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Production of a Murine Monoclonal Antibody That Recognizes an Epitope Specific to *Coccidioides immitis* Antigen 2

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Antigen 2 (Ag2) has been implicated as a T-cell-reactive component of the pathogenic fungus *Coccidioides immitis*. We report the production of a murine monoclonal antibody (MAb) of the immunoglobulin G2a isotype that recognizes an epitope specific to *C. immitis* Ag2. This specificity was evidenced by the finding that the MAb did not recognize other antigens present in coccidioidin or spherulin and did not show reactivity with antigenic extracts from *Histoplasma capsulatum* or *Blastomyces dermatitidis*. The epitope was labile to enzymatic digestion with pronase but resistant to treatment with glycolytic enzymes and to periodate oxidation. This peptide epitope appears to require conformational structure on the basis that it was not recognized by the MAb in immunoblots of antigen that had been electrophoresed in polyacrylamide gels under denaturing, reducing conditions. Immunoaffinity chromatography of spherulin on columns containing the MAb established that the MAb was effective as a ligand for isolating Ag2 from heterogeneous extracts. The production of a MAb which recognizes an Ag2-specific epitope and its utility as a ligand for isolating Ag2 will provide a valuable reagent for studies of this immunologically important antigen.

Cumulative investigations have implicated antigen 2 (Ag2) as a major T-cell-reactive component of the dimorphic fungus *Coccidioides immitis* (2, 6, 11, 17). This large-molecular-weight antigen is present in the cell walls of mycelia and spherules and can be extracted from the walls, along with the antigen that reacts with the tube precipitin (TP) antibody, by treatment of the walls with alkali (3, 4, 7). The alkali-soluble, water-soluble extract, designated C-ASWS, was shown to be reactive in eliciting cell-mediated immune responses in coccidioidomycosis patients and infected animals (6, 8, 17), in inducing protection against lethal challenge in mice (15), and in detecting the serodiagnostic TP antibody response (7). Since Ag2 has not been purified to homogeneity, its identification as the T-cell-reactive component of C-ASWS has been inferred from the finding that the TP polysaccharide antigen was purified (4) and shown to be nonreactive in eliciting T-cell responses (2). Direct support that Ag2 bears T-cell-reactive epitopes has been obtained in recent studies of a 33-kDa peptide which was isolated from a chemically deglycosylated spherule lysate (11). The 33-kDa peptide showed antigenic identity with the anodal precipitin peak of the Ag2 polymeric precipitinogen in tandem two-dimensional immunoelectrophoresis (2D-IEP) with coccidioidin (CDN) and elicited proliferation responses in mononuclear cells from healthy, skin-test-positive persons.

We report here the development of a murine monoclonal antibody (MAb) of the immunoglobulin G2a (IgG2a) isotype which recognizes a peptide epitope that is specific to Ag2 and present on the Ag2-derived 33-kDa peptide.

MATERIALS AND METHODS

Antigens and antisera. CDN and spherulin (SPH) were prepared as toluene-induced lysates of mycelial- and spherule-phase cells, respectively, of *C. immitis* Silveira (5). The purified TP antigen, the hyperimmune goat antisera to CDN or SPH, and a murine IgG1 MAb to a carbohydrate epitope on the TP antigen were the same preparations used in earlier studies (4, 5, 10). The 33-kDa peptide subunit of Ag2 was obtained by chromatographic fractionation of a spherule lysate that had been chemically deglycosylated by treatment of the lysate with anhydrous hydrogen fluoride as described by Dugger et al. (11). Blastomycin, histoplasmin, rabbit antiblastomycin serum, and rabbit antihistoplasmin serum were purchased from Scott Laboratories, Inc. (Carson, Calif.).

Production of MAb. Splenocytes were isolated from BALB/c mice (Charles River Laboratories, Raleigh, N.C.) which had been immunized by three monthly intramuscular injections of SPH (100 μg) in incomplete adjuvant, followed by an intravenous boost of the antigen (400 μg) 3 days before sacrifice. Hybridomas were produced by the fusion of the splenocytes with SP2/0 Ag14 myeloma cells as detailed previously (10). The hybridomas were cloned twice by limiting dilution and then injected into pristane-primed BALB/c mice for ascites production. A hybridoma producing an irrelevant murine IgG1 MAb was obtained from the American Type Culture Collection (ATCC TIB 8) and similarly injected into pristane-primed mice. The MAbs were purified from the ascites by immunoadsorption on an Affi-Gel protein A MAPS II column (Bio-Rad Laboratories, Richmond, Calif.). Ig isotype was determined by using an enzyme-linked immunosorbent assay (ELISA) purchased from Zymed (South San Francisco, Calif.).

