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Preparation of Species-Specific Murine Monoclonal Antibodies against the Yeast Phase of *Paracoccidioides brasiliensis*

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A panel of four murine monoclonal antibodies showing species specificity for the yeast phase of the pathogenic dimorphic fungus *Paracoccidioides brasiliensis* was produced by using a modification of the standard monoclonal antibody technology. This involved the use of the immunosuppressive drug cyclophosphamide to suppress the immune response of test animals to fungi showing cross-reactivity, i.e., to *Histoplasma capsulatum*. One monoclonal antibody, P4, which had a high titer by enzyme-linked immunosorbent assay, was shown to recognize a linear antigenic epitope of *P. brasiliensis* at a molecular size of 70,000 to 75,000 daltons by Western blot (immunoblot) analysis. The potential use of these monoclonal antibodies, which are the first species-specific probes to *P. brasiliensis* that have been produced, in the field of serodiagnosis is discussed.

Paracoccidioidomycosis is a major systemic mycosis in Central and South America (7, 12, 25). The causative agent is the thermally dimorphic fungus *Paracoccidioides brasiliensis*. Its mode of transmission and habitat have not yet been determined (13, 23). The condition is a chronic granulomatous disease characterized by primary pulmonary lesions with dissemination to visceral organs, particularly the reticuloendothelial system, and by ulcerative granulomas of the skin and mucous membranes (9).

Because of the wide range of clinical presentations, the diagnosis of paracoccidioidomycosis is based on the finding of characteristic multiple budding yeasts in sputum, biopsy material, or samples taken from cutaneous ulcers by direct examination (10, 19, 20) or by culture of *P. brasiliensis* and serology (3, 6, 11, 13, 17). Antibodies to *P. brasiliensis* can be detected by a number of serological techniques such as immunodiffusion (3, 21), complement fixation (18), indirect immunofluorescence (1, 16, 24), immunoelectrophoresis, and enzyme-linked immunosorbent assay (ELISA) (5, 19). However, antigenic similarities between the dimorphic fungi result in serological cross-reactivities when existing *P. brasiliensis* antigens are used in these tests, particularly between *P. brasiliensis* and *Histoplasma capsulatum* (12, 16), often leading to false-positive results. There is a similar problem in using existing antigens as skin test reagents to study the epidemiology of the infection (5, 12, 18, 21).

The problem of cross-reactivity has, in turn, hindered the production of species-specific monoclonal antibodies which could be used to define species-specific epitopes. The major components of fungal cross-reactivity are immunodominant carbohydrates which are present in the cell wall of different fungi. Recently, Hamilton and co-workers (11a; A. J. Hamilton, M. A. Bartholomew, L. E. Fenelon, J. I. Figueroa, and R. J. Hay, Trans. R. Soc. Trop. Med. Hyg., in press) have used a modification of an immunosuppressive technique directed against immunodominant epitopes (15, 27) to raise species-specific monoclonal antibodies aganist *H. capsulatum* var. *capsulatum* and, so, to overcome the difficulties provided by these carbohydrate epitopes. Here we report the successful use of this technique to produce species-specific *P. brasiliensis* monoclonal antibodies.

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MATERIALS AND METHODS

Antigenic preparation. Three mycelial isolates of *P. brasiliensis* were obtained from the National Collection of Pathogenic Fungi (NCPF) Mycological Reference Laboratory, Public Health Service, Colindale, United Kingdom (NCPF 3285, 4115, and 4095), and were transformed to the yeast phase on brain heart infusion agar slopes supplemented with 0.2 mM L-cysteine at 37°C. Cultures were then expanded in liquid brain heart infusion broth at 37°C. Yeasts were harvested by filtration of the culture through Whatman no. 2 filter paper, and they were then washed twice in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) (11a; Hamilton et al., Trans. R. Soc. Trop. Med. Hyg., in press). Yeast samples were split into two, and a mixture of protease inhibitors (2) was added to one of the portions; this sample was used to prepare antigen for Western blotting (immunoblotting). Both samples were then homogenized by using a bead beater (Biospec Products), and the cell extract homogenate was collected and centrifuged at 1,500 × *g* for 7 min. The supernatant was retained, a determination of its protein content was performed by the Coomassie blue method (22), and it was then separated into portions and frozen at −70°C.

