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Production and Characterization of Monoclonal Antibodies to a 58-Kilodalton Antigen of *Aspergillus fumigatus*

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Eight monoclonal antibodies that recognize a serodiagnostically important 58-kDa antigen of *Aspergillus fumigatus* were produced and partially characterized. 2-7, 2-12, and 2-14 are of the immunoglobulin M class, and 2-2-1, 2-2-4, 2-2-6, 2-2-9, and 2-2-13 are all immunoglobulin G1(κ) antibodies. Immunoblot analysis with *A. fumigatus* mycelial extract demonstrated that all of the monoclonal antibodies recognize a major 58-kDa antigen. The antigen was also detected by immunoblot analysis of 4- and 7-day culture filtrate preparations. 2-2-1, 2-2-4, and 2-2-6 cross-reacted with an antigen of approximately 55 kDa from an extract of *Candida albicans*. 2-7, 2-12, 2-14, and 2-2-4 formed a precipitin band with immunoaffinity-purified 58-kDa antigen by immunodiffusion. Results from indirect immunofluorescence assays with 2-7 and 2-2-9 showed fluorescent staining mainly on the surfaces of conidia and hyphae, indicating that the 58-kDa antigen may be cell wall associated. 2-2-9 and 2-2-13 and antibodies in patient and immune rabbit sera precipitated the [^{35}S]methionine-labeled 58-kDa antigen. The 58-kDa antigen immunoprecipitated by each of the antibodies was enzymatically cleaved by *Staphylococcus aureus* V8 protease; one cleavage product, a 35-kDa fragment, was generated, indicating that the precipitated antigens share primary structure. Immunoblot analysis with an immunoaffinity-purified 58-kDa antigen showed that sera from patients with invasive aspergillosis reacted with the same antigen as that recognized by the monoclonal antibodies.

Since their development, monoclonal antibodies (MAbs) have served as useful research tools and have dramatically improved the specificity of immune procedures. Some of the many applications of MAbs have included immunochemical characterization (3, 7) and purification (4, 12, 14) of bacterial, fungal, or viral antigens, localization of viral and fungal glycoproteins (1, 9), and development of antibody (14) and antigen (6, 11) detection assays. Invasive aspergillosis is a serious problem in individuals with compromised host defenses, and presently there are no assays or reagents available for diagnostic applications. The production of MAbs to clinically important *Aspergillus* antigens would greatly facilitate the purification of these antigens and allow the development of an immunoassay with increased sensitivity and specificity. A 58-kDa *Aspergillus fumigatus* antigen recognized by antibodies in the sera of patients with invasive aspergillosis has been identified (5). In this report we describe the production and partial characterization of eight MAbs directed against the immunodominant 58-kDa antigen. Two MAbs, an immunoglobulin M (IgM) and an IgG1(κ), were used to locate this antigen on the organism; another, an IgG1(κ), was used to purify it.

MATERIALS AND METHODS

Organism and culture conditions. *A. fumigatus* F92 was obtained from the culture collection of the Department of Microbiology, The Thomas Jefferson University, Philadelphia, Pa. It was isolated from a sputum specimen from a patient with an aspergilloma. The organism was grown on Sabouraud plates for 5 to 10 days at 25°C. Conidia were harvested from mycelia by washing the plates with sterile 0.5% Tween 80. The suspension was inoculated into Czapek-Dox broth (Difco Laboratories, Detroit, Mich.) (approximately 8×10^6 conidia per ml of broth) and incubated for 4 days at 37°C on a gyratory shaker.

Preparation of MAbs. (i) Antigen preparation. The mycelial portion was recovered and was washed with phosphate-buffered saline (PBS; 0.05 M sodium phosphate containing 0.8% [wt/vol] NaCl) (pH 7.4). The mycelial fragments in PBS (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride were mechanically disrupted with glass beads (diameter, 0.25 mm) in a Braun homogenizer (B. Braun, San Francisco, Calif.). Cellular debris was removed by centrifugation at 17,000 × *g* for 20 min. The supernatant was centrifuged again (80,000 × *g*, 90 min, 4°C) and filtered through Nalgene (Nalge Co., Rochester, N.Y.) 0.45-μm-pore-size filter units. Protein was measured by the method of Lowry et al. (13). A portion of the mycelial extract (ME) was passed through concanavalin A-Sepharose (Pharmacia, Uppsala, Sweden) affinity columns. The equilibration buffer was 0.05 M Tris hydrochloride (pH 7.4) with 0.02% (wt/vol) NaN_3, 0.001 M $MnCl_2 \cdot 4H_2O$, 0.001 M $MgCl_2 \cdot 6H_2O$, and 0.001 M $CaCl_2 \cdot 2H_2O$. The unbound fraction was collected, and the column was washed with equilibration buffer. The bound fraction (BF) was eluted from the column with 0.4 M α-methyl-D-mannoside (Sigma Chemical Co., St. Louis, Mo.) in equilibration buffer. The BF was dialyzed overnight and then concentrated by using Centricell ultrafilter units (Polysciences, Inc., Warrington, Pa.) with a molecular weight cutoff of 10,000. Components in the BF were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (9). The 58-kDa antigen present in the concanavalin A BF was purified by cutting out the band from gels and then electroeluting them from the gel pieces.

