Production and Characterization of a Hybridoma-Derived Antibody to *Blastomyces dermatitidis*

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A hybridoma cell line was isolated which produced monoclonal antibody to one protein component of a yeast-phase cytoplasmic antigenic complex of *Blastomyces dermatitidis*. The immunoglobulin M antibody product was characterized by immunodiffusion, autoradiography of polyacrylamide gels, and cellulose acetate electrophoresis. By attaching the antibody to an affinity gel, one major protein band was identified by polyacrylamide gel electrophoresis as the antigen for which the antibody was specific.

In the past few years, a technique has been developed which allows the production of large quantities of monoclonal antibodies (10, 11, 13). Such antibodies are extremely pure and are specific for only one antigenic determinant. They can, therefore, be used as extremely sensitive probes or isolation tools for their respective antigens.

The production of these hybridoma-derived antibodies for pathogenic fungi was first reported for *Blastomyces dermatitidis* (K. D. Young and H. W. Larsh, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, F1, p. 319) and for *Cryptococcus neoformans* (N. K. Hall and R. Blackstock, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, F2, p. 319). The former presentation dealt with optimum conditions for production of *B. dermatitidis*-specific hybridomas. The latter reported isolation of nine hybridomas producing antibodies having differing specificities to *C. neoformans*, *Histoplasma capsulatum*, and *Coccidioides immitis*. Neither of these reports included characterization of the antigens for which the antibodies were specific.

We report the isolation of a hybridoma line secreting antibody to a cytoplasmic preparation of *B. dermatitidis*. By immobilizing this antibody to a solid support, the protein for which the antibody is specific was isolated and subsequently characterized by polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Antigen preparation. A cytoplasmic fraction was prepared by mechanical disruption of *B. dermatitidis* yeast cells in a Braun homogenizer as previously described (16) and was obtained from Sharon Harris and Stan Bauman (Immunomycologics, Inc., Norman,

Okla.). This 009BY preparation was derived from Merthiolate-killed yeast cells of the Centers for Disease Control isolate A 373 and contained 1.0 mg of protein per ml. Purified A antigen was a gift of W. K. Harrell (8). Alkali-soluble, water-soluble (ASWS) antigen was a gift of F. Deighton and was prepared as described previously (4).

Production of hybridomas. C57BL/6J mice (Jackson Laboratories, Bar Harbor, Maine) were injected with 5×10^5 Formalin-killed *B. dermatitidis* yeast cells at 2-week intervals for 8 weeks. The first three injections were intraperitoneal, and the last was intravenous in the tail vein. Mice were sacrificed 3 days after the final injection, and their spleens were removed.

The hybridization procedure was a composite drawn from Kennett et al. (9), Shulman et al. (15), and Geffer et al. (7). Spleens were placed in sterile Eagle minimal essential medium (EMEM) without calcium and magnesium and minced through wire mesh screens. The cell suspension was gently drawn into a syringe, dispensed through 20-, 22-, and 26-g needles, and centrifuged at 400 to 500 $\times g$ for 10 min. The cell pellet was suspended in 5 ml of cold 0.17 M NH_4Cl for 10 min, diluted with 10 ml of Dulbecco modified Eagle medium (DMEM), centrifuged as above, washed twice with DMEM, and resuspended in 5 ml of EMEM-fusion medium.

NS-1 myeloma cells (Hybritech, Inc., La Jolla, Calif.) were maintained in DMEM plus 8-azaguanine (10^{-4} M), and cells in mid-log were used for fusions. NS-1 and spleen cells were combined in approximately a 1:10 ratio, centrifuged, and washed twice with EMEM. A 30% polyethylene glycol 1000 solution in EMEM-fusion medium was adjusted to pH 7.4 to 7.6 and equilibrated at 37°C. The centrifuged cells were covered with 0.2 ml of this 30% polyethylene glycol 1000 solution for 8 min and centrifuged at 400 to 500 $\times g$ for 3 to 6 min of that time. The cells were then gently suspended in 5 ml of EMEM-fusion medium, mixed in an additional 5 ml, centrifuged, and washed once in the same medium. The pellet was suspended in 15 to 16 ml of DMEM plus hypoxanthine (10^{-4} M) and thymidine (1.6×10^{-4} M), and 50 μl was distributed to each of the 60 interior wells of five microtiter plates (no. 1-220-24B; Linbro, Hamden, Conn.). After incubating for 24

