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NOTES

Prevalence in *Cryptococcus neoformans* Strains of a Polysaccharide Epitope Which Can Elicit Protective Antibodies

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Monoclonal antibody (MAb) 2H1 binds to an epitope in the capsule of *Cryptococcus neoformans* that can elicit protective antibodies. The binding of MAb 2H1 to *C. neoformans* strains was studied by agglutination, immunofluorescence, and phagocytosis assays. The MAb 2H1 epitope was present in all 21 isolates studied, including those recovered from patients with recurrent infections.

Cryptococcus neoformans infections in patients with AIDS are accompanied by high mortality (9). In patients with AIDS, *C. neoformans* infection has a high relapse rate and present management involves lifetime maintenance therapy with antifungal agents (30). The difficulty of treating *C. neoformans* infections in patients with AIDS has led to an interest in antibody-based strategies for the prevention (11) and therapy (14, 17, 20, 29) of cryptococcosis.

Monoclonal antibody (MAb) 2H1 is a murine immunoglobulin G1κ (IgG1κ) antibody which binds *C. neoformans* capsular glucuronoxylomannan (GXM) (4, 5). MAb 2H1 can modify the course of lethal *C. neoformans* infection in mice (18, 19, 21) and potentiates amphotericin B against *C. neoformans* (20). Thus, MAb 2H1 defines an epitope which can elicit potentially useful antibodies for host defense and belongs to a class of murine MAbs under development as potential human therapeutic reagents (29). The exact polysaccharide structure recognized by MAb 2H1 has not been determined, but GXM O-acetyl groups appear to be important in binding (4). MAb 2H1 binds to the capsular polysaccharide of the four *C. neoformans* serotypes (4). However, the prevalence of the MAb 2H1 epitope in recent clinical isolates is unknown. Isolates from AIDS patients have been reported to have small nonmucoid capsules (1, 2). There is extensive genetic diversity among strains (3, 25), and functional and structural differences in the capsule have been demonstrated by MAb binding (24), complement activation (27, 28), nuclear magnetic resonance (8), and resistance to phagocytosis (23). Other broadly reactive MAbs to *C. neoformans* GXM bind some strains but not others (24). Therefore, it is important to establish that potential MAb therapeutic reagents bind to a variety of strains, including recent clinical isolates, in preclinical testing.

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J9, J11, J22, SB4, SB6, J14, J15, J20, and J26 are genetically distinct *C. neoformans* var. *neoformans* strains which have been recently recovered from patients (6, 10, 26). For the J9, J11, J22, SB4, and SB6 strains, the A isolate is the initial isolate recovered when the diagnosis of cryptococcal meningitis was made, and the B, C, and D isolates were recovered during clinical relapses (6, 26). For the J11, SB4, and SB6 strains, the initial and relapse isolates were not distinguishable by restriction fragment length polymorphism analysis or electrophoretic karyotyping (26). For the J9 isolate, the restriction fragment length polymorphism analyses with two probes were identical but karyotyping revealed a minor difference between J9A and the relapse isolates (26). For J22, the initial and relapse isolates were not distinguishable by restriction fragment length polymorphism analysis (6). With the exception of J11, all strains were isolated from patients with AIDS. *C. neoformans* var. *gattii* ATCC 24065 (serotype B) and *C. neoformans* var. *neoformans* ATCC 24067 (serotype D), ATCC 34870 (serotype A), ATCC 34873 (serotype D), and ATCC 48183 (serotype A) were obtained from the American Type Culture Collection (Rockville, Md.). MAb 2H1 binding to strains ATCC 24065 and ATCC 24067 was studied previously by enzyme-linked immunosorbent assay (ELISA) (4, 5). *C. neoformans* var. *neoformans* 371 (serotype A) was obtained from J. Bennett (Bethesda, Md.). MAb 2H1 was generated from a BALB/c mouse (4) immunized with a GXM-tetanus toxoid vaccine made from the GXM of strain 371 (11). Strains were maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) at 4°C and grown with moderate shaking at 30°C in Sabouraud dextrose broth (Difco Laboratories).

MAb 2H1 ascites fluid was obtained by paracentesis after intraperitoneal injection of hybridoma cells into pristane-primed mice. Antibody concentrations were determined by ELISA, relative to IgG1 standards of known concentration. The ability of MAb 2H1 to agglutinate *C. neoformans* suspensions was determined by microscopic examination. Briefly, serial dilutions of MAb 2H1 (ranging from 250 to 0.0019 μg/ml) were made in 96-well microtiter polystyrene plates (Corning Glass Works, Corning, N.Y.) in 75 mM NaCl–10 mM sodium phosphate buffer (pH 7.2)–0.5% bovine serum albumin–1 mM azide, 5×10^4 yeast cells were added per well, and the plate was then shaken for 15 min on a shaker (Fisher clinical rotator; Fisher Scientific, Pittsburgh, Pa.) at 100 rpm

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and incubated at 4°C overnight. Although agglutination occurred rapidly, the plates were read after overnight incubation because this produced more reproducible endpoints. The agglutination endpoint was the lowest dilution of MAb 2H1 that agglutinated *C. neoformans* relative to control wells containing no MAb. All measurements were repeated at least four times.