Immunoassays. The ELISA was performed by adsorbing target antigens, diluted in 0.1 M bicarbonate buffer (pH 9.6), onto polystyrene wells (Immunlon II; Dynatech Laboratories, Inc., Alexandria, Va.) overnight at 4°C. The wells were blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (TW) and reacted with MAb, goat anti-CDN serum, rabbit antihistoplasmin serum, or rabbit antiblastomycin serum for 90 min at

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room temperature. Nonreactive components were removed by washing with PBS-TW, and the wells were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG, rabbit anti-goat Ig, or goat anti-rabbit Ig diluted in PBS-TW (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). Enzymatic reactions were developed after a 90-min incubation at room temperature by the addition of substrate (*p*-nitrophenyl phosphate diluted in 10% diethanolamine buffer), and the A_{410} values were read.

Immunoblotting was performed with antigens that had been electrophoresed in polyacrylamide gels under denaturing, reducing or nondenaturing, nonreducing conditions (9). For denaturing, reducing polyacrylamide gel electrophoresis (PAGE), the antigens were diluted in buffer containing 4% sodium dodecyl sulfate (SDS), 4% 2-mercaptoethanol, 0.001% bromophenol blue, 0.05 M Tris hydrochloride, and 10% glycerol, and then heated for 5 min in a boiling water bath. The samples were electrophoresed through a 12% SDS-polyacrylamide gel under reducing conditions and then electrophoretically transferred to nitrocellulose acetate membranes. The membranes were blocked with PBS-TW, and individual lanes were reacted with murine MAb or goat anti-CDN serum for 90 min at room temperature. After removal of nonreactive components, the membranes were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG or rabbit anti-goat Ig, respectively, for 90 min at room temperature. The reactions were developed by the addition of 5-bromo-4-chloro-3-indolylphosphate–Nitro Blue Tetrazolium (Kirkegaard and Perry). Nonreducing, nondenaturing PAGE was performed as described above except that the antigens were diluted in buffer without SDS and 2-mercaptoethanol and were not heat treated before electrophoresis.

For 2D-IEP, CDN or SPH was electrophoresed in the first-dimension gel for 1 h at 10 V/cm. A 7-mm-wide agarose strip containing MAb (as ascites) or, for a control, ascites fluid from pristane-treated BALB/c mice injected with the SP2/0 Ag14 myeloma cell line, was poured parallel to the first-dimension gel. The intermediate gel was allowed to solidify, and agarose containing goat anti-CDN or goat anti-SPH serum was applied to the remainder of the second-dimension gel area. The gel was electrophoresed overnight at 3 V/cm and then stained with Coomassie blue.

Epitope treatments. The effect of enzyme treatments and periodate oxidation on epitope reactivity was evaluated by using a modified ELISA in accordance with published methods (9, 16, 18). For this procedure, CDN was immobilized onto polystyrene wells (500 ng per well) and treated with pronase, mannosidase, glucosidase, galactosidase, endoglycosidase H, *N*-glycanase, neuraminidase, or sodium metaperiodate, all from Sigma Chemical Co. (St. Louis, Mo.). Enzymatic digestion in PBS or Tris buffer was performed according to the manufacturer's directions. After incubation overnight at 37°C, the wells were washed and assayed for reactivity with the MAb by ELISA. Preliminary experiments were done to establish that treatment of CDN with the buffers alone did not alter epitope recognition by the MAb.