(ii) Immunization. Two groups of BALB/c mice (three mice per group) were immunized with either ME or purified 58-kDa antigen. Mice immunized against ME proteins received 200 μg of protein subcutaneously in RIBI MPL + TDM emulsion (RIBI ImmunoChem Research, Inc., Hamil-

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ton, Mont.), and those immunized against the 58-kDa antigen received 60 μg of protein subcutaneously in RIBI. Both sets of mice received three booster injections; the last injection was administered 4 days before the fusions were performed.

(iii) Fusion and screening. Cell fusion was performed by a modification of the procedure of Strockbine et al. (17). The method was modified by substituting a spleen cell feeder layer with a supernatant from a lipopolysaccharide-induced mouse monocyte-macrophage cell line, RAW 264.7 (ATCC TIB 71) (16).

The cell culture supernatants were screened by enzyme-linked immunosorbent assay (ELISA) for MAbs to *Aspergillus* antigens. Dynatech Immunolon I ELISA plates (Dynatech Laboratories, Inc., Alexandria, Va.) were coated overnight at 4°C with 100 μl of a solution of 25 μg of ME protein per ml diluted in 0.015 M carbonate-0.035 M bicarbonate buffer (pH 9.2). The second antibody was either peroxidase-labeled goat anti-mouse IgG, IgA, or IgM (H+L chains) (0.1 mg/ml) or peroxidase-labeled goat anti-γ chain (Kirkegaard and Perry Laboratories, Gaithersburg, Md.). The substrate (200 μl) was 0.4 M 2,2′-azino-di-(3-ethylbenzthiazoline sulfonic acid) in 0.05 M citrate buffer containing 0.05% (vol/vol) hydrogen peroxide. Positive hybrids were subcloned twice by limiting dilution and retested by ELISA for antibody production. Immunoglobulin isotypes were determined by ELISA with class-, subclass- and light chain-specific antisera (Fisher Scientific, Orangeburg, N.Y.).

(iv) MAb production and purification. Ascites fluid was produced by injecting 10^6 hybridoma cells intraperitoneally into pristane-primed BALB/c mice. Ascites fluid was collected and clarified by centrifugation. Immunoglobulins were precipitated with 50% saturated ammonium sulfate [$(NH_4)_2SO_4$] (pH 7.8) and then dialyzed against PBS (pH 7.4). The final solution was centrifuged at 4°C for 30 min at 3,000 rpm, and the supernatant was stored at −70°C.

Characterization of MAbs. (i) Western immunoblot analysis. Components of the ME, samples of *A. fumigatus* culture filtrates, immunoaffinity-purified 58-kDa antigen, and a *Candida albicans* extract were separated by electrophoresis on 12.6% SDS–polyacrylamide gels (10). The separated proteins were electrophoretically transferred to nitrocellulose paper (Hoefer Scientific Instruments, San Francisco, Calif.) by the method Towbin et al. (18). Gels (0.75 mm thick) were transferred for 35 min at 0.5 A in a Transphor transfer cell (model TE 52; Hoefer). After transfer, the nitrocellulose paper was blocked with 5% (wt/vol) nonfat dry milk in PBS. It was incubated overnight with mouse ascites fluid or human serum, both diluted 1:250 in PBS containing 2% normal goat serum. The second antibody was either goat anti-mouse IgG, IgA, or IgM (H+L chains) or goat anti-human IgG, IgA, or IgM (H+L) conjugated to alkaline phosphatase (Kirkegaard and Perry). The blots were developed with a 5-bromo-4-chloro-3-indolyl-phosphate–Nitro Blue Tetrazolium substrate.

(ii) Culture filtrate preparations. Czapek-Dox medium was inoculated with conidia, and the cultures were incubated for either 4, 7, or 14 days at 37°C. The cultures were harvested, and the culture filtrate broth was separated from the mycelia by filtration. The filtrates were dialyzed overnight against 0.001 M NH_4HCO_3 and concentrated with polyethylene glycol (PEG 8000).