The fluorescence titer was determined by indirect immunofluorescence. Cells from a 24- to 48-h culture of *C. neoformans* were washed twice in distilled water, and 0.1 ml of a 10^7 suspension was placed on polylysine-coated slides (Sigma Chemical, St. Louis, Mo.) and allowed to air dry. The slides were gently heat fixed by rapidly passing them over an open flame. The slides were incubated with 5 to 0.01 μg of MAb 2H1 per ml for 2 h and washed with distilled water. Rhodamine-conjugated goat anti-mouse IgG1 was added to each slide at 8 μg/ml, and the slides were incubated at room temperature in darkness for 40 min, washed, and examined with fluorescence microscopes (Diapshot-TMP [Nikon, Tokyo, Japan] or Axiophot Photomicroscope [Carl Zeiss, Oberkochen, Germany]). The fluorescence endpoint was the lowest concentration of MAb 2H1 at which a fluorescence intensity difference was detected by rhodamine-labelled goat anti-mouse IgG1 relative to yeast cells without MAb 2H1.

Phagocytosis was studied in the murine macrophage-like cell line J774.16 (7). MAb 2H1 has been previously shown to promote phagocytosis and to enhance the activity of J774.16 cells against strain 24067 (20). Phagocytosis assays used MAb purified by protein G (Pierce, Rockford, Ill.) affinity chromatography. J774.16 cells were grown in Dulbecco's modified Eagle medium–10% fetal calf serum–10% NCTC 109 (Gibco), plated at 10^5 cells per well, stimulated with 500 U of gamma interferon per ml, and incubated overnight. Prior to the assay, the supernatant was removed and replaced with fresh medium containing 1,000 U of gamma interferon per ml and 3 μg of lipopolysaccharide per ml. *C. neoformans* cells from a 24-h culture were washed three times in sterile PBS and were added to achieve an approximate yeast cell/macrophage ratio of 20:1. The plates were incubated with various concentrations of MAb 2H1 for 2 h at 37°C, after which the wells were washed several times with PBS, fixed with ice-cold methanol, stained with Giemsa (Sigma) 1:20, and then examined with a microscope. Phagocytosis was measured by determining the phagocytic index, which is the number of ingested and attached yeast cells per macrophage. The vast majority of yeast cells were ingested and could be clearly seen within phagosomes. The murine IgG1 MAb 36-65 to the *p*-azophenylarsonate hapten (22) was used as a negative control in agglutination, fluorescence, and phagocytosis experiments. MAb 36-65 does not bind *C. neoformans* polysaccharide (21).

Agglutination is a classical technique for determining antibody binding and was useful in the preantibiotic era in guiding the choice of type specificity sera for pneumococcal pneumonia (12, 13). MAb 2H1 agglutinated all 21 isolates from 15 strains of *C. neoformans* at concentrations ranging from 31.2 to 1.0 μg/ml (Table 1). Thus, the MAb 2H1 epitope was present in all isolates of *C. neoformans* tested, including genetically diverse strains. The majority of isolates (20 of 21) agglutinated at MAb 2H1 concentrations of 15.6 μg/ml or less. However, agglutination endpoints revealed significant (up to 30-fold) differences in the amount of MAb 2H1 required for agglutination. For the J11, J22, SB4, and SB6 strains, the initial (A) and relapse (B, C, and D) isolates agglutinated at the same MAb 2H1 concentration or within a 1-dilution difference. The J9A strain required a higher concentration of MAb 2H1 for agglutination than the relapse isolates J9B and J9D. The serotype A strains 371, ATCC 34870, and ATCC 48183 differed in their agglutination endpoints, confirming an earlier observation that MAbs can distinguish strains grouped within a serotype (24).