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h in a 5% CO₂ atmosphere, 50 µl of this suspension medium was supplemented with aminopterin (8×10^{-7} M) and added to each well. After 7 days, 100 µl of DMEM plus hypoxanthine and thymidine was added. Colonies appeared 10 to 20 days after the last addition of medium and were transferred to new medium in microtiter dishes and then to larger culture dishes if they were found to be producing antibody.

Ascites fluid containing hybridoma antibodies was produced by injecting 10⁶ hybridoma cells intraperitoneally into pristane-primed BALB/c mice. Fluid was drawn after 10 to 14 days. Hybridoma cells were frozen by suspending to 5×10^6 to 10×10^6 cells per ml in cold 5% dimethyl sulfoxide in fetal calf serum and cooling first to -20°C for 1 h and then transferring to a -70°C freezer. Antibodies from selected hybridoma culture supernatants were precipitated with 50% saturated ammonium sulfate, resuspended in 7 ml of phosphate-buffered saline, pH 7.2, and dialyzed against the same buffer. The precipitate from 500 ml of supernatant yielded 170 mg of antibody.

Maintenance medium was DMEM containing NaHCO₃, glutamine, 10% fetal calf serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. EMEM-fusion medium was EMEM without calcium and magnesium, pH 7.4 to 7.6. All tissue culture media were obtained from GIBCO Laboratories, Grand Island, N.Y. Polyethylene glycol 1000 was obtained from Koch-Light, Ltd., Colnbrook, Bucks, England. All other chemicals were from Sigma Chemical Co., St. Louis, Mo.

ELISA procedure. Hybridoma antibodies specific for *B. dermatitidis* were detected by enzyme-linked immunosorbent assay (ELISA). Antigen was suspended in carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C in polyvinyl microtiter plates (no. 1-220-24B; Dynatech Laboratories, Inc., Alexandria, Va.). Plates were rinsed three times with phosphate-buffered saline. Supernatants (0.1 ml) from presumptive hybridoma cultures were added to the antigen-primed wells, incubated for 1 h at room temperature, and washed three times in phosphate-buffered saline. Peroxidase-labeled goat anti-mouse immunoglobulin G (IgG) (heavy and light chain-specific; Bionetics Laboratory Products, Litton Bionetics, Kensington, Md.) was diluted 1:100, and 0.1 ml was added to each well, incubated for 1 h at room temperature, and washed three times in phosphate-buffered saline. The reagent solution of 0.15% H₂O₂ and 0.1% *o*-phenylenediamine was added in 0.1-ml amounts to each well, incubated for 1 h at room temperature, and read spectrophotometrically at 492 nm. Controls consisted of wells containing no antigen or antigen plus uninoculated medium, medium for NS-1 cells alone, or normal mouse serum.

Autoradiography of polyacrylamide gels. Localization of immunoglobulins in polyacrylamide gels was accomplished by the method of Bonner and Laskey (1). Hybridoma or myeloma cells were suspended to a final concentration of 0.1×10^6 to 1.0×10^6 cells per ml in 1.0 ml of DMEM plus hypoxanthine and thymidine. [³H]leucine was added to give 1.0 to 4.0 µCi/ml. The suspension was incubated for 24 h at 37°C in 5% CO₂. The cells were removed by centrifugation, and the supernatant was stored at -20°C.