(iii) Immunodiffusion. Samples of 5 ml of 1% (wt/vol) Noble agar (Difco) in 0.05 M sodium borate buffer (pH 8.6) were placed in 50- by 9-mm plastic petri dishes (no. 1006; Falcon Plastics, Oxnard, Calif.). The center well cut into the agar contained 6 μg (protein) of 58-kDa antigen, and the surrounding wells contained 30 μl of ascites fluid. The dishes were incubated for 96 h at room temperature in a humid chamber, and bands were visualized after they were washed with PBS and stained with 0.1% (wt/vol) amido black.

(iv) Immunofluorescence. Czapek-Dox medium was inoculated with *A. fumigatus* conidia, and the culture was incubated for 8 h, 24 h, or 4 days at 37°C on a gyratory shaker. Hyphae and germinating conidia were dried onto microscope slides and fixed by immersing into 95% ice-cold ethanol for 10 min. The slides were incubated for 30 min at room temperature either with MAb 2-7 or 2-2-9 or with an MAb directed against a heterologous antigen (*Streptococcus faecium* cell wall antigen). The slides were rinsed in slide dishes two times for 10 min with PBS. Samples of 1 ml of a 1:20 dilution of fluorescein isothiocyanate-labeled goat anti-mouse IgG-IgM (H+L) (0.5 mg/ml; Kirkegaard and Perry) in PBS containing 1% normal goat serum were placed on the slides, and the slides were incubated in the dark for 30 min at room temperature. They were rinsed in slide dishes several times and blotted dry. The mounting medium contained 2 ml glycerol to 1 ml of PBS, and 3% (wt/vol) *n*-propyl gallate (Sigma) was added to prevent quenching. Photographs were taken with Kodak Ektachrome film (ASA 400) on a Nikon Optiphot microscope equipped with a Nikon FX-35A camera.

Immunoprecipitation. Immunoprecipitation was performed by a modification of the method of Kessler (8). The washing and dilution buffer was RIPA (0.01 M Tris hydrochloride [pH 7.4], 0.5 M NaCl, 0.001 M EDTA, 1% [wt/vol] SDS, 0.02% [wt/vol] NaN_3, 1% [wt/vol] Triton X-100, 1% [wt/vol] sodium deoxycholate). ^{35}S-labeled ME was precleared with protein A-Sepharose beads (Pharmacia) prepared in 25 mM Tris hydrochloride (pH 7.5) containing 0.02 M NaCl and 0.01% (wt/vol) NaN_3. Then 50 μl of mouse ascites fluid, 30 μl of human serum, 20 μl of rabbit serum, or 30 μl of normal human serum was added to precleared labeled antigen (5×10^5 trichloroacetic acid-precipitable cpm). A second antibody (rabbit anti-mouse immunoglobulin; Accurate Chemical and Scientific Corp., Westbury, N.Y.) (8 μl) was added to the tubes containing mouse antibody. All tubes were incubated on a rotator for 18 h at 4°C. This was followed by the addition of 100 μl of protein A-Sepharose and incubation for 2 h at 4°C. The mixture was placed through Quik Sep columns with plastic filter disks (Isolab, Inc., Akron, Oh.), and the beads in each column were washed extensively with washing buffer. To release antigen-antibody complexes from the beads, SDS sample buffer (10) was placed in each tube; after a 15-min incubation, it was collected. After elution from the beads, the immunoprecipitated products were completely dissociated by immersing the samples in a boiling water bath for 6 min. The samples were then applied to 12.6% SDS–polyacrylamide gels, and the components were separated. The gels were fixed and stained with 1% (wt/vol) Coomassie blue R-250. After treatment with En^{3}Hance autoradiography enhancer (New England Nuclear, Boston, Mass.), the gels were dried and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.).

Limited proteolytic digestion. The 58-kDa antigen identified by immunoprecipitation was cleaved by partial enzymatic proteolysis by the method of Cleveland et al. (2).

Purification of 58-kDa antigen and immunoblot analysis. The 58-kDa antigen was purified by immunoaffinity chromatography. 2-2-9, an IgG1(κ) MAb directed against the 58-kDa antigen, was purified from mouse ascites fluid with the

TABLE 1. Characterization of MAbs to *A. fumigatus*[a]

Cell line	Isotype	Immunoblot analysis			Immunodiffusion	Immunofluorescence
		A. fumigatus ME	*A. fumigatus* CF	*C. albicans* extract		
2-7	IgM	Major band (58 kDa)	35 to 94 kDa	No bands	PB	Positive
2-12	IgM	Major band (58 kDa)	NT	No bands	PB	NT
2-14	IgM	Major band (58 kDa)	35 to 94 kDa	No bands	PB	NT
2-2-1	IgG1(κ)	Major band (58 kDa)	Major band (58 kDa)	55 kDa	Negative	NT
2-2-4	IgG1(κ)	Major band (58 kDa)	58 to 94 kDa	55 kDa	PB	NT
2-2-6	IgG1(κ)	Major band (58 kDa)	Major band (58 kDa)	55 kDa	Negative	NT
2-2-9	IgG1(κ)	Major band (58 kDa)	Major band (58 kDa)	No bands	Negative	Positive
2-2-13	IgG1(κ)	Major band (58 kDa)	Major band (58 kDa)	No bands	Negative	NT

[a] PB, Precipitin band; NT, not tested.

