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Production of Agglutinating Monoclonal Antibody against Antigen 8 Specific for *Cryptococcus neoformans* Serotype D

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A hybridoma (clone CRND-8) that produced agglutinating monoclonal antibody (MAb) against *Cryptococcus neoformans* serotype D was established by using a soluble capsular polysaccharide-keyhole limpet hemocyanin conjugate for immunization. The isotype was immunoglobulin M(κ). Specificity was determined by cell slide agglutination and enzyme-linked immunosorbent assay (ELISA). In both tests, the MAb reacted to serotypes D and A-D but not to serotypes A, B, and C. Furthermore, the specificity of the MAb determined by ELISA was the same as that of polyclonal antibody factor serum (PAb factor) 8, which showed high-level reactivity with serotypes D and A-D. These results supported the deduced specificity of the PAb-based antigenic factor 8. A total of 15 isolates of serotypes D and A-D but no serotype A isolates reacted with the MAb in cell slide agglutination tests. CRND-8 MAb can be used in place of PAb factor 8 for serotyping *C. neoformans* isolates and for the analysis of the antigen 8 epitope.

Cryptococcus neoformans is a fungus which causes serious infection in humans and animals. About half of the cases of cryptococcosis occur in immunocompromised hosts. Recently cryptococcosis was recognized as an AIDS-related infection. Indeed, cryptococcal meningitis is the second most common mycosis in AIDS (4).

C. neoformans is classified into two varieties and five serotypes (11, 18, 22, 27), the latter on the basis of the polysaccharide (PS) capsule, which was shown to be a virulence factor. *Cryptococcus neoformans* var. *neoformans* has serotypes A, D, and A-D, and *Cryptococcus neoformans* var. *gattii* has serotypes B and C. Differentiation between the two varieties in clinical and environmental isolates is performed by using canavanine glycine-bromthymol blue agar medium (22). The structures of the capsular PSs of all five serotypes have been solved, and structural differences between serotypes are the basis for the serological specificities (2, 6, 15). The serotyping of *C. neoformans* isolates was first carried out by a slide agglutination test with factor sera prepared by adsorption of anti-*C. neoformans* rabbit sera with heterologous heat-killed cells. Its interpretation is based on the antigenic patterns of the five serotypes (11). One such kit, consisting of five factor sera, is commercially available and has been evaluated (14).

Monoclonal antibodies (MAbs) recognizing antigens 1, 2, 3, 7, and 8 have been obtained by several investigators (3, 7, 8, 15, 19, 25). The properties of these MAbs support the antigenic patterns reported in our previous study (11). Specificities of the MAbs have been variously determined by enzyme-linked immunosorbent assay (ELISA), agglutination, precipitation, or indirect immunofluorescence assay. In our experience the results of ELISA or indirect immunofluorescence assay are sometimes too ambiguous to be interpreted. Since the antigenic pattern was originally based upon the results of cell slide agglutination (CSA) tests, we decided to screen MAb-secreting hybridomas with that same test (25). Although CSA is a rapid and simple immunochemical test, it requires high-titered antibody; however, the capsular PS of *C. neoformans* is poorly immunogenic in mice (20). Because of these problems, agglutinating MAb specific for serotype D has not yet been reported. In this communication we describe the production of *C. neoformans* serotype D-specific MAb which is useful for the slide agglutination test.

MATERIALS AND METHODS

Strains used. *C. neoformans* serotype D M9019 (=NIH 52) was used for immunization. Standard serotype strains M9022 (=CDC551, serotype A), M9010 (=CBS 132, serotype A-D), M9017 (=NIH 112, serotype B), and M9018 (=NIH 18, serotype C) and 20 isolates were used for comparative study. Serotype A was kindly supplied by L. Kaufman (Centers for Disease Control, Atlanta, Ga.), serotypes B, C, and D were obtained from J. E. Bennett (National Institutes of Health, Bethesda, Md.) by our former professor Y. Fukazawa in 1977, and serotype A-D was obtained from Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. Most of the 20 strains were clinical isolates, and 10 of 12 serotype D strains were isolated from dermatologic lesions.

Preparation of antigen. Soluble capsular PS was isolated from cultural supernatant as described previously (10). Briefly, the strain was grown in yeast nitrogen base broth (Difco) containing 2% glucose, 1% Casamino Acids, and 100 μg of streptomycin per ml. After 5 days of incubation at 27°C with shaking, cells were removed by centrifugation and the supernatant was concentrated with an evaporator. Crude PS was precipitated by ethanol, dissolved in water, dialyzed against water, and lyophilized. Since the antibody response of mice against cryptococcal PS is weak, the crude PS was conjugated with keyhole limpet hemocyanin (KLH) (25). Forty milligrams of crude PS was dissolved in 4 ml of physiological saline solution (PSS). One milliliter of 1% cyanuric chloride in dimethylformamide was added to the PS solution and stirred for 1 h at 4°C. KLH in PSS (100 mg/2ml) was added and stirred for 1 h at 25°C, and the solution was then incubated at 4°C for 18 h. The solution was dialyzed against water for 30 h and centrifuged. The protein concentration in the supernatant was adjusted to 2 mg/ml, 1/9 volume of 10% aluminum potassium sulfate solution was added, and the pH was adjusted to 6.5 with 10% sodium carbonate. The precipitated antigen was removed by centrifugation and resuspended in PSS.