Mycelial isolates of *Histoplasma capsulatum* var. *capsulatum* (two isolates), *H. capsulatum* var. *duboisii* (two isolates), *Blastomyces dermatitidis* (one isolate), and *Sporothrix schenckii* (two isolates) (NCPF 4100 and 4088, 4094 and 4077, 4076, and 3268 and 3181, respectively) were obtained from the Mycological Reference Laboratory and were transformed and processed as described above for *P. brasiliensis* mycelial isolates to yield cytoplasmic antigens.

Animals. Adult male BALB/c mice (age, 2 to 4 months) were used throughout this study for the immunization protocol, macrophage feeder production, and ascites production. Mice were obtained from the Medical Research Council Laboratories, Mill Hill, London, United Kingdom.

Immunization protocol. The modification by Hamilton and co-workers (11a; Hamilton et al., Trans. R. Soc. Trop. Med. Hyg., in press) of the protocol immunization of Matthew and Sandrock (15) was used in this study. On day 0, *H. capsulatum* var. *capsulatum* cytoplasmic yeast antigen, which was made up in an emulsion of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), in a ratio of 1:1 (by volume), was used to inoculate intraperitoneally 10

adult BALB/c mice at a final protein concentration of 50 μg and a total volume of 100 μl per mouse. Cyclophosphamide (Sigma Chemical Co., St. Louis, Mo.) in PBS was then injected intraperitoneally at a dose of 50 mg/kg per mouse at intervals of 10 min, 24 h, and 48 h.

On day 12, an intraperitoneal inoculation with the *P. brasiliensis* antigen preparation (50 μg of protein per mouse) made up in a 1:1 ratio in Freund incomplete adjuvant was given. An identical inoculation was given on day 17. Five days later a blood sample was taken from the tail vein of each mouse, and their sera were screened by ELISA (described below), to find the individual mouse with the highest differential polyclonal response to *H. capsulatum* and *P. brasiliensis* antigens. This individual mouse received an intravenous inoculation of *P. brasiliensis* antigen in PBS (50 μg of total protein) and was then used for the fusion protocol on day 25.

Fusion protocol. Cells of the murine myeloma line sp 2/0 were fused with spleen cells from the test mouse in a ratio of 1:10 by the modified protocol of Zola and Brooks (30) by using polyethylene glycol 4000. Hybridomas were then distributed to 96-well plates (Linbro), and at 10 days postfusion colonies were screened by a differential ELISA. *P. brasiliensis* and *H. capsulatum* var. *capsulatum* antigen in 0.06 M carbonate buffer (pH 9.6) was used to coat 96-well microdilution plates at a protein concentration of 1 μg per well (100 μl per well) overnight at 4°C. Wells were washed in PBS–Tween 20 (0.05%), blocked for 1 h at 37°C with 1% bovine serum albumin in PBS–Tween 20, and then incubated for 1 h with culture supernatants. An immunoglobulin peroxidase-linked conjugate of goat anti-mouse immunoglobulin G (IgG) (Jackson Immuno-Research) was then added at a dilution of 1:5,000 in PBS–Tween 20, and plates were incubated for an additional 1 h at 37°C. The substrate *o*-phenylenediamine (0.2 mg/ml and 0.005% H_2O_2 in 0.01 M sodium citrate buffer [pH 5.0]) was used to visualize positive reactions, after washes in PBS–Tween 20 and PBS. Cells from wells showing absolute discrimination between the two antigens were then expanded into 24-well plates and subcloned twice by limiting dilution before a final expansion into a 25-ml culture flask. Culture supernatants were also assayed for reactivity against the other fungal cytoplasmic antigens by ELISA, as appropriate. BALB/c mice which were primed with pristane were inoculated with 10^4 cells from each monoclonal antibody line, and ascitic fluid was collected over a period of 1 week after the development of ascites.

Polyacrylamide gel electrophoresis, electroblotting, and immunoenzyme development. A modification of the method of Tsang and co-workers (28) was used for polyacrylamide gel electrophoresis, electroblotting, and immunoenzyme development. The original preparations of dimorphic fungal antigens containing protease inhibitors were boiled for 2 to 5 min and treated with 2-mercaptoethanol and sodium dodecyl sulfate. In addition, a standard preparation of histoplasmin (L. Kaufman, Mycotic Diseases Division, Centers for Disease Control, Atlanta, Ga.) was prepared in this way for electrophoresis.