Affi-Gel Protein A MAPS II Kit (Bio-Rad Laboratories, Richmond, Calif.). Purified MAb was then coupled to Affi-Gel 10 (Bio-Rad) in 0.1 M $NaHCO_3$ buffer (pH 8.5), and a column was prepared. After equilibration with 0.05 M $NaHCO_3$ buffer (pH 8.0), the concanavalin A BF was passed through the column. After the column was washed with equilibration buffer to remove unbound material, 0.1 M glycine (pH 2.5) was passed through the column to collect the bound fraction. The bound material was concentrated by using Centricell ultrafilter units (molecular weight cutoff, 10,000), dialyzed against distilled water, and stored at −70°C.

Reagents. (i) Rabbit serum. Hyperimmune rabbit serum against *A. fumigatus* ME was raised by three subcutaneous injections of ME (0.7 to 1.0 mg of protein) in incomplete Freund adjuvant.

(ii) Human sera. The human sera were provided by George Talbot, Hospital of the University of Pennsylvania, Philadelphia, William Merz, Johns Hopkins University Hospital, Baltimore, Md., and Thomas Walsh, National Cancer Institute, Bethesda, Md.

Serum JH-7 came from a patient diagnosed as having myelodysplastic syndrome. A Hickman catheter became infected with *Aspergillus flavus*, and the organism eroded into the lung. Serum W-9 was from a neutropenic cancer patient who developed invasive pulmonary aspergillosis due to *A. fumigatus*. Serum P3-2 was from a patient with a chest tube graft infection due to *A. fumigatus*, and serum P31 was from a patient with acute leukemia who developed invasive pulmonary aspergillosis due to *A. flavus*. The normal human serum came from healthy, nonhospitalized men and women.

RESULTS

MAb isolation and characterization. From the first fusion, with mice immunized against the purified 58-kDa antigen, 50% (380 of 760) of the wells contained hybrid cells. Then 144 hybrids, which when tested by ELISA for production of antibodies to *Aspergillus* species gave the highest absorbance values, were transferred, and the supernatants were screened again by ELISA. Cells in 22 wells containing positive hybrids (confirmed by ELISA) were cloned by limiting dilution, and one clone was selected from each of 15 subcloned hybrid cell lines on the basis of reactivity by ELISA and immunoblotting. Ascites fluid was produced with five clones that recognized the 58-kDa antigen by immunoblotting; three of these clones, 2-7, 2-12, and 2-14, were selected for further characterization. All three secreted antibodies of the IgM class (Table 1).

The same screening procedure was used in selecting clones produced from the second fusion. From the fusion with mice immunized against ME, approximately 95% (722 of 760) of the wells contained hybrid cells. The 278 hybrids that produced the highest absorbance values by ELISA were retested, and 90 hybrids were selected. These were tested again by ELISA with goat anti-mouse γ chain as the second antibody to select only IgG-producing clones. Thirty-four hybrid cell lines were cloned by limiting dilution, and one clone was selected from each of the subcloned hybridomas. Ascites was produced with five of the clones that reacted with the 58-kDa antigen by immunoblotting. The MAbs produced were 2-2-1, 2-2-4, 2-2-6, 2-2-9, and 2-2-13, and they secreted antibodies of the IgG1(κ) class.

The immunoblots shown in Fig. 1 demonstrate that all of the antibodies react with an ME antigen, forming a broad band at 58 kDa. In the blots reacted with 2-7, 2-12, and 2-14 (Fig. 1, lanes 1 through 3), some higher-molecular-weight material is also detected, but no distinct bands are visible. In the blots reacted with the IgG MAbs (lanes 4 through 8), a band greater than 94 kDa is also recognized. As a negative control, blot 9 was incubated with an MAb [IgG1(κ)] against an antigen of *C. albicans*. No bands are visible on the immunoblot.