Immunization protocol. Male 4-week-old BALB/c mice were used for immunization. PS-KLH conjugate (0.4 μg, as mannose) suspended in PSS (0.2 ml) was intraperitoneally injected three times at 4-day intervals (on days 1, 5, and 9). On day 25 the same amount of conjugate in Freund complete adjuvant was injected intraperitoneally and, starting on day 50, antigen in Freund incomplete adjuvant was injected at 25-day intervals six times. Booster injections were given on day 303 and day 317. In each booster immunization a total of 10 μg of antigen in Freund complete adjuvant was inoculated into the footpads and base of the tail. Serum antibody levels were monitored by ELISA and CSA.

Hybridoma production. The procedure for the production of hybridoma was as described by Furuya et al. (9). Spleen cells from immunized mice and murine myeloma cells (P3X63Ag8U1; a gift of T. Watanabe, Saga Medical College, to Y. Furuya, Kanagawa Prefectural Public Health Laboratory) were hybridized by using polyethylene glycol 1000. Hybridoma cell lines were selected by using hypoxanthine-aminopterin-thymidine in RPMI 1640 medium containing 10% fetal calf serum, glutamine, and 2-mercaptoethanol. Cultural supernatant was

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screened for antibody production by ELISA and CSA. A hybridoma that secreted agglutinating antibodies was cloned by a single-cell manipulation method. Ascites was generated by intraperitoneal injection of hybridoma cells into BALB/c mice primed with pristane. The MAb isotype was determined by ELISA with a mouse typer kit (Bio-Rad).

Serological characterization of MAb. CSA and ELISA were used for characterization of MAb. A heat-killed cell suspension adjusted to McFarland no. 10 turbidity was used as the antigen in the CSA test. To perform the test, twofold serial dilutions of cultural supernatant or ascitic fluid were mixed with equal volumes of cell suspension on a glass slide. After rotation for 5 min, agglutination reactions were observed (24).

For ELISA, either formalin-killed cells or deproteinized PS was used as the antigen. The cellular antigen consisted of a 50-μl suspension of *C. neoformans* (2×10^8 cells per ml) placed in a well of a polystyrene microplate (Maxisorp Immunoplate; Nunc). After incubation at 65°C for 4 h, 0.25% glutaraldehyde (50 μl) was added to each well, and the plates were incubated for 15 min at room temperature and washed with 0.01 M phosphate-buffered saline, pH 7.2 (PBS). The plates were postcoated with 3% bovine serum albumin (BSA) in PBS (350 μl) at 4°C overnight. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and stored in a desiccator at 4°C. The hybridoma supernatant was added to the antigen-coated plates and incubated for 2 h at 37°C. The plates were washed with PBS-T and incubated for 2 h at 37°C with alkaline phosphatase-labeled antibodies specific for mouse immunoglobulins G and M (heavy and light chains) (Tago). After incubation with the second antibody, the plates were washed and incubated for 30 min with 0.1% *p*-nitrophenyl phosphate disodium salt in diethanolamine buffer, pH 9.8. The reaction was stopped by addition of 50 μl of 3 M NaOH, and the A_{410} was determined.

Alternatively, PS deproteinized with pronase, as described by Kozel and Cazin (16), was used as the antigen. The PS was fixed to the plate by the method described by Belay et al. (1). Thus, PS was oxidized with 0.5 M sodium periodate for 5 min and was reacted with the adipic acid hydrazide derivative of BSA. Hybridoma supernatant containing MAb was added to the antigen-coated well, and detection was as described above. Polyclonal antibody (PAb) factor serum 8 in Crypto Check (Iatron, Tokyo, Japan) was used to compare the specificities of MAb and PAb. Background reaction values were determined by using the complete system without antigen. These values were always less than 0.1.