Immunoaffinity chromatography. Solid-phase immunoadsorption was performed on columns containing MAbs covalently coupled to Sepharose 4B. Coupling was performed by adding 28 mg of the MAPS II-purified MAbs dissolved in 0.1 M bicarbonate buffer (pH 8.3) containing 0.5 M NaCl to 5 g of cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The suspensions were tumbled overnight at 4°C and then washed by centrifugation in bicarbonate buffer. Nonreactive groups were blocked with 0.2 M glycine, and the coupled gels were washed three times with alternating cycles of 0.1 M acetate buffer (pH 4.0) and 0.1 M borate buffer (pH 8.0), each containing 1 M NaCl. The immunosorbent gels were transferred to columns (2 by 10 cm) and equilibrated with PBS. SPH (5 mg) diluted to 1 mg of Tris-buffered saline (pH 8.6) per ml was eluted over the columns at a descending flow rate of 12 ml/h. The column effluents were collected until the A_{280} returned to the base line, at which time the eluates were eluted in 0.01 M acetic acid (pH 2.3). The solid-phase immunosorbent effluent and eluate fractions were collected, dialyzed against distilled water, and lyophilized. The lyophilized preparations were redissolved in the original volume of SPH applied to the columns so that the reactivity of the fractions in an ELISA with anti-Ag2 MAb could be compared directly with that of the untreated antigen. To exclude any deleterious effects of the desorbing reagent, a 5-mg aliquot of SPH was diluted in glycine-HCl buffer, and, after dialysis and lyophilization, the sham-treated antigen was assayed for reactivity by the ELISA.

RESULTS

Five IgG-secreting hybridomas were produced, but only one secreted a MAb that showed reactivity with Ag2. The MAb was the IgG2a isotype, and although it did not precipitate Ag2, its reactivity was demonstrable by intermediate gel 2D-IEP. Incorporation of the MAb in a gel interposed between the first-dimensional electrophoresis of CDN and a second-dimensional gel containing goat anti-CDN serum effected the in situ adsorption of Ag2 (compare Fig. 1A and B). An identical pattern of reactivity was demonstrable when the MAb was interposed in a gel between SPH and goat anti-SPH serum (results not shown). None of the other precipitin peaks in CDN or SPH were affected.

We initially observed that the MAb was only weakly reactive in an ELISA with coccidioidal antigens, an anomalous result considering that the antibody effected the in situ adsorption of Ag2 in intermediate gel 2D-IEP. Since the 2D-IEP procedure is performed at pH 8.6, i.e., the isoelectric point of most Igs, the incubation phase of the MAb with antigen-coated wells was performed in PBS-TW buffer at pH 8.6. As shown in Table 1, the MAb was reactive with CDN, SPH, and the Ag2-derived 33-kDa peptide. The MAb did not recognize the purified TP antigen when assayed in a buffer at either pH 8.6 or 7.2. For a comparison, the target antigens were also assayed with a murine anti-TP MAb and with hyperimmune goat anti-CDN serum. The anti-TP MAb recognized CDN, SPH, and the TP antigen but not the 33-kDa antigen. Polyvalent goat anti-CDN serum showed strong reactivity with all four target antigens.

Immunoblot analyses of CDN and the 33-kDa peptide, using goat anti-CDN serum and the anti-Ag2 MAb as probes, are depicted in Fig. 2. Goat anti-CDN detected multiple bands in blots of CDN that had been electrophoresed in PAGE under denaturing, reducing conditions (Fig. 2A). None of these were recognized by the anti-Ag2 MAb. When used to probe blots of CDN performed under nondenaturing conditions, the MAb showed weak but discernible reactivity, with a diffuse band present at the top of the gel (Fig. 2A). Immunoblots of the 33-kDa peptide electrophoresed under denaturing conditions yielded two bands, having molecular sizes of 33 and 59 kDa, in blots probed with goat anti-CDN serum (Fig. 2B). Neither of these bands was detected by the anti-Ag2 MAb. By contrast, nondenaturing blots of the 33-kDa peptide yielded three strongly reactive bands and a