When culture filtrates (CFs) of *A. fumigatus* were analyzed by immunoblotting, a 58-kDa antigen was recognized by all of the MAbs. MAb 2-2-9 reacts with an antigen in the CF that forms a broad band at 58 kDa (Fig. 2). The antigen

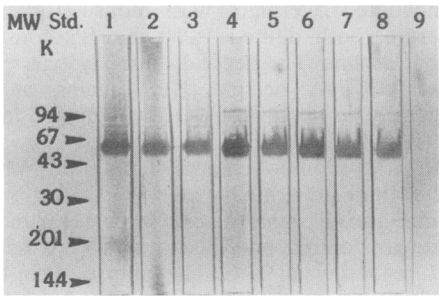

FIG. 1. Representative immunoblots of *A. fumigatus* ME incubated with MAbs against the 58-kDa antigen. Samples of 40 μg of ME protein were applied to lanes of a 12.6% SDS gel, and the separated components were transferred to nitrocellulose paper. Blots were incubated with the following: 1, 2-7; 2, 2-12; 3, 2-14; 4, 2-2-1; 5, 2-2-4; 6, 2-2-6; 7, 2-2-9; 8, 2-2-13; 9, MAb [IgG1(κ) subclass] against an antigen of *C. albicans* (16). MW Std., Molecular weight standards.

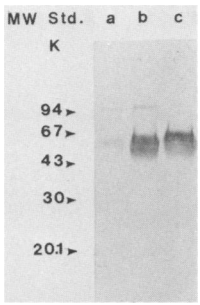

FIG. 2. Immunoblot analysis of *A. fumigatus* CF antigens. Samples of 30 μg of CF protein were applied to 12.6% SDS gels. After electrophoresis, the CF components were transferred to nitrocellulose paper. The blot was incubated with MAb 2-2-9. The lanes marked a, b, and c contained CF components recovered from cultures grown for 14, 7, and 4 days, respectively. MW Std., Molecular weight standards.

is present in both 4- and 7-day CFs; however, by 14 days only a faint band was visible on the blot (Fig. 2, lane a). At 14 days it is likely that *Aspergillus* proteases in the CF had digested the antigen. The other MAbs also recognized the 58-kDa antigen in the CFs. 2-2-4 reacted strongly with an antigen greater than 94 kDa, and there was diffuse staining above the 58-kDa band (Table 1). 2-7 and 2-14 reacted with material ranging in molecular weight from 40,000 to greater than 94,000 and reacted with bands at approximately 35 and 37 kDa when the 14-day CF was used. Since candidiasis and aspergillosis are the two most common fungal infections affecting immunocompromised individuals, the MAbs were also reacted by immunoblotting with antigens present in an extract of *C. albicans*. 2-2-1, 2-2-4, and 2-2-6 reacted with an antigen of *C. albicans* of approximately 55 kDa (Table 1). No bands were detected on the blots after incubation with 2-7, 2-12, 2-14, 2-2-9, and 2-2-13.

MAbs were tested by double immunodiffusion for reactivity against immunoaffinity-purified 58-kDa antigen. Antibodies 2-7, 2-12, 2-14, and 2-2-4 reacted with the purified 58-kDa antigen to form a precipitin band. An indirect immunofluorescence assay was performed with MAbs 2-7 and 2-2-9 to determine whether the 58-kDa antigen was cell wall associated. There was cell surface immunofluorescent staining with *A. fumigatus* cultured for 8 h, 24 h, and 4 days. Figure 3 shows results obtained with *A. fumigatus* cultured for 8 h (Fig. 3A), when the conidia were beginning to germinate, and with the organism grown for 24 h (Fig. 3B), when the culture was almost 100% hyphae. Both were incubated with MAb 2-7.

Immunoprecipitation with human and rabbit antibodies and MAbs. Immunoprecipitation was used to compare the antigens recognized by patient serum, serum from a rabbit immunized against ME, and MAbs 2-2-9 and 2-2-13. The autoradiograph of an SDS-polyacrylamide gel containing the labeled ME (Fig. 4, lane 1) shows that the 58-kDa antigen was a predominant component in the antigen preparation. Immune rabbit serum, the two patient sera, and the MAbs all recognized an antigen forming a broad band at 58 kDa. Polyclonal rabbit serum precipitated several components besides the 58-kDa antigen of molecular weights of approximately 74,000, 80,000, 38,000, and 40,000. Normal rabbit serum did not precipitate the 58-kDa antigen in any appreciable amount (data not shown). The 38-kDa component was also precipitated by P3-2 (Fig. 4, lane 3), 2-2-9 (lane 6), and

