Production of Species-Specific Murine Monoclonal Antibodies against *Cryptococcus neoformans* Which Recognize a Noncapsular Exoantigen

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Three monoclonal antibodies (MAbs), designated 7C5, 7C9, and 5G8, against a cytoplasmic antigen of *Cryptococcus neoformans* were produced. MAbs 7C5 and 7C9 recognize culture filtrate antigen (exoantigen) of both encapsulated and nonencapsulated isolates of this pathogen, which suggests that they do not recognize capsular polysaccharide material. This is supported by immunofluorescence data which show reactivity of all 3 MAbs to cytoplasm and cell membranes only. MAb 7C9 also recognized *C. neoformans* var. *gattii* antigens but no other fungal pathogens tested in an enzyme-linked immunosorbent assay, while 7C5 and 5G8 recognized antigens of the cross-reactive pathogen *Trichosporon beigelii* but did not recognize either *C. neoformans* var. *gattii* isolates or any other fungal antigens. By Western blot (immunoblot), 7C9 detected antigen at 110 to 120, 65 to 70, 45 to 50, and 36 to 38 kDa; in addition to the latter band, the other two MAbs recognized a band at approximately 30 kDa. All three MAbs were of the immunoglobulin G1 subclass. The two MAbs which are capable of reacting with noncapsular culture supernatant antigen have possible uses in serodiagnosis, particularly in AIDS patients infected with *C. neoformans*, since in this group the present latex agglutination test has some limitations.

Cryptococcus neoformans is an encapsulated yeast which causes the disease cryptococcosis usually characterized in its disseminated form by meningitis. The disease has become an important complication in human immunodeficiency virus (HIV) infection, particularly in Africa, where, for example, one recent survey in Zaire showed that 11% of AIDS patients had active infections, as indicated by the presence of circulating capsular antigen (19). Serodiagnosis relies on a latex agglutination test in which latex particles are coated with polyclonal antisera directed against capsular polysaccharide material (10, 17). Although this has proved to be a useful test, it has a number of drawbacks, particularly with regard to false-positives generated either through the presence of rheumatoid factor or infection with the cross-reactive pathogen *Trichosporon beigelii* (3, 5, 12). However, the major limitation of the test in AIDS patients is its detection of persistent high carbohydrate capsular antigen titers which may reflect failure to eliminate polysaccharide as well as the organisms themselves (4). This makes the test of lesser value in determining the outcome of the disease and the correct course of therapy. Therefore, there is a need for species-specific antibodies that recognize noncarbohydrate exoantigens for use in diagnosis and follow-up of the infection in AIDS patients.

Although several groups have produced monoclonal antibodies (MAbs) against *C. neoformans*, they have all been directed against capsular carbohydrate (6, 7). Recently our group has been able to produce highly specific MAbs against noncarbohydrate antigens of several fungal pathogens (9, 11), using a modification of the technique of cyclophosphamide ablation of B-cell responses to common antigenic determinants (15). Preliminary data on these MAbs suggest that they will be diagnostically useful. In this paper we

describe a similar approach designed to produce MAbs against noncarbohydrate exoantigens of *C. neoformans*.

MATERIALS AND METHODS

Antigen preparation. Isolates of capsulated *C. neoformans* var. *neoformans* (NCPF 3171 [serotype D], 3168 [serotype A], 3409, 3081, and B3501), *C. neoformans* var. *gattii* (NCPF 3169 and 3170), acapsular *C. neoformans* (B4131), *Trichosporon beigelii* (NCPF 4874 and 5246), *Aspergillus fumigatus* (NCPF 2010 and 2078), *Aspergillus flavus* (NCPF 2208 and 2617), *Candida albicans* (NCPF 3343), *Histoplasma capsulatum* var. *capsulatum* (NCPF 4100 and 4088), *Paracoccidioides brasiliensis* (NCPF 3285 and 4115), *Sporothrix schenckii* (NCPF 3181 and 3286), and *Blastomyces dermatitidis* (NCPF 4076) were obtained from the National Collection of Pathogenic Fungi, Mycological Reference Laboratory, Colindale, London, United Kingdom (C. Campbell); the Mycology Laboratory, St. Johns Hospital for Diseases of the Skin, London, United Kingdom (M. Moore); and Laboratory of Clinical Investigations, National Institutes of Health, Bethesda, Md. (K. J. Kwon Chung). The dimorphic fungi were obtained as mycelial isolates, which were transformed to the yeast phase on slopes of brain heart infusion (BHI) agar (Difco, East Moseley, United Kingdom) supplemented with 0.2 mM L-cysteine at 37°C and then subcultured in BHI broth at 37°C. The other fungi were grown first on Sabouraud agar (Oxoid, Basingstoke, United Kingdom) slopes at either room temperature or 37°C and then subcultured in liquid Sabouraud medium under the same conditions. Each culture was then collected by filtration (Whatman paper no. 2) and washed twice in phosphate-buffered saline (PBS; 0.01 M, pH 7.4) prior to division into two subsamples; to one subsample, a cocktail of protease inhibitors was added (1, 2) for subsequent use as antigen for Western blotting (immunoblotting). A bead beater (Biospec

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Products, Bartlesville, Okla.) was used to homogenize all samples, each of which was subsequently centrifuged at $2,000 \times g$ for 7 min. The supernatants were then collected to yield a cytoplasmic antigen which was stored at -70°C . Culture filtrates of the various *Cryptococcus* isolates were retained (3 and 5 days after inoculation of 2.5-liter cultures) and dialyzed overnight at 4°C against polyethylene glycol 8000 to produce a concentrate (1:100th of original volume), which was in turn dialyzed against PBS (24 h at 4°C) for use as antigen in an enzyme-linked immunosorbent assay (ELISA). The Coomassie blue method (18) was then used to determine the protein content of each sample.

Immunization protocol. Four male BALB/c mice were injected intraperitoneally with *T. beigeli* (NCPF 4874) antigen (50 μg of protein per mouse) in Freund complete adjuvant (day 1). An intraperitoneal inoculation of cyclophosphamide (Sigma, Poole, Dorset, United Kingdom) in PBS (40 mg/kg of body weight) was given 15 min later to each mouse, followed by the same treatment 24 and 48 h later. On day 15 the mice were given an intraperitoneal inoculation with *C. neoformans* (nonencapsulated strain B4131, 50 μg per mouse) in Freund incomplete adjuvant, and a similar booster injection was given on day 22. On day 25 mice were test bled and their sera were screened by ELISA to determine the individual with the highest polyclonal response to *C. neoformans* antigens (capsulated and noncapsulated) and the lowest response to *T. beigeli*. This mouse was inoculated intravenously with *C. neoformans* (unencapsulated strain, 50 μg of protein total) in PBS and used 3 days later for the fusion.

Fusion protocol. Polyethylene glycol 4000 was used to fuse cells of the murine myeloma line sp 2/0 (a gift from J. Raynes, Clinical Sciences Department, London School of Hygiene and Tropical Medicine, London, United Kingdom) with spleen cells from the chosen mouse at a ratio of 1:10 by a modified version of an existing protocol (20). The resulting hybridomas were then plated onto 10 96-well microtiter plates.

Seven days later, colonies were screened by ELISA. Three antigens, *C. neoformans* (capsulated B3501 and nonencapsulated B4131) and *T. beigeli* (NCPF 4874, in 0.06 M sodium carbonate buffer (pH 9.6) were used to coat 96-well microtiter plates overnight at 4°C and a protein concentration of 1 μg per well (100 μl per well). After washes in PBS plus 0.05% Tween (PBS-Tween), nonspecific binding was blocked by incubating the plates for 30 min at 37°C in 1% (wt/vol) bovine serum albumen in PBS-Tween. After a further PBS-Tween wash, wells were incubated for 1 h at 37°C in culture supernatants (100 μl per well). Immunoglobulin (Ig) peroxidase-linked conjugated goat anti-mouse IgG (G MlgG P₀) (Jackson, West Grove, Pa.), at a dilution of 1:5,000 in PBS-Tween was added, and the plates were again incubated for 1 h at 37°C . After washes in PBS-Tween and in PBS, the substrate *o*-phenylenediamine (OPD) (0.2 mg ml⁻¹, with 0.005% H₂O₂ in 0.01 M sodium citrate [Sigma] buffer, pH 5) was used to visualize positive reactions. The reaction was terminated after 10 min with 0.2 M H₂SO₄. Clones showing recognition of the two *C. neoformans* antigens with little or no reactivity to *T. beigeli* were subcloned twice by limiting dilution, prior to a final expansion into 25-ml culture flasks. Ascitic fluid was produced by inoculation of pristane-primed BALB/c mice with 10^4 cells of each MAb-producing line. MAbs were then assigned to subclasses, using the Serotec subclassing kit (Serotec, Kidlington, Oxford, United Kingdom); the MAbs were first bound to *C. neoformans* antigen (capsular positive) on microtiter plates and were

then recognized by using the appropriate swine anti-mouse IgG subclass probe. The latter was in turn recognized by a peroxidase-linked donkey anti-swine IgG probe which was visualized using OPD as described above.

The MAbs were purified using a protein A-Sepharose column (Pharmacia, Milton Keynes, United Kingdom) (8). Citric acid buffer (0.1 M; pH 6.0) was effective at eluting bound MAbs from the column. Protein determinations were carried out as detailed previously. Purified MAbs were then made up in PBS-Tween (initial concentration, 100 μg ml⁻¹), and their specificity was assessed by ELISA (100 μl per well) when reacted with the different fungal cytoplasmic antigens described earlier (1 μg per well). MAbs were also assessed for reactivity to *C. neoformans* culture supernatant (exoantigen) by ELISA at an initial antigen concentration of 1 μg per well, which was progressively diluted 1:10. Negative controls were provided by using either the species-specific anti-*H. capsulatum* MAb C69 (11) or PBS-Tween.

Polyacrylamide gel electrophoresis, electroblotting, and immunoenzyme development. Each fungal antigen preparation containing protease inhibitors (total of 100 μg per gel) was first boiled for 2 to 5 min with 2-mercaptoethanol and sodium dodecyl sulfate (SDS) and then electrophoresed in a Tris-glycine-SDS running buffer using a mini Protean II cell (Bio-Rad, Hemel Hempstead, Hertfordshire, United Kingdom) on a 10% (wt/vol) polyacrylamide gel at 200 V for 1 h. Gibco BRL (Paisley, Scotland) molecular weight markers were run simultaneously after similar pretreatment. Separated antigens were then transferred onto nitrocellulose paper in a Tris-glycine buffer using a semidry multigel electroblotter (Ancos, Paisley, Scotland) at 200 mA for 30 min. For immunoenzyme development, nitrocellulose strips were first blocked overnight at 4°C with PBS containing 3% casein (Marvel, Ashford, Kent, United Kingdom), washed in PBS-Tween, and then incubated with the various MAbs (25 μg of protein per strip in PBS-Tween containing Marvel) for 1 h at 37°C . After washes in PBS-Tween, the strips were probed with G MlgGP₀ diluted 1:200 in PBS-Tween (1-h incubation at 37°C), and then washed in PBS-Tween and in PBS; a color reaction was produced by the addition of the substrates 3,3'-diaminobenzidine tetrahydrochloride and 4-chloro-1-naphthol in PBS. A tap water wash and treatment with 0.01 M sulfuric acid were used to stop the reaction. Negative controls were as described for the ELISAs.

Immunofluorescence studies. Frozen sections of both the encapsulated and nonencapsulated isolates of *C. neoformans* were mounted on 3-aminopropyltriethoxysilane-covered slides (13), air dried, and fixed in absolute acetone for 5 min at room temperature. After a brief wash in PBS, the slides were incubated for 1 h at 37°C in the appropriate MAb made up at a concentration of 100 μg of protein per ml of PBS-Tween. After three further washes in PBS-Tween, the slides were then incubated under the same conditions as described above in fluorescein-conjugated goat anti-mouse IgG (Ortho Diagnostics, Raritan, N.J.) diluted 1:10 in PBS-Tween. Slides were mounted in Citifluor medium (agar aids; Stansted, Essex, United Kingdom) after final washes in PBS-Tween. Negative controls were as described above.

RESULTS

The ELISA performed on polyclonal sera from the test mice showed that three mice showed higher reactivity to the two *C. neoformans* antigens than to the *T. beigeli* antigen. Somewhat surprisingly, all three mice showed greater polyclonal recognition of the encapsulated rather than the non-

FIG. 1. Reactivity in ELISA of polyclonal sera from mouse used in fusion against *C. neoformans* (encapsulated, B3501) (■), *C. neoformans* (nonencapsulated, B4131) (○), and *T. beigelii* (NCPF 4874) (△). A_{492} values were measured.

encapsulated *C. neoformans* antigen, despite the fact that the mice had not been immunized with the former. This is illustrated in Fig. 1, which shows the differential activity of the sera from the mouse chosen for the fusion. Control mice which did not receive cyclophosphamide treatment between immunizations with *C. neoformans* and *T. beigelii* antigens showed no differential activity by ELISA (data not shown). The initial screening of the fusion by ELISA revealed 15 wells that were reactive to both *C. neoformans* var. *neoformans* strains (capsule positive and negative), with little or no recognition of *T. beigelii*. From these, three MABs were produced after successive subcloning and designated 7C9, 7C5, and 5G8; each was found to be of the IgG1 subclass. When tested in an ELISA, 7C9 showed greatest recognition of the acapsular isolate of *C. neoformans*, with lower reactivity to the encapsulated *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* isolates. There was no recognition of the *T. beigelii* antigens or any of the other fungal pathogens tested (Fig. 2). In contrast, MABs 7C5 and 5G8 showed highest reactivities to the encapsulated isolates of *C. neoformans* var. *neoformans*, with a lower value for the acapsular mutant. These MABs showed some slight recognition of *T. beigelii* antigens, although there was no reactivity to *C. neoformans* var. *gattii* isolates or to any other fungal cytoplasmic antigens (Fig. 2).

MAB 7C9 was reactive to the culture supernatants of both *Cryptococcus* variants, at a minimal protein concentration of approximately 1 pg, while 7C5 was reactive only to the *C. neoformans* variant at a minimal concentration of approximately 1 ng (Fig. 3). MAB 7C9 exhibited a prozone effect in its reactivity to antigen. Compared with MAB C69 or PBS-Tween controls, MAB 5G8 exhibited no recognition of antigens.

Western blot analysis of both capsular and acapsular *C. neoformans* isolates as well as *C. neoformans* var. *gattii* and culture filtrate antigens showed that 7C9 recognized bands at 110 to 120, 65 to 70, 45 to 50, and 36 to 38 kDa (Fig. 4), with no recognition of *T. beigelii* (on immunoblots) or of the other fungal pathogens (data not shown). Both 5G8 and 7C5 recognized a band at 36 to 38 kDa and a fainter band at approximately 30 kDa in blots of *C. neoformans* (encapsulated and nonencapsulated) and *T. beigelii* antigens. 7C5 also showed very faint reactivity to these bands when reacted

FIG. 2. Reactivities of MABs 7C9, 7C5, and 5G8 against a range of antigens, as defined by absorbance values in ELISA. Nonencapsulated *C. neoformans* (B4131 [○]), encapsulated *C. neoformans* (B3501 [■]), NCPF 3171 [▲], NCPF 3081 [▼], NCPF 3168 [◆], and NCPF 3409 [●]), *C. neoformans* var. *gattii* (NCPF 3169 [◇] and NCPF 3170 [□]), and *T. beigelii* (NCPF 4874 [△]) are shown. Isolates of all other fungi tested gave values below 0.1 (data not shown).

with blots of culture filtrate antigen. There was again no recognition of other fungal antigens (data not shown).

Indirect immunofluorescence studies revealed that both 5G8 and 7C5 were strongly reactive to both the cytoplasm and cell membrane of the capsular and acapsular isolates of *C. neoformans*, with no staining of the cell wall or capsule.

FIG. 3. Reactivities of three MABs to culture supernatant antigens by ELISA (using antigen derived from *C. neoformans* var. *neoformans* B3501). Symbols: ▲, MAB 7C9; ■, MAB 7C5; ●, MAB 5G8. Other *C. neoformans* encapsulated isolate culture supernatant antigens showed a pattern similar to that shown.

FIG. 4. Western blot reactivities of three MAbs. Reactivities of MAb 7C9 (lanes 1 to 4), 7C5 (lanes 5 to 7), and 5G8 (lanes 8 to 10) against encapsulated *C. neoformans* B3501 (lanes 1, 5, and 8), nonencapsulated *C. neoformans* B4131 (lanes 2, 6, and 9), B3501 (culture supernatant) (lane 3), and *T. beigelii* NCPF 4874 (lanes 4, 7, and 10) are shown.

In contrast, 7C9 was poorly reactive but showed the same pattern of reactivity as the two other MAbs (Fig. 5).

DISCUSSION

The successful production of three MAbs which show little or no cross-reaction with antigens other than those targeted extends the effectiveness of the cyclophosphamide modification of conventional immunization protocols which we had previously applied to the tropical dimorphic fungi (9, 11). The choice of the acapsular mutant in the immunization protocol was intended to increase the chances of ensuring the maximum antibody responses to noncarbohydrate capsule antigens, but it also served to reduce possible problems due to immunosuppression arising from the use of capsular material as an immunogen (16). Although most unencapsulated mutants have a thin layer of glucuronoxylomannan, this does not seem to have affected the outcome of the fusion. The choice of *T. beigelii* as the antigen to which the mouse immune response was ablated was designed to minimize the production of MAbs against epitopes shared with other fungal pathogens, and this rationale seems to have proved effective. It was somewhat surprising that the polyclonal antibody response of the mouse chosen for the fusion, and indeed the responses of the other animals which were not used, were greater against the antigen from encapsulated *C. neoformans* to which they had never been exposed than to the acapsular antigen used in the protocol. From this, it can only be assumed that the effect of the cyclophosphamide had been to select not only B-cell clones which produced antibodies reactive to nonimmunodominant epitopes (15) but also those producing antibodies reactive to epitopes which in terms of absolute concentration were more prevalent in the encapsulated isolates. The recognition of the nonencapsulated isolate of *C. neoformans* by the three MAbs strongly suggests that they are directed against noncapsular antigens, a view that is supported by the fluorescence studies which

FIG. 5. Immunofluorescence reactivities of MAbs. (a) 7C5. Bar, 50 µm. (b) 7C9. Bar, 50 µm. (c) 5G8. Bar, 300 µm.

demonstrated that they were directed at a cytoplasmic constituent of *C. neoformans*. Their reactivity by this method has potential application in the detection of this pathogen in histological material, an area which is presently being investigated.

The Western blot data on these MAbS suggest that they may recognize different determinants on the same antigen; most obviously both the ELISA and Western blot data for 7C5 and 5G8 suggest that the same antigen is being recognized by these MAbS, and indeed the recognition of a common 36- to 38-kDa band tends to link all three MAbS.

The major potential of MAbS 7C9 and 7C5, which have been clearly shown to detect noncapsular material which is both present in the cytoplasm and also readily detectable in sera or cerebrospinal fluid of HIV patients infected with *C. neoformans*, since the present latex agglutination test would appear to be of limited prognostic value in these individuals. It is envisaged that more sensitive tests such as a two-site ELISA (14) will be developed for laboratory testing of HIV-positive sera, to be used along with the existing latex agglutination test. Such a test would ideally be in the form of a complete supplied kit for routine testing. Work is presently in hand both to identify the antigen and to develop the best means of using MAbS 7C9 and 7C5 in serodiagnosis.

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