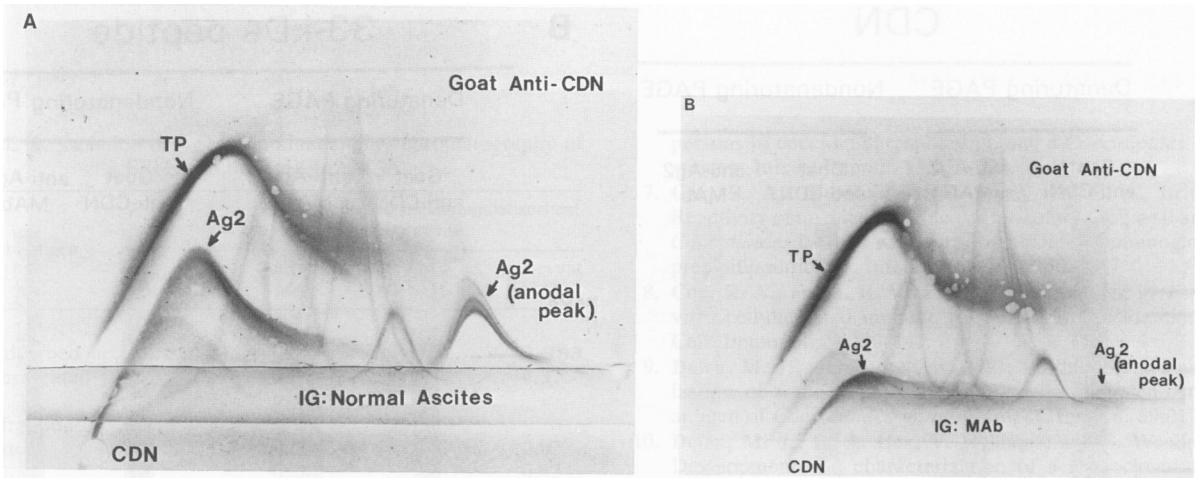

FIG. 1. Intermediate gel 2D-IEP of CDN versus goat anti-CDN serum with control ascites prepared by injecting pristane-primed BALB/c mice with the SP2/0 myeloma cell line (A) or ascites containing the MAb-secreting hybridoma produced in a fusion of splenocytes from SPH-immunized BALB/c mice with the SP2/0 Ag14 myeloma cell line (B).

fourth band that was weakly reactive with the MAb. All four bands were detected in nondenaturing blots that were probed with goat anti-CDN serum. It is noteworthy that the bands detected in the denaturing and nondenaturing blots of the 33-kDa peptide were not well delineated but rather were characterized by a diffuse pattern of reactivity, suggesting polymerization of the peptide.

Further experiments showed that the loss of epitope recognition in denaturing, reducing PAGE was not attributed to heat treatment alone or to treatment with SDS and 2-ME alone but rather resulted from the combined effects of these procedures (data not shown), indicating that the epitope requires conformational structure. To further characterize the epitope recognized by the MAb, CDN was immobilized onto polystyrene wells, treated with enzymes or sodium metaperiodate, and then assayed for reactivity with the anti-Ag2 MAb. As shown in Table 2, pronase treatment of CDN effected a 47% reduction in the reactivity of the epitope recognized by the MAb, whereas periodate oxidation had no effect. Glucosidase and galactosidase treatment resulted in a slight reduction of epitope reactivity, but this was not considered to be significant since the differences in the A_{410} values of the enzyme- and buffer-treated controls were ≤ 0.06. The susceptibility of the epitope to enzymatic digestion with pronase and its resistance to periodate oxidation were confirmed by treating CDN that was dissolved in buffer (as opposed to being immobilized) and then, after dialysis and lyophilization, evaluating the reactivity of the antigen in an ELISA. Pronase treatment resulted in a 54% reduction in epitope recognition, whereas periodate oxidation effected a reduction of less than 14%.

To determine whether the epitope recognized by the MAb was specific to *C. immitis* or common to antigens of *Histoplasma capsulatum* or *Blastomyces dermatitidis*, the MAb was evaluated for reactivity in an ELISA using histoplasmin and blastomycin. As shown in Table 3, neither of these heterologous extracts was reactive with the anti-Ag2 MAb.

The efficacy of the MAb as a ligand for isolating Ag2 was examined by eluting SPH over a solid-phase immunosorbent containing the anti-Ag2 MAb or, for a comparative control, an irrelevant IgG1 MAb (Table 4). Quantitative comparisons of the reactivity of the solid-phase immunosorbent fractions and the nonadsorbed antigen with the anti-Ag2 MAb in the ELISA established that the anti-Ag2 MAb effected the adsorption of approximately 70% of Ag2. By contrast, the SPIA eluate from the immunosorbent prepared with an irrelevant MAb was void of Ag2.

TABLE 1. Reactivity of anti-Ag2 MAb with *C. immitis* antigens by ELISA

Antibody probe[a]	A_{410} of target antigen[b]			
	CDN	SPH	33-kDa peptide	TP
Anti-Ag2 MAb	0.34	0.35	1.78	0
Anti-TP MAb	0.60	0.95	0	0.38
Goat anti-CDN serum	2.14	2.15	2.37	0.79

[a] The MAbs were used at a concentration of 10 μg of MAPS II-purified antibody per ml; goat anti-CDN serum was used at a 1:10,000 dilution. Incubation of the primary antibodies was performed in PBS-TW at pH 8.6 for the anti-Ag2 MAb and at pH 7.2 for the anti-TP MAb and goat anti-CDN serum.