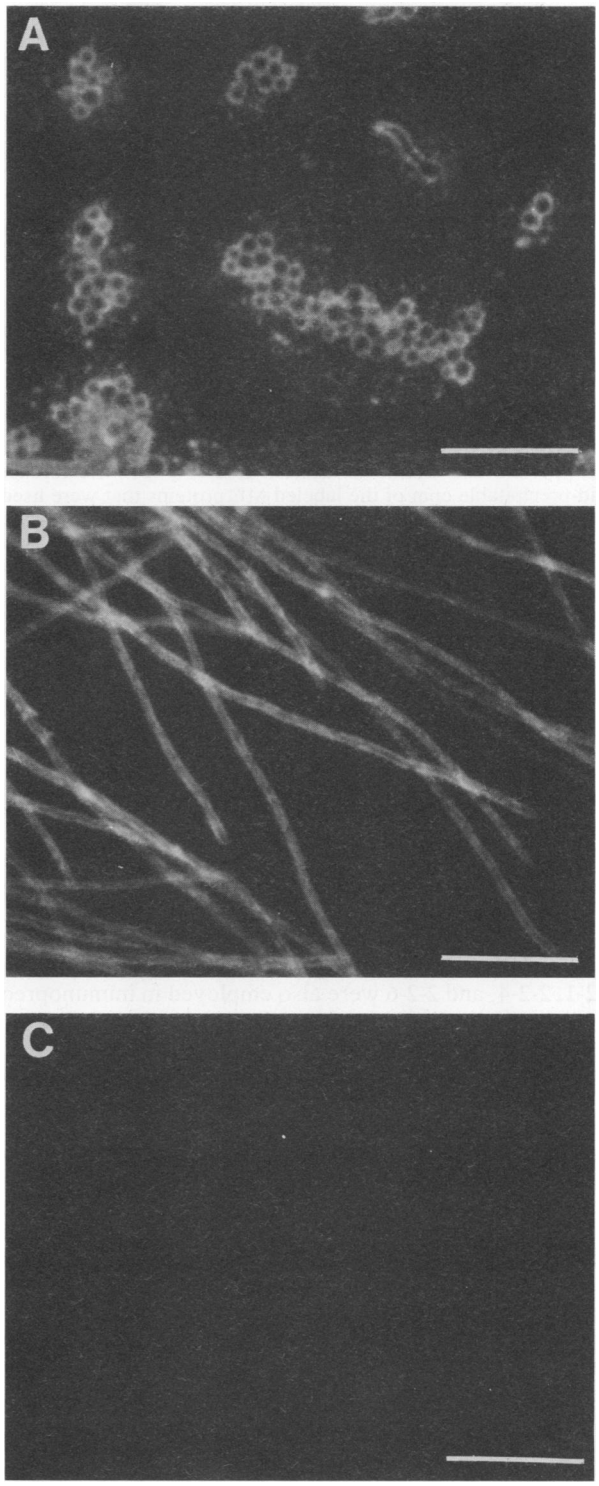

FIG. 3. Immunofluorescent staining of *A. fumigatus* conidia and hyphae. (A and B) Cultures were grown for 8 and 24 h, respectively, and the slides were incubated with MAb 2-7. (C) Only background staining is visible after incubation of a heterologous MAb with a 4-day culture. Negative results were also obtained after incubation of 8- and 24-h cultures with the heterologous antibody. Bars, 10 μm.

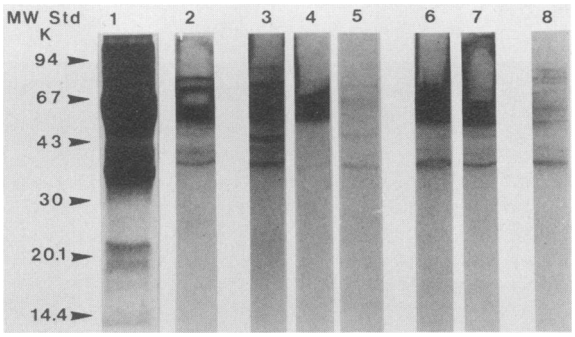

FIG. 4. Protein A-Sepharose immunoprecipitation with MAbs 2-2-9 and 2-2-13, antibodies in the serum of two patients with invasive aspergillosis, and antibodies in the serum of a rabbit immunized against ME. *Aspergillus* conidia were inoculated into Czapek-Dox medium and incubated at 37°C for 22 h. Then 1 mCi of [^{35}S]methionine (SJ.1015; Amersham) was added, and the culture was incubated for an additional 44 h. ME was prepared as described in Materials and Methods. Lanes: 1, 1.7×10^4 trichloroacetic acid-precipitable cpm of the labeled ME proteins that were used as the antigen in the immunoprecipitation; 2 to 8, 24-h exposures; 2, antigens precipitated by immune rabbit serum; 3 and 4, antigens precipitated by antibodies in the serum of patients with invasive aspergillosis, P3-2 and P31, respectively; 5, antigens precipitated by normal human serum; 6 and 7, antigens precipitated by antibodies in ascites fluid from mice inoculated with hybridomas 2-2-9 and 2-2-13, respectively; 8, proteins precipitated by protein A-Sepharose beads incubated with ME alone. MW Std., Molecular weight standards.

