

Murine Monoclonal Antibodies to Glycoprotein Antigens of *Aspergillus fumigatus* show Cross-reactivity with other Fungi

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ABSTRACT

Six monoclonal antibodies produced against *Aspergillus fumigatus* were studied for their cross-reactivity against other fungal antigens from related and unrelated organisms. Five of the monoclonal antibodies reacted with glycoprotein antigens as evidenced by their binding to concanavalin A, although one did not react with concanavalin A. The reactivities of the monoclonal antibodies with various antigens were studied by biotin-avidin linked immunosorbent assay and Western blots. The results indicate that two monoclonal antibodies (Asp D1 and Asp C9) react with antigens from aspergilli as well as other fungi including *Penicillium notatum* and *Candida albicans*, whereas three of six antibodies (Asp H10, Asp ILB8, and Asp C2B1) react with *A. fumigatus* antigen only. Hence, these monoclonal antibodies can be used to obtain group specific and species specific antigens for various immunodiagnostic assays. (Allergy Proc 14: 189-193, 1993)

Aspergillus species have been implicated as the causative agents of a number of different clinical conditions in humans. These diseases include invasive aspergillosis (IA), aspergilloma, allergic asthma, allergic

bronchopulmonary aspergillosis (ABPA), and hypersensitivity pneumonitis.¹⁻⁴ *A. fumigatus* has been recognized as the predominant causative agent in these diseases.^{1, 2, 5} The diagnosis of *Aspergillus*-induced diseases is based on clinical and laboratory findings, particularly immunological findings.^{1, 2, 5-9} Several major clinically relevant and immunologically significant antigens from *A. fumigatus*, having clinical relevance have been identified and characterized. However, there is still a lack of dependable antigens for use in the serodiagnosis of aspergillosis.^{4, 10-13}

The availability of monoclonal antibodies (mAbs) to clinically relevant antigens has greatly facilitated the purification and characterization of antigens, and the development of immunoassays with increased sensitivity, specificity, and reproducibility. Several mAbs to *A. fumigatus* have been produced and characterized in the recent years.¹⁴⁻¹⁸ The relevancy and specificity of these mAbs have not been investigated in detail. Low levels of antibodies against commonly occurring fungi have been frequently detected in normal individuals when sensitive methods were employed.^{2, 19} However, in patients with aspergillosis usually higher levels of antibodies have been demonstrated. Although the mAbs are usually specific, they may also be directed against identical epitopes present in the antigens of different fungi. Hence, it is essential to have detailed knowledge on the shared epitope reactivity of different fungal antigens to the mAbs. Such studies will aid in identifying highly specific monoclonal antibodies that can be reliably used in immunodiagnosis. In the present report we describe

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the production and partial characterization of mAbs produced against *A. fumigatus* antigens and the reactivity of these mAbs with antigens of other fungi as well as of different *Aspergillus* species. The results indicate that some of the mAbs demonstrate unique reactivity with *A. fumigatus* glycoproteins whereas others cross-react with glycoproteins from other fungi as well.

MATERIALS AND METHODS

(I) Preparation of Antigens

Culture filtrate (CF) antigens from *A. fumigatus* (AF-102; ATCC-42202), *A. niger* (Ag 512), *A. flavus* (Ag 525), *A. fischeri* (Ag 516), *A. clavatus* (B165-13), *A. glaucus* (B 1178), *A. terreus* (Ag 510), *Penicillium notatum* (Ag 538), and *Trichoderma viride* (ATCC-13631) were prepared according to the method described previously.¹¹ The organism was grown in synthetic broth (AOAC, Difco, Detroit, MI) with 1% glucose for 3 weeks in stationary cultures at 30°C.¹¹ The broth was separated from the mycelium, dialyzed extensively against deionized water, and lyophilized. The antigen was evaluated for the quality and reactivity against specific antibodies using methods described earlier.¹² These methods include protein and carbohydrate contents, enzyme and protein profile, and reactivity against positive and negative sera by agar gel double diffusion, crossed immunoelectrophoresis (CIE), and biotin-avidin linked immunosorbent assay (BALISA).^{8, 12, 20} The antigen of *C. albicans* used in the study was an extract obtained commercially (Miles Laboratories Inc., Elkhart, IN).