Portions of the supernatants were separated on 14% polyacrylamide gels containing 10% sodium dodecyl

sulfate, and the gels were fixed in 50% trichloroacetic acid for 30 min. The gels were then soaked in two successive 20× volumes of dimethyl sulfoxide for 30 min each. The gels were next placed in a 4× volume of 22.2% (wt/vol) 2,5-diphenyloxazole in dimethyl sulfoxide for 3 h, after which they were soaked in a 20× volume of distilled water for 1 h. The gels were dried on no. 4 paper (Whatman, Inc., Clifton, N.J.) in a Bio-Rad gel dryer for 1 to 2 h and exposed to Kodak X-OMAT R film at -70°C for 3 to 7 days. The X-ray film is more sensitive if pretreated with light before exposing the gels (12). Pretreatment consisted of a 20-s exposure to a yellow 20-W bulb placed 20 ft (6.096 m) from the film. The bulb was shielded with cardboard containing a 1-in. (2.54-cm) slit through which the light could pass. The film was developed according to manufacturer's directions (6).

Cellulose acetate electrophoresis. Electrophoresis was performed in a Microzone apparatus (Beckman Instruments, Inc., Fullerton, Calif.), using membrane filters (Millipore Corp., Bedford, Mass.). One-microliter samples were applied and electrophoresed at 250 V for 20 min in 0.05 M barbital buffer, pH 8.6. Proteins were stained in 0.4% amido black in 7% acetic acid, destained with 5% acetic acid, and cleared with 30% cyclohexanone in 95% ethanol.

Additional procedures. Polyacrylamide gel electrophoresis, Ouchterlony immunodiffusion, and affinity chromatography have been described previously (14, 16). Rabbit anti-mouse IgG, anti-mouse IgM, and pure mouse IgG and IgM from myeloma lines MOPC-21 and MOPC-104C were obtained from Bionetics Laboratory Products.

RESULTS

Of 14 hybridomas isolated, culture supernatant from 1 (OU32/B6) produced absorbance readings six times that of control values in the indirect ELISA procedure with *B. dermatitidis* extract 009BY as the solid phase. No reaction was observed when histoplasmin was used as the solid phase. This clone was selected for further characterization. Another clone, OU32/A6, reacted with both the *B. dermatitidis* and histoplasmin antigens but was not further characterized.

The tissue culture supernatant of OU32/B6 and its ammonium sulfate-precipitated fractions were analyzed by immunodiffusion, cellulose acetate electrophoresis, and autoradiography. These methods indicated that OU32/B6 produced only IgM, since heavy IgG chains were absent and heavy IgM chains were present in the supernatant. The hybridoma cells secreted antibody in much greater amounts than that produced by the original myeloma line (Fig. 1, lanes 2 and 4). Use of [³H]leucine in amounts greater than 1 µCi/10⁶ cells reduced the amount of antibody secreted (Fig. 1, lanes 3 and 5).

Mice inoculated with OU32/B6 produced ascites fluid containing IgM and IgG as determined by immunodiffusion. A precipitin band formed between this preparation and the 009BY antigen.

FIG. 1. Autoradiography of hybridoma antibodies in polyacrylamide gels. Antibodies were separated in a 14% polyacrylamide gel containing 10% sodium dodecyl sulfate. Cells of myeloma NS-1 and hybridomas OU32/B6 (*B. dermatitidis*-reactive antibody) and OU32/C1 (*B. dermatitidis*-unreactive antibody) were incubated in various concentrations of [3 H]leucine as follows: Lane 1, NS-1 plus 1.0 μ Ci; lane 2, OU32/C1 plus 1.0 μ Ci; lane 3, OU32/C1 plus 2.0 μ Ci; lane 4, OU32/B6 plus 1.0 μ Ci; lane 5, OU32/B6 plus 4.0 μ Ci. Film was exposed to the gel for 165 h before developing. The top, middle, and lower arrows locate the position of IgM heavy chains, IgG heavy chains, and light chains, respectively, as determined in a separate gel stained for protein.

No bands appeared when tested against purified A antigen or ASWS antigen (data not shown). Neither this band nor IgG was present when the ammonium sulfate-precipitated antibody from tissue culture supernatant of OU32/B6 was tested, and thus they may be artifacts due to other components in the ascites fluid. Therefore, only the precipitated antibody from tissue culture supernatants was used in subsequent procedures.

Affinity chromatography with 35 mg of the precipitated monoclonal antibody as an immunoadsorbent was successful in purifying one component from the 009BY antigen preparation (data not shown). Of 1.25 mg of crude antigen applied to the affinity column, less than 1% was retained. This portion was eluted with glycine hydrochloride as previously described (16) and concentrated to almost 50 μ g/ml, and 5 μ l was examined by polyacrylamide gel electrophoresis. The location of this band by polyacrylamide gel electrophoresis relative to the other protein components of the antigen preparation is shown in Fig. 2. The location of the slowest-moving

specific component of the A antigen is noted for comparison (Fig. 2 and reference 16) and can be seen to be distinct from the affinity-isolated protein.

DISCUSSION

We report the development of a hybridoma cell line which produces IgM antibody against a cytoplasmic protein of *B. dermatitidis*. The specificity of this antibody was investigated by indirect ELISA, Ouchterlony immunoprecipitation, and affinity chromatography.

The antigen for which this antibody is specific is not shared with a closely related preparation, histoplasmin, as determined by indirect ELISA. This characteristic is important for any diagnostically specific antigen, since positive blastomycosis case sera most often cross-react with antigens of *H. capsulatum*.

The relationship of this antigen to the A and ASWS preparations of *B. dermatitidis* was investigated by Ouchterlony immunoprecipitation. The results were dependent on the source of the hybridomal antibody. Antibody from mouse ascites fluid reacted with the test 009BY preparation but not with the A or ASWS material. When hybridomal antibody was isolated from tissue culture supernatant, however, no reaction occurred with any of the three preparations. The absence of immunoprecipitation could be simply a reflection of the often poor precipitation capabilities of monoclonal antibodies. This would

FIG. 2. Gel scan of 009BY antigen preparations separated on a 14% native polyacrylamide gel, stained with Coomassie blue, and destained in 10% acetic acid. The small dot indicates a nonspecific, opaque band which appears in unstained gels. The solid arrow designates the protein which corresponds to the band eluted from an OU32/B6 antibody-linked affinity column. The open arrow marks the position of the slowest-moving specific component of purified A antigen (16). DF, Dye front; C, protein bands which also stain for carbohydrate by the periodic acid-Schiff procedure.

explain the discrepancy between the positive results of the ELISA and affinity chromatography data and the negative results of precipitation tests, all of which were done with supernatant-purified antibody. Since the antibody imparts specificity for the test antigen and not for the A and ASWS preparations when unpurified ascites antibody is used, a helping function may be present which promotes precipitation.

Identification of the protein for which the antibody is specific has been made, using affinity chromatography and gel electrophoresis. This protein is not shared with components of the A or ASWS preparations and thus may represent a new specific antigen. The immunological and diagnostic potential of this protein has not yet been investigated, so it is not known if the protein is expressed in vivo or if humans recognize it.

Although the A and ASWS preparations have improved the diagnosis of blastomycosis (4, 8), they still contain numerous separate proteins (5, 16; K. D. Young and H. W. Larsh, *Mycopathologia*, in press). It is possible that these polyvalent preparations may lack or obscure important protective or diagnostic components (2). That multiple antigens are probably important in the immunology of fungal diseases has recent support in work with cross-reactions between skin tests and lymphocyte transformation assays (3).

Hybridoma technology has allowed us to isolate a single antigenic component of *B. dermatitidis* which is recognized by mice but which differs from components present in the current A or ASWS preparations. This is a first step toward the ideal goal as expressed by Campbell, that "accurate serologic analysis . . . can not be achieved except by the isolation and characterization of each antigenic component an organism produces, related as well as unrelated, and the further correlation of these components' activities with antibodies produced throughout all stages of an infection" (2).

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