2-2-13 (lane 7) and by the protein A-Sepharose beads (lane 8). The beads also bound several other proteins present in ME; however, the 58-kDa antigen was not bound in appreciable amounts. Antibodies in patient serum P3-2 precipitated components with approximate molecular weights of 86,000, 75,000, 43,000, 40,000, and 38,000. Serum P31 (lane 4) precipitated predominantly the 58-kDa antigen. MAbs 2-2-1, 2-2-4, and 2-2-6 were also employed in immunoprecipitation experiments (data not shown), and the profiles of precipitated antigens obtained were similar to those of 2-2-9 and 2-2-13. The 58-kDa antigen was the most abundant, and the minor components that were detected also bound nonspecifically to the beads.

Analysis by limited proteolysis. To determine the relatedness of the precipitated antigens, the 58-kDa antigen immunoprecipitated by patient sera P3-2 and P31, MAbs 2-2-9 and 2-2-13, and the immune rabbit serum was subjected to limited proteolytic digestion. If the antibodies precipitated the same antigen, similar-molecular-weight fragments would be obtained after digestion with *Staphylococcus aureus* V8 protease. A single radioactive fragment with a molecular weight of approximately 35,000 was visible on the autoradiograph after digestion of each of the precipitated antigens (Fig. 5).

Immunoblot analysis with purified 58-kDa antigen. The 58-kDa antigen was purified by immunoaffinity chromatography employing MAb 2-2-9. The bound material (purified 58-kDa antigen) contained one major Coomassie blue- and periodic acid-Schiff-stained 58-kDa component (5). Immunoblot analysis with the purified 58-kDa antigen showed that MAbs 2-7, 2-2-4, 2-2-9, and 2-2-13 (Fig. 6, lanes 1 to 4, respectively) and patient sera JH-7, W-9, P3-2, and P31 (lanes 5 to 8, respectively) reacted with a broad 52- to 62-kDa (58-kDa) band.

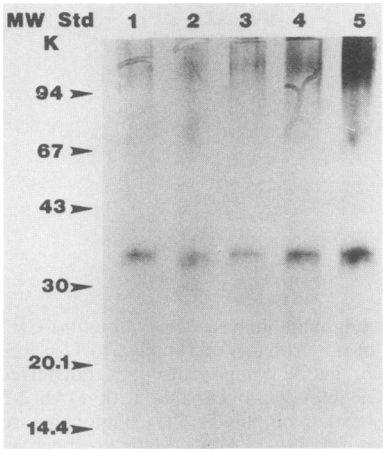

FIG. 5. Autoradiograph of an SDS gel after limited proteolysis of the antigens precipitated by two human sera, immune rabbit serum, and MAbs 2-2-9 and 2-2-13. The ^{35}S-labeled 58-kDa band precipitated by each of the antibodies was cut out of the original gel, applied to wells of a 12.6% SDS–polyacrylamide gel, and treated with 0.045 μg (0.03 U) of *S. aureus* V8 protease (ICN Biomedicals). The substrate (58-kDa antigen) was digested for 20 min at the interface of the stacking and separating gels, and the peptide fragments were then separated by electrophoresis. Lanes: 1, radioactive fragment generated by digestion of the 58-kDa antigen precipitated by P31; 2, fragment generated by digestion of the 58-kDa antigen precipitated by P3-2; 3, fragment generated by digestion of the 58-kDa antigen precipitated by the immune rabbit serum; 4 and 5, fragments generated by digestion of the 58-kDa antigen precipitated by 2-2-13 and 2-2-9, respectively. The gel was exposed to the X-ray film for 7 weeks. MW Std., Molecular weight standards.

DISCUSSION

A 58-kDa antigen present in ME of *A. fumigatus* is able to elicit an antibody response in mice and rabbits and in patients with invasive aspergillosis. The carbohydrate moiety of this antigen contains mannose, galactose, and glucose residues. The ratio of protein to carbohydrate is 1.16:1, and the antigen also contains phosphate groups (5). Cassone et al. (1) produced an IgM MAb to a glucomannoprotein

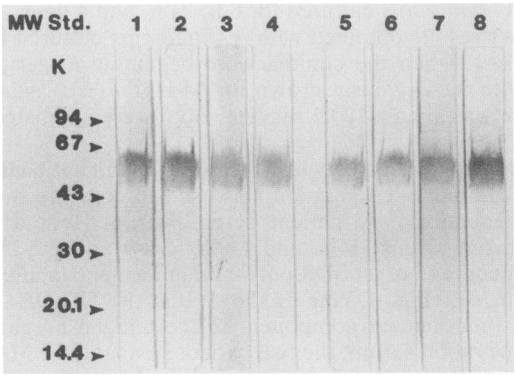

FIG. 6. Immunoblot analysis of immunoaffinity purified 58-kDa antigen. Samples containing 2.2 μg of purified 58-kDa antigen protein were applied to SDS gels. After electrophoresis and transfer to nitrocellulose paper, the blots were incubated with MAbs or with patient sera. Blots were incubated with the following: 1, 2-7; 2, 2-2-4; 3, 2-2-9; 4, 2-2-13; 5 to 8, patient sera (JH-7, W-9, P3-2, and P31, respectively). MW Std., Molecular weight standards.