[b] Wells were coated with 0.1 μg of CDN or SPH, 0.02 μg of the 33-kDa peptide, or 1 μg of the purified TP antigen.

DISCUSSION

MAbs have provided valuable tools for use in the isolation and characterization of immunoreactive antigens (13). We report here the development of a murine IgG2a MAb which recognizes a conformational peptide epitope that is specific to Ag2 and an Ag2-derived peptide and, when used as a ligand in immunoaffinity chromatography, was effective in isolating Ag2 from a crude, heterogeneous extract of *C. immitis*.

Previous studies have established that Ag2 bears carbohydrate epitopes that are common to those on the TP antigen (3, 4, 10). Thus, attempts to produce polyclonal antisera specific for Ag2 have yielded antibodies reactive with both Ag2 and the TP antigen, even when the precipitin arcs obtained in 2D-IEP of CDN or C-ASWS against homologous goat antisera were used for immunization (2). Ag2 has also been reported to bear epitopes that are common to heterologous fungi, notably *H. capsulatum* and *B. dermatitidis* (1,

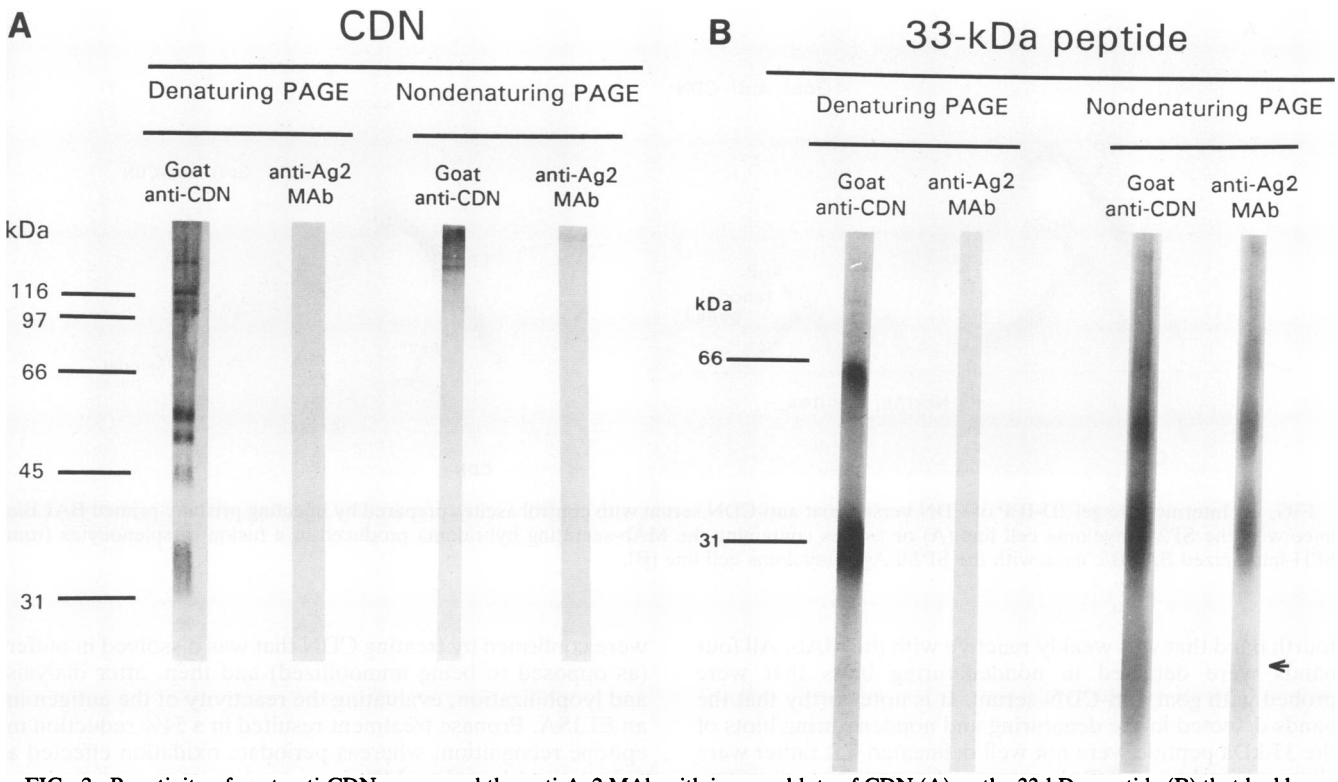

FIG. 2. Reactivity of goat anti-CDN serum and the anti-Ag2 MAb with immunoblots of CDN (A) or the 33-kDa peptide (B) that had been electrophoresed under either denaturing, reducing or nondenaturing, nonreducing conditions. Molecular size markers are shown to the left of the gels. The arrow (B) denotes a weak but discernible band detected by the anti-Ag2 MAb.

14). Although the deglycosylation procedure used to isolate the 33-kDa peptide is effective in removing carbohydrate epitopes that may be common to the TP antigen (11), the peptide showed reactivity in an ELISA with serum from a histoplasmosis patient (12), indicating the presence of an epitope which is shared by *H. capsulatum*. The identification of an Ag2-specific epitope in this investigation and the production of a MAb that recognizes the epitope will enable studies to develop recombinant or synthetic peptides that bear the Ag2-specific epitope.