(II) Production and Characterization of mAbs to *A. fumigatus*

The method employed for the production of mAbs was the same as that described previously.^{15, 16} Splenocytes from 8 to 10 weeks-old mice immunized with CF antigens of *A. fumigatus* were fused with mouse myeloma cells (P3-NS-1/Ag4-1).^{15, 16, 21} Hybrids selected using hypoxanthine, aminopterin, and thymidine medium were screened by BALISA method and significant antibody-secreting cells were cloned by limiting dilution. Hybridomas thus obtained after repeated cloning and testing for antibody production were injected into pristane-primed BALB/c mice for ascitic fluid production. The ascitic fluids were evaluated for anti-*A. fumigatus* antibodies by BALISA method. The animal studies were approved by the Institutional Animal Review Committee.

Five of the mAbs against *A. fumigatus* antigens obtained from different fusions were selected for the present study. The immunoglobulin isotypes of these mAbs were determined by BALISA with class-, subclass-, and light chain-specific antisera (Sigma Chemical Co., St.

Louis, MO). The reactivity of these mAbs to *A. fumigatus* were studied by BALISA, antigen-antibody crossed electrophoresis, rocket electrophoresis, and agar gel double diffusion.^{8, 12, 20}

(III) Reactivity with Different Fungal Antigens

The reactivity of these mAbs with various fungal antigens detailed above were studied using BALISA and by Western blot.

BALISA. The BALISA method used was the same as described previously.^{6, 8} Dynatech Immulon II ELISA plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with different antigens at 1 µg/mL concentration in carbonate buffer (pH 9.6). The antigen-coated plates were incubated for 3 hours at 37°C and then at 4°C overnight. The wells were washed with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST). After blocking the free sites with bovine serum albumin (BSA, 1% w/v) in PBS (PBS-BSA); 100mL of different ascitic fluids diluted in PBS-BSA were added to the wells and the plates were incubated for 3 hours at room temperature. This was followed by the addition of goat anti-mouse IgG₁, IgA, or IgM; biotinylated rabbit anti-goat IgG, streptavidin peroxidase, and the substrate to the wells of the plates. All these reagents were incubated for 1 hour at 37°C, and the wells were washed three times with PBST in between the addition of reagents. The color developed with the substrate (orthophenylene diamine and 0.015% H₂O₂ in citrate buffer) was read in an automatic ELISA plate reader at 490nm. The optical density (OD) values obtained with different antigens and mAbs were compared. All dilutions of the reagents were selected empirically after checkerboard titrations using positive and negative controls.

The BALISA titer was calculated from a computerized program that plots a curve from the OD of four serum dilutions and derives a titer of serum expressed as log₁₀ titer.²² A cut-off value of 0.1 was used to calculate the titer.

Western blot analysis. The antigen extracts (CF) from *A. fumigatus*, *A. flavus*, and *A. niger* were subjected to electrophoresis in a 0.5% SDS-polyacrylamide vertical gel with a 7.5 to 20% linear gradient.^{13, 22} Approximately 1 mg of protein was loaded onto a 160 × 160 × 1 mm gel and electrophoresed at 30mA/gel for 2-2.5 hours at 10°C. After electrophoresis, the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA) by using a semidry electrotransfer system (Integrated Separation Systems, Natick, MA), according to manufacturer's instructions. The transfer efficiency was determined by staining strips of gels with Coomassie brilliant blue R250 for proteins before and after blotting and comparing them with colloidal gold-stained PVDF

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membranes containing the blotted proteins. The free protein-binding sites on the membrane were then blocked with Tris-buffered saline (TBS) containing 3% BSA for 1 hour.²² After drying, the membranes were cut into small strips and stored at -20°C until used. The activity of the antigens were stable at least for 2 months of storage at this temperature.

The procedure followed was the same as described earlier with minor modifications.¹⁶ Briefly, the strips were incubated overnight at 4°C with 1:100 dilutions of mouse mAbs made in TBS-BSA (BSA, 1% w/v) and were washed with TBST (TBS containing 0.05% Tween-20). This was followed by incubating with 1:500 dilution of goat anti-mouse IgG or IgM for 1 hour at room temperature and washing in TBST. The strips were then incubated with biotinylated rabbit anti-goat IgG at a dilution of 1:1000 for 30 minutes and washed as before. The blots were then incubated with 1:1000 dilution of streptavidin peroxidase for 30 minutes and again washed. The antigen-antibody complexes were visualized with a chromogenic substrate (60 mg 4-chloro-1-naphthol, 20 mL methanol, 100 mL TBS, and 0.015% H₂O₂).²² The lectin-binding characteristics of the antigen bands were determined by the reactivity with biotinylated concanavalin A (con A).

