (Fig. 3). One can't exclude the possibility, however, that this antigen is present also inside the sporozoite at this moment. Biotinylation of cell surface proteins substantiates this observation. Western blots against sporozoite antigens employing immune human, equine, and bovien sera (data not shown) demonstrated distinct reactivity to a 20-kDa band matching the antigen identified by monoclonal antibodies Kor1 and Ea2.

Cryptosporidium parvum differs from other coccidian parasites in that it recycles sporozoites through the generation of thin-walled oocysts (15). It is therefore not surprising that the host mounts a humoral response to this life cycle stage. Antibody cross-reactivity between sporozoites and merozoites (anther major developmental stage) may also exist should these stages share antigenic determinants. In this regard, the monoclonals Kor1 and Ea2, which bind the 20-kDa surface antigen of sporozoites, react whit merozoites recovered from the ileum of infected mice through indirect immunofluorescence, resulting

in surface labeling similar to that of sporozoites (Fig. 3). Western blots of merozoite membrane antigens have not yet been performed, therby limiting the characterization of this cross-reactive antigen.

요 약

AIDS 환자의 치명적인 2차 감염을 유발하는 *Cryptosporidium parvum*의 infective stage인 sporozoites의 단일군 항체를 분리하였다.

Oocysts를 효소처리하여 sporozoites를 excystation시킨 후 isopycnic percoll gradients를 이용하여 sporozoites를 순수분리한 후 단일군 항체 생산을 위한 항원으로 사용하였다.

두 달된 BALB/c 쥐를 immunize한 후 splenocytes와 P3-X63-Ag8 myeloma cells를 융합시킨 후 hybridoma 기술을 이용해 Korl(IgGl), Ea2(Ig2a) 두 clones을 분리하였으며 정제된 sporozoites를 SDS-PAGE로 분리한 후 Western blot을 이용하여 단일군 항체 Korl과 Ea2는 20,000 daltons 크기의 항원을 인식하였다.

Immunofluorescent assay에서 단일군 항체가 sporozoites 표면에 반응하는 것으로 보아 20-kDa 단백질 항원은 sporozoites 표면에 위치하는 항원으로 밝혀졌으며 *C. parvum*에 감염되었을 때 항체생성에 관여하는 중요 항원 중 하나일 것으로 추정되었다.

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