The loss of epitope recognition in blots of CDN or the 33-kDa peptide that had been subjected to electrophoresis under denaturing, reducing conditions provides evidence that the epitope requires a conformational structure. A conformational epitope might account for our paradoxical finding that the MAb is only weakly reactive with C-ASWS (results not shown). That is, we reported previously that the alkali extraction procedure used to isolate C-ASWS may have effected a partial denaturation of Ag2 as suggested by an immunoelectrophoretic profile of the polymer in 2D-IEP (3). Recognition of an epitope having conformational structure might also account for the finding that the MAb was optimally reactive when assayed in a buffer at a pH of 8.6 as opposed to a pH of 7.2 to 7.6.

Solid-phase immunoadsorption of SPH on columns containing the anti-Ag2 MAb established that the MAb is an effective ligand for isolating Ag2 directly from a heterogeneous extract. This method can be used to purify large quantities of the Ag2, in its native form, for use in the development of immunologic assays and for studies of host-fungus interaction in coccidioidomycosis.

TABLE 2. Effect of enzyme treatments and periodate oxidation on epitope recognition by MAb[a]

Enzyme treatment (amt)	A_{410} by ELISA	% Inhibition
None	0.34	
Pronase (2 U)	0.18	47
Periodate (0.2 M)	0.42	0
Mannosidase (0.1 U)	0.33	4
Glucosidase (0.5 U)	0.30	12
Galactosidase (0.1 U)	0.28	19
Endoglycosidase H (0.01 U)	0.33	3
N-glycanase (0.15 U)	0.32	6
Neuramidase (1 mU)	0.38	0

[a] CDN was adsorbed onto polystyrene wells (500 ng of antigen per well), and, after being washed with PBS-TW, the wells were incubated with enzyme or sodium metaperiodate at 37°C for 18 to 24 h and then evaluated for reactivity with the anti-Ag2 MAb.

TABLE 3. Lack of reactivity of anti-Ag2 MAb with histoplasmin and blastomycin[a]

Antibody probe	A_{410} of target antigen		
	CDN	Histoplasmin	Blastomycin
Anti-Ag2 MAb	1.34	0.01	0.01
Rabbit antihistoplasmin serum	0.57	0.59	0.41
Rabbit antiblastomycin serum	0.60	0.30	0.26

[a] Wells were coated with 0.1 μg of CDN or a 1:1,000 dilution of histoplasmin or blastomycin. The anti-Ag2 MAb was used at a concentration of 10 μg/ml; rabbit antihistoplasmin and antiblastomycin sera were each diluted 1:1,000.

TABLE 4. Isolation of Ag2 by immunoaffinity chromatography of SPH with the anti-Ag2 MAb

Target antigen	Antibody ligand for immunoadsorbent columns	
	Anti-Ag2 MAb	Irrelevant MAb
SPH		
Unadsorbed	0.88[a]	0.40
Sham treated	0.85	0.37
SPIA fraction		
Eluate	0.60	0.02
Effluent	0.24	0.42

[a] Values (A_{410}) were determined by an ELISA with the anti-Ag2 MAb as a probe.

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