Samples were then electrophoresed in a Tris-glycine-sodium dodecyl sulfate running buffer at a concentration of 100 μg of protein per gel by using a Mini Protean II cell (Bio-Rad Laboratories, Richmond, Calif.) on a 10% polyacrylamide gel at 200 V for 1 h; molecular weight markers (GIBCO Laboratories, Grand Island, N. Y.) were electrophoresed simultaneously. Separated antigens were transferred to nitrocellulose paper by using a semidry multigel electroblotter (Ancos) at 200 mA for 30 min. Blots were then cut into strips and kept at 4°C until they were needed.

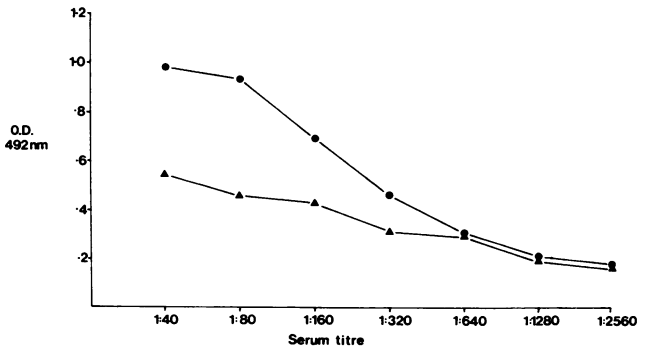

FIG. 1. Differential reactivity of polyclonal serum from a mouse selected for fusion protocol as a plot of the optical density (O.D.) at 492 nm determined by ELISA against the titer in serum. ●, Reactivities to *P. brasiliensis* NCPF 3285; ▲, reactivities to *H. capsulatum* NCPF 4100.

Immunoenzyme development was performed after blocking (1 h of incubation at 37°C in PBS containing casein at 30 g/liter) by first washing the strips in PBS-Tween 20 once and then incubating them with the culture supernatants of monoclonal antibodies P4, 7E2, 7B9, and 2E2, which were made up in PBS–Tween 20 containing casein (1:1), at 37°C for 1 h. The strips were then washed three times in PBS–Tween 20 and were probed by incubation with an immunoglobulin peroxidase-linked conjugate of goat anti-mouse IgG, which was diluted 1:250 in PBS–Tween 20 for 1 h at 37°C. Strips were subsequently washed twice with PBS–Tween 20 followed by a final wash in PBS. A color reaction was then generated by the use of the substrates 3,3'-diaminobenzidine tetrahydrochloride at 0.2 mg/ml and 4-chloro-1-naphthol at 0.06 mg/ml in PBS with 0.005% H_2O_2. The reaction was stopped after 10 min by washing the nitrocellulose strips with 0.001 M H_2SO_4 followed by a final wash with tap water.

RESULTS

The ELISA carried out with sera from 10 inoculated mice indicated that 1 mouse produced antibody with high differential activities against *P. brasiliensis* and *H. capsulatum* antigens, with a much higher reactivity toward *P. brasiliensis*. This was the mouse that was selected for the fusion protocol. Figure 1 shows the optical densities at 492 nm plotted against the dilutions of sera for the reactivity of the sera of this mouse against the two antigens.

The ELISA was performed 7 days postfusion and revealed more than 50 colonies with a high specificity toward *P. brasiliensis* antigens, when read by visual inspection. After consecutive subcloning, four monoclonal antibodies were obtained; these were designated P4, 7E2, 7B9, and 2E2. Figure 2 shows the differential reactivities of these monoclonal antibodies toward *P. brasiliensis*, *H. capsulatum*, *B. dermatitidis*, and *S. schenckii* antigens by ELISA. Monoclonal antibody P4 showed a strong recognition of all three *P. brasiliensis* isolates but no reactivity against any of the other isolates of fungal species antigens. Negative controls showed a cutoff optical density value of 0.2. Monoclonal antibodies 7E2, 7B9, and 2E2 showed no cross-reactivity with the other fungal antigens, although they exhibited much lower titers to *P. brasiliensis*.

Western blot (immunoblot) analysis of monoclonal anti-

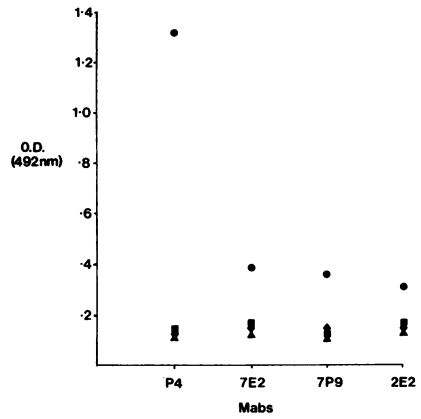

FIG. 2. Reactivity as measured by optical density (O.D.) in ELISA to *P. brasiliensis* NCPF 3285 (●), *H. capsulatum* NCPF 4100 (▲), *B. dermatitidis* NCPF 4276 (■), and *S. schenckii* NCPF 3181 (◆) antigens. Negative controls indicate a cutoff optical density of 0.2 (culture supernatants). Other yeast isolates of each species exhibited very similar reactivities (data not shown). Mabs, Monoclonal antibodies.