constituent of *C. albicans* and localized the antigen by immunofluorescence to the cell surface of *Candida* species. In the present study, 2-7 and 2-2-9 bound to a cell surface component of *A. fumigatus* conidia and hyphae (Fig. 3) and 2-2-9 recognized the 58-kDa antigen in 4- and 7-day CF preparations (Fig. 2). Cell surface components can be sloughed off from the cells into the medium during incubation of vigorously shaken cultures. The presence of the 58-kDa component in the medium is probably not due to cell lysis, since it was detected in the CF of young 4-day cultures and its presence was greatly decreased in 14-day CF.

The antigenic specificities of the eight MAbs generated in the present study were studied by immunoblot analysis by using ME. They all recognize the 58-kDa antigen (Fig. 1); however, the IgG antibodies also recognize an antigen with a molecular weight greater than 94,000. This band may represent a precursor protein, which is later cleaved and glycosylated to form the 58-kDa antigen. The band with the molecular weight greater than 94,000 may also be due to the formation of aggregates of the 58-kDa antigen, which may not be dissociated after SDS-polyacrylamide gel electrophoresis. This could account for reactivity with the MAbs.

Antibodies raised against *A. fumigatus* and other fungal antigens have been shown to cross-react in serological assays (15, 19). By immunoblot analysis with a *C. albicans* extract, MAbs 2-2-1, 2-2-4, and 2-2-6 showed reactivity with an antigen of approximately 55 kDa (Table 1). These antibodies may recognize epitopes containing mannose residues, since mannans are a principal component of unfractionated *C. albicans* extracts. The antibodies may recognize the same epitope on both antigens. It is also possible that MAbs may recognize two nonidentical epitopes. Therefore, it is possible to have a similar but not identical epitope on a *Candida* antigen that still allows detectable binding of MAbs against the *Aspergillus* 58-kDa antigen by immunoblotting.

The IgM MAbs and 2-2-4 formed precipitin bands when reacted with the 58-kDa antigen by immunodiffusion (Table 1), indicating that the epitopes recognized by each of the antibodies is present at two or more sites on the molecule, allowing cross-linking and the formation of a precipitin band. The results from the immunoblot analyses and immunodiffusion demonstrate that several of the MAbs probably do not recognize the same antigenic determinant (Table 1). The reactions of the IgM antibodies resulted in a pattern that was different from that of the IgG antibodies. 2-2-1, 2-2-4, and 2-2-6 recognized the 55-kDa *Candida* antigen, whereas the IgM antibodies 2-2-9 and 2-2-13 did not. 2-2-4 was the only IgG antibody that reacted with the 58-kDa antigen by immunodiffusion.

Results from the immunoprecipitation experiment (Fig. 4) demonstrate that the major component of ME recognized by the antibodies was the 58-kDa antigen. Normal human serum did not precipitate the antigen in an appreciable amount. The 38-kDa component precipitated by the antibodies also bound nonspecifically to the protein A-Sepharose beads. The relationship of the 38-kDa component to the 58-kDa antigen, if any, has not been determined. Limited proteolytic digestion of the 58-kDa antigen precipitated by each of the antibodies (Fig. 5) resulted in one cleavage product of molecular weight 35,000, indicating that the antigens recognized by each of the antibodies shared similar primary structure. It is possible that other smaller fragments were generated that were too small to remain on the gel or that the amount of radioactivity of the fragments was insufficient for detection.

The immunoblots shown in Fig. 6 demonstrate that the MAbs and antibodies in patient sera recognize the same immunoaffinity-purified 58-kDa antigen. These results indicate that the MAbs are directed against an epitope present on an antigen that is recognized by antibodies in the serum of patients with invasive aspergillosis.

Although the humoral immune response in immunosuppressed patients who develop invasive aspergillosis is weak, the 58-kDa antigen was recognized by antibodies in all of the sera that were tested by immunoblotting, and it was the only antigen recognized by approximately 90% of the sera (5). The MAbs produced in this study may be used for the development of a sensitive diagnostic test for invasive aspergillosis. The antibodies may be used as reference antisera and/or for the purification of the antigen employed in the test, allowing for standardization of the immunoassay. Since 2-7 and 2-2-9 bound to hyphal surfaces, these antibodies may be used to produce specific fluorescent antibody conjugates for the detection of *Aspergillus* species in tissue and other specimens.

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