TABLE 1. MAb 2H1 agglutination and fluorescence endpoints for 15 *C. neoformans* strains

Isolate[a]	MAb 2H1 agglutination			MAb 2H1 fluorescence endpoint (μg/ml)[b]
	n	Endpoint (μg/ml)		
		Range	Median	
371[d]	4	31.2	31.2	0.25–0.1
ATCC 34870[d]	4	1.9–3.9	2.0	ND[e]
ATCC 48183[d]	4	1.0	1.0	ND
ATCC 34873[f]	4	7.8	7.8	(0.05)
ATCC 24067[f]	5	1.9–3.9	1.9	0.3, (0.03)
ATCC 24065[g]	4	15.6–31.2	23.4	0.3
J9A	4	15.6	15.6	1.0
J9B	4	1.9–3.9	2.9	0.1
J9D	5	1.0–3.9	1.0	0.2–0.15
J11A	6	7.8–15.6	11.7	0.3
J11B	4	7.8	7.8	0.3–0.25
J22A	5	1.9–3.9	3.9	0.2
J22B	5	3.9–7.8	7.8	0.25–0.20
SB4A	6	7.8–15.6	15.6	0.1
SB4C	5	15.6	15.6	0.1
SB6A	5	7.8–15.6	7.8	0.2
SB6B	4	7.8	7.8	0.25
J15	4	3.9–15.6	3.9	0.25
J17	4	15.6–31.2	15.6	0.25
J20	4	1.9	1.9	0.3
J26	4	15.6–31.2	15.6	0.1

[a] For the J9, J11, J22, SB4, and SB6 strains, the A isolate is the initial isolate recovered when the diagnosis of *C. neoformans* meningitis was first made and the B, C, and D isolates are relapse isolates.
[b] The endpoint in parentheses was measured with an Axiophot Photomicroscope, which differed in sensitivity from the other fluorescent microscope used, the Nikon Diapshot-TMP.
[c] *n*, the number of replications done.
[d] Serotype A strain.
[e] ND, not done.
[f] Serotype D strain.
[g] Serotype B strain.

In contrast to agglutination, the fluorescence endpoints for 18 of 19 isolates were within the narrow range of 0.1 to 0.3 μg/ml. The difference between the ranges of agglutination and fluorescence endpoints may be due to differences in the distribution of the MAb 2H1 epitope in the capsule. Agglutination is caused by antibody cross-linking through the Fab arms and presumably requires epitopes near or at the surface of the capsule. In contrast, fluorescence measures the distribution of the epitope throughout the capsule. No qualitative differences in indirect immunofluorescence were evident for the 21 isolates studied.

Phagocytosis by J774.16 cells in the presence and in the absence of MAb 2H1 was used as a functional measure of the prevalence of this epitope. In the absence of MAb 2H1, there was little or no phagocytosis by the J774.16 cell for 10 strains tested (Table 2). This finding is consistent with previous observations that *C. neoformans* strains are not phagocytosed in the absence of opsonins (15, 16). The addition of MAb 2H1 produced a significant increase in the number of ingested *C. neoformans* yeast cells for all 10 strains tested. There was no obvious correlation between the agglutination and indirect fluorescence endpoints and the phagocytic index. This may reflect differences in the abilities of strains to resist phagocytosis (23). No agglutination, immunofluorescence, or phagocytosis was observed with the negative control IgG1 MAb 36-65.

TABLE 2. Phagocytic indices for *C. neoformans* incubated with J774.16 cells in the presence and in the absence of MAb 2H1

Isolate	Phagocytic index[a] at the following concn of MAb 2H1 (μg/ml):				
	20	10	5	1	0.0
371			0.32	0.02	0.00
ATCC 34870		0.201	0.07	0.04	<0.01
ATCC 34873		0.476	0.21	0.17	<0.01
ATCC 48183		0.402	0.13	0.13	0.00
ATCC 24065	1.26	0.28	0.08	0.08	0.00
J9A	3.1	2.3	0.68	0.25	0.00
J11A			0.63	0.51	0.00
J22A			0.46	0.33	0.00
SB4A			2.01	0.28	0.00
SB4C		0.35	0.33	0.37	<0.01
SB6A			0.55	0.17	0.0

[a] Average number of *C. neoformans* cells per macrophage.

Establishing that MAb 2H1 binds to relapse isolates is important because the MAb 2H1 epitope could be subject to in vivo immune selection. For strain J9, the agglutination and indirect immunofluorescence endpoints suggest quantitative differences in the MAb 2H1 epitope between J9A and relapse isolates J9B and J9D. In contrast to J9, the J11, J22, SB4, and SB6 initial and relapse isolates either had the same agglutination and fluorescence endpoints or differed by, at most, 1 twofold dilution. The MAb 2H1 epitope was present in both initial and relapse isolates.

The results are relevant for the development of antibody-based therapies against *C. neoformans* infections. The occurrence of the MAb 2H1 epitope among genetically distinct strains indicates that this antigenic determinant is prevalent among clinical strains. Since MAb 2H1 was generated using the GXM-tetanus toxoid vaccine, the results suggest that GXM-tetanus toxoid immunization (11) is likely to elicit antibodies to an epitope prevalent in clinical strains. Similarly, the prevalence of the MAb 2H1 epitope suggests that protective antibodies binding to this epitope may be useful for passive therapy.

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