RESULTS

Isotype-specific BALISA showed that three of the monoclonal antibodies belong to the IgM class, whereas three were of the IgG1 class, and all had the κ -light chains. The results of the BALISA are given in Table 1. The monoclonal antibody Asp C9 reacted with all 10 fungal antigens tested, although the titer was highest with homologous antigen. Similarly, 8/10 antigens reacted with Asp D1 antibody whereas, 3 mAbs (Asp H10, Asp ILB8, and Asp C2B1) failed to show

any reactivity with heterologous antigens. Monoclonal antibody Asp C1E6 reacted with both *A. fischeri* and *A. fumigatus* antigens.

Western blots of mAbs reacted with *A. fumigatus* antigen showed 1-3 prominent bands (Fig. 1). Four of the mAbs (Asp D1, Asp C9, Asp H10, and Asp C1E6) reacted with an antigenic component of approximately 29 kDa. One mAb (Asp ILB8) reacted with a 22 kDa band of *A. fumigatus* and Asp C2B1 reacted with 3 antigenic components in the molecular weight range of 25-29 kDa. All the monoclonal antibodies except Asp ILB8 reacted with antigens having con A binding activity indicating their glycoprotein nature (Fig. 1, g). Reactivity of monoclonal antibodies Asp D1 and Asp C9 with *A. fumigatus* antigen showed identical bands, whereas their reactivity with other fungal antigens were different (Table 1; Fig. 1).

Strong reactivity to *A. flavus* was seen with only Asp H10 showing two bands (Fig. 2, c), although it reacted with only a single band of *A. fumigatus* antigen (Fig. 1, c). None of the other monoclonal antibodies reacted with *A. flavus* antigen. Two of the mAbs (Asp D1 and Asp C9) reacted with a number of con A binding components of *A. niger* on the western blots and Asp H10 failed to show any reactivity (data not shown). Monoclonal antibodies Asp ILB8, Asp C1E6, and Asp C2B1 were not reactive with any of the components of *A. niger* antigen.

DISCUSSION

The demonstration of *A. fumigatus*-specific antibodies is an important criterion in the diagnosis of Aspergillus-induced diseases. A number of antigens have been used in the serodiagnosis of these diseases. However, these antigens mostly constitute crude preparations and frequently demonstrate nonspecific reac-

TABLE I

BALISA Titers of Monoclonal Antibodies Tested Against Different Fungal Antigens*

Antigen	Asp D1	Asp C9	Asp H10	Asp ILB8	Asp C1E6	Asp C2B1
<i>A. fumigatus</i>	3.051	2.798	2.661	9.693	9.745	5.277
<i>A. niger</i>	1.878	2.09	0	0	0	0
<i>A. flavus</i>	2.06	2.105	0	0	0	0
<i>A. glaucus</i>	0	2.208	0	0	0	0
<i>A. clavatus</i>	2.209	2.537	0	0	0	0
<i>A. fischeri</i>	0	2.285	0	0	0	0
<i>A. terreus</i>	2.191	2.194	0	0	2.975	0
<i>A. notatum</i>	1.878	2.09	0	0	0	0
<i>C. albicans</i>	1.71	2.074	0	0	0	0
<i>T. viride</i>	2.099	2.183	0	0	0	0

*A cut off value of 0.100 was used in determining the titer using a computer program and expressed as log₁₀ titer (See text for details).

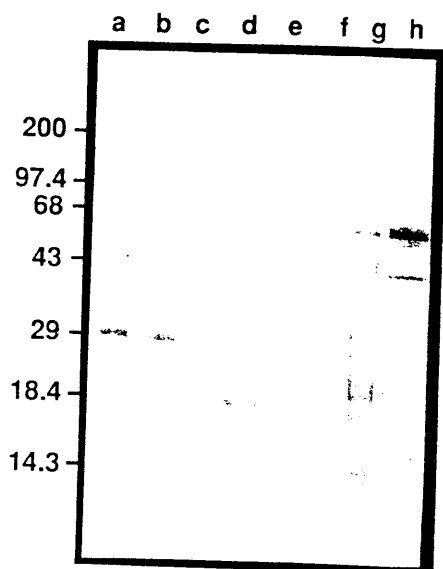


Figure 1. Reactivity of monoclonal antibodies with *A. fumigatus* antigen on Western blots. Lanes a-f: the blots reacted with monoclonal antibodies Asp D1, Asp C9, Asp H10, Asp ILB8, Asp C1E6, and Asp C2B1, respectively. Lane g: blot reacted with con A and Lane h: antigen transferred to Immobilon and stained with colloidal gold.

tivity and/or nonreactivity. Such crude preparations of antigens from the cytosol or culture filtrate frequently contain over 30 components that can be demonstrated in antigen-antibody crossed electrophoresis using rabbit anti-*A. fumigatus* sera and ABPA or aspergilloma sera.^{4, 11, 23-25} Several of these antigenic components