TABLE 1. Slide agglutination titers of CRND-8 MAb and PAb factor 8 against *C. neoformans* isolates

Strain (source)	Serotype identified with factor sera	CSA titer	
		CRND-8 MAb	PAb factor 8
M9022 (CDC551)	A	<1:2	<1:2
M9019 (NIH 52)	D	1:128	1:16
M9010 (CBS 132)	A-D	1:128	1:4
M9017 (NIH 112)	B	<1:2	<1:2
M9018 (NIH 18)	C	<1:2	<1:2
M9112	D	1:128	1:8
M9113	D	1:64	1:8
M9114	D	1:128	1:8
M9115	D	1:64	1:8
M9116	D	1:128	1:8
M9117	D	1:64	1:8
M9118	D	1:64	1:8
M9119	D	1:128	1:8
M9120	D	1:64	1:8
M9196	D	1:64	1:16
M9214	D	1:64	1:16
M9215	D	1:64	1:16
M9125	A-D	1:256	1:8
M9123	A-D	1:64	1:4
M9124	A-D	1:64	1:8
M9188	A	<1:2	<1:2
M9189	A	<1:2	<1:2
M9190	A	<1:2	<1:2
M9191	A	<1:2	<1:2
M9192	A	<1:2	<1:2

RESULTS

Production of hybridoma. Several immunization procedures were tested. The one described above produced a high level of antibody in three mice. Spleen cells from these mice gave one hybridoma that secreted agglutinating MAb against *C. neoformans* serotype D. The hybridoma was designated CRND-8. The isotype of the MAb was identified as immunoglobulin M(κ).

Serological characterization by CSA. CSA titers of CRND-8 in ascitic fluid against the five standard serotypic strains (11) are shown in Table 1. The ascitic fluid showed an agglutination titer of 1:128 against serotypes D and A-D, whereas it showed no detectable reaction with serotypes A, B, and C. This pattern corresponds to the agglutination pattern of PAb factor 8, which is specific for serotypes D and A-D. However, the agglutination titer of CRND-8, at 1:128, was higher than that of PAb factor 8, which was less than 1:32.

Serological characterization by ELISA. PS and cell antigens were used to determine the specificity of the MAb secreted by CRND-8 (Fig. 1a and b). For both preparations, the reactivities against serotypes D and A-D were higher than those against serotypes A, B, and C. When killed cells were used, the cross-reaction against serotype A was lower. The specificity of PAb factor 8 was studied with the same antigens. As shown in Fig. 1c the reactivities of PAb factor 8 against PSs from serotypes D and A-D were higher than those against PSs from serotypes A, B, and C. With cell antigens, the reactivities against serotypes A, B, and C cells were negligible, as shown in Fig. 1d. Because the CSA titers of the cultural supernatant of CRND-8 and PAb factor 8 were 1:2 and 1:8, respectively, the level of reactivity of PAb factor 8 was higher than the level of reactivity of the CRND-8 MAb in this experiment. These properties were similar to those of the CRND-8 MAb. Thus, both CSA and ELISA showed that the CRND-8 hybridoma was secreting a MAb which recognized cell surface antigen 8. In addition, inhibition of indirect immunofluorescence was studied. The pretreatment of *C. neoformans* serotype D cells with CRND-8 MAb decreased the binding of PAb factor 8, and vice versa.

Application of CRND-8 MAb to serological identification of isolates. To determine the reactivity of clinical isolates of *C. neoformans* var. *neoformans*, 20 strains were studied by using the CSA test. The serotypes had previously been identified by CSA with PAb factor sera. All of the 12 strains of serotype D and 3 strains of serotype A-D reacted with CRND-8 MAb with agglutinin titers ranging from approximately 1:64 to 1:256. However, none of the five strains of serotype A reacted. These results suggest that CRND-8 MAb can be used in place of PAb factor 8 for serotyping *C. neoformans* isolates.

DISCUSSION

MAbs against the *C. neoformans* capsule have been produced on several occasions (3, 7, 8, 19, 25). In general, their specificities have supported the validity of the PAb-based antigenic factors 1, 2, 3, 7, and 8 reported in our previous papers (11, 19, 25). *C. neoformans* serotypes A, B, C, D, and A-D have both common and serotype-specific antigens. For analysis of the epitopes of each antigen, MAbs should be more useful than polyclonal factor sera.

ELISA is frequently used for immunochemical analyses. This assay is a sensitive test which is performed in ordinary laboratories, and the results are recorded objectively. However, the results of ELISA are sometimes ambiguous in that we find it difficult to decide on the cutoff point.

Antigenic structures of the five serotypes were deduced from results of cross-absorption and slide agglutination tests. These are simple and rapid methods, although high-titered antibody