body P4 (Fig. 3) showed that it recognizes a linear epitope on a molecule with a molecular size of 70,000 to 75,000 daltons on the nitrocellulose strip containing *P. brasiliensis* cytoplasmic yeast antigen, while there were no reactions on strips containing *H. capsulatum* var. *capsulatum*, *H. capsulatum* var. *duboisii*, histoplasmin, *B. dermatitidis*, or *S. schenckii* antigens. The other three monoclonal antibodies that were raised showed no appreciable reaction to any antigen by Western blot analysis.

DISCUSSION

The results of the differential ELISA performed with the polyclonal mouse serum, which showed a much higher reactivity toward *P. brasiliensis*, established the effectiveness of immunomodulation with cyclophosphamide in the production of monoclonal antibodies. The possible mechanisms underlying this immunomodulation have previously been described in detail (11a, 15, 27; Hamilton et al., Trans. R. Soc. Trop. Med. Hyg., in press). The success of the polyclonal response was mirrored in the 50 or more different clones that were detected in the first screening after fusion as colonies with high reactivities toward the *P. brasiliensis* antigen but with very low or no reactivity toward the *H. capsulatum* antigen; that, in itself, is remarkable, given the previous attempts to raise species-specific monoclonal antibodies against fungi.

Monoclonal antibodies P4, 7E2, 7B9, and 2E2 are the first species-specific monoclonal antibodies toward *P. brasiliensis* demonstrated by ELISA and, in the case of monoclonal antibody P4, Western blot analysis. It is important to emphasize that these antibodies do not react with *H. capsulatum* antigen, since these two fungi share the same endemic regions in Latin America, and until now, there has been a high degree of cross-reactivity between them in serological and intradermal tests (12).

These monoclonal antibodies may be directly suitable for diagnostic systems in which polyclonal or cross-reactive monoclonal antibodies against *P. brasiliensis* are used. For example, such monoclonal antibodies may supersede the existing hyperimmune sera used in direct immunofluorescence testing, which has known cross-reactive properties

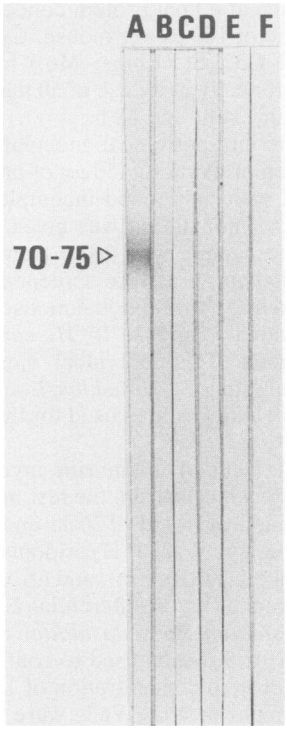

FIG. 3. Western blot (immunoblot) analysis of monoclonal antibody P4. Lane A, *P. brasiliensis* NCPF 3285 antigen; lanes B and C, *H. capsulatum* var. *capsulatum* NCPF 4100 and *H. capsulatum* var. *duboisii* NCPF 4094 antigens, respectively; lane D, histoplasmin antigen; lane E, *B. dermatitidis* NCPF 4076 antigen; lane F, *S. schenckii* NCPF 3181 antigen. The numbers 70–75 indicate the protein of 70 to 75 kilodaltons.

(8). In addition, these monoclonal antibodies can be used as positive controls in immunodiffusion and immunoelectrophoresis techniques (3, 24). However, their most important use may come in the development of a two-site ELISA for the detection of circulating antigen (4, 14, 29).

Recently, much of the work defining species-specific epitopes for *P. brasiliensis* has focused on a 43-kilodalton glycoprotein (26). Although this molecule has been shown to be recognized by sera from patients, data on its cross-reactivity with other dimorphic fungi are not definitive (21). If the species-specific epitope(s) defined by our monoclonal antibodies is recognized by sera from patients, it may well complement, or improve on, the use of the 43-kilodalton molecule.

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