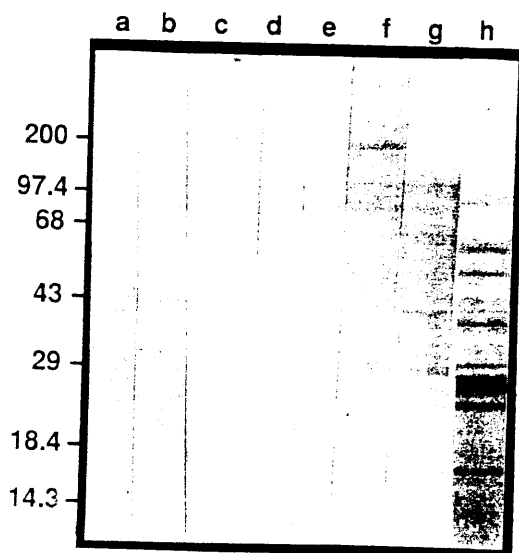


Figure 2. Reactivity of monoclonal antibodies with *A. flavus* antigen on Western blots. Lanes a-f: the blots reacted with monoclonal antibodies Asp D1, Asp C9, Asp H10, Asp ILB8, Asp C1E6, and Asp C2B1, respectively. Lane g: blot reacted with con A and Lane h: antigen transferred to Immobilon and stained with colloidal gold.

have been shown to react significantly with sera from aspergillosis patients and hence may be relevant in the disease.^{4, 10, 13, 20, 25}

Low levels of antibodies to commonly occurring fungal antigens have been reported in a majority of normal individuals.¹⁹ However, the levels of antibodies to specific antigens will be much higher in patients than in normal individuals and hence can be used to delineate patients from normal individuals. The occurrence of such low levels of antibodies in the sera of normal individuals may be due to the presence of cross-reacting antigens among different fungi.^{23, 25, 26} The presence of such cross-reactivity may adversely affect the interpretation of the results of immunologic methods employing the monoclonal antibodies. Monoclonal antibodies are directed against specific antigenic determinants and they react consistently with such components. Because of the shared antigenic components among different fungi results of immunoassays employing monoclonal antibodies need careful evaluation and interpretation. To the contrary, monoclonal antibodies that show cross-reactivity with several fungi will be helpful in initial screening, whereas those produced against unique epitopes may aid in detecting the specific antigens. The two monoclonal antibodies Asp C9 and Asp D1 represent the former, whereas the remaining four monoclonals represent the latter group (Table 1).

Glycoproteins are the major antigens of *A. fumigatus*. They have been shown to exhibit consistent reactivity in the detection of specific circulating antibodies in aspergillosis, although protein antigens were found to be more specific in some forms of the disease.^{12, 13} However, the presence of antibodies to glycoprotein antigens in some normal individuals may be a limitation in the use of such monoclonal antibodies in immunodiagnostic tests. Lymphocyte proliferative studies from our laboratory have shown that ABPA patients as well as normal subjects exhibit proliferative response to glycoprotein antigens.²⁸ The results of the western blot analysis in this study indicate that the majority of the cross-reactive antigens found among the different fungi are con A binding. Although not specific, these predominant antigens may be involved in the pathogenesis of the disease and hence may be significant in the disease process.

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REFERENCES

1. Bardana EJ Jr. The clinical spectrum of aspergillosis. Part 1. Epidemiology, pathogenicity, infection in animals and immunology of aspergillosis. *CRC Crit Rev Clin Lab Sci* 13:21-83, 1980.

2. Bardana EJ Jr. The clinical spectrum of aspergillosis. Part 2. Classification, diagnosis, and treatment. *CRC Crit Rev Clin Lab Sci* 13:85-117, 1980.
3. Kurup SP. Microbiology of aspergillosis. *Clin Microb Rev* 1:1-10, 1988.
4. Long SP, Kurup SP. Identification of aspergillus antigens by immunoblotting. *J Clin Microbiol* 23:1173-1175, 1980.
5. Green DE. The role of aspergillus in the pathogenesis of aspergillosis. *Clin Microb Rev* 1:11-20, 1988.
6. Brumfield J. The role of aspergillus in the pathogenesis of aspergillosis. *Clin Microb Rev* 1:21-30, 1988.
7. Fink G. The role of aspergillus in the pathogenesis of aspergillosis. *Clin Microb Rev* 1:31-40, 1988.
8. Kurup SP. The role of aspergillus in the pathogenesis of aspergillosis. *Clin Microb Rev* 1:41-50, 1988.
9. Wilson J. The role of aspergillus in the pathogenesis of aspergillosis. *Clin Microb