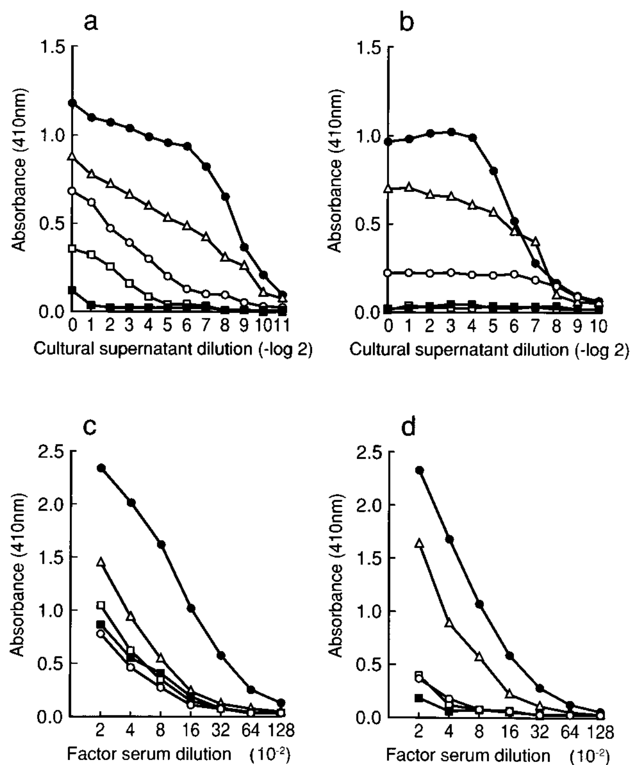

FIG. 1. ELISA reactivities of MAb CRND-8 and PAb factor 8 against five serotypes. Symbols: ●, serotype D; △, serotype A-D; ○, serotype A; ■, serotype B; □, serotype C. (a) MAb against PS; (b) MAb against formalin-killed cells; (c) PAb against PS; (d) PAb against formalin-killed cells.

is necessary. Though serotypes A and D have specific antigens 7 and 8, respectively, the chemical structures of the PSs from serotypes A and D are very similar (2, 6). Therefore, to differentiate between the specific epitopes of antigen 7 and 8, highly specific antibodies are needed. PAb factor 8 is made by adsorption of anti-*C. neoformans* serotype D rabbit serum with serotype A cells. The agglutination titers of available PAb factor 8 sera are between 1:4 and 1:16. While investigators have requested factor sera with higher titers, the antibody response to *C. neoformans* PS is low in rabbits, so that it is not easy to supply high-titered factor serum consistently. Thus, the availability of an agglutinating MAb with a CSA titer of 1:128 will be useful for the immunochemical analysis of *C. neoformans* serotypes A, D, and A-D.

Immunization of mice with *C. neoformans* PS was as difficult as the immunization of rabbits because the mice produced the desired level of antibodies only after almost 1 year of immunization with PS-KLH conjugate. The problem of immunological unresponsiveness (20) in the production of anticryptococcal PS MAbs has been variously solved. Dromer et al. used 3-month-old mice (7), Eckert and Kozel used PS antigen coupled to sheep erythrocytes (8), Casadevall and Scharff infected mice with a live clinical isolate (3), and Shinoda et al. used PS-KLH conjugate antigen (25). The respective prefusion serum antibody titers in these studies were over 1:1,000 by indirect immunofluorescence assay, at least 1:20,000 by ELISA, more than 1:200 by ELISA, and 1:8 to 1:32 (approximately) by CSA. In the present experiment the titer was less than 1:2 by CSA but more than 1:256 by ELISA.

We used two ELISA antigens for serological characterization of CRND-8 MAb. When killed *C. neoformans* cells were used as the antigen, the specificity of the reactivities against serotypes D and A-D versus serotypes A, B, and C was distinct. The same result was obtained in ELISA with PAb factor 8. Indeed, since in the preparation of PAb factor 8, anti-*C. neoformans* serotype D rabbit serum was adsorbed by serotype A cells, one would expect the cross-reactivity against cells of serotype A, as well as B and C, to be low.

C. neoformans serotype D is ubiquitous. In the United States 74.6, 4.8, 10.3, 4.0, and 4.0% of clinical isolates were serotype A, D, B, C, and A-D, respectively (17). In Japan 86.6% of clinical isolates were serotype A, 9.7% were serotype D and 3.7% were serotype A-D (24). Interestingly, serotype D isolates are often isolated from dermatologic lesions in Japan (21). In Italy the dominant serotype from all patients, AIDS as well as non-AIDS, is serotype D (12, 17). The pathogenicity of *C. neoformans* has been evaluated by biosynthesis and molecular biological studies of capsular PS performed with acapsular mutants derived from the serotype D wild type (5, 13, 26). Thus, the establishment of an agglutinating serotype D-specific MAb could be useful for both research and clinical laboratories.

Preliminary experiments were done to determine the epitope of antigen 8 (data not shown). Deproteinized PS was fractionated by DEAE-Toyopearl 650M column chromatography. The glucuronoxylomannan fraction had the highest reactivity with CRND-8 MAb in the ELISA. However, de-O-acetylated glucuronoxylomannan failed to react. These results suggest that glucuronoxylomannan containing the O-acetyl group has the epitope of antigen 8. Thus, the CRND-8 MAb established here would be a useful tool to precisely analyze the antigen 8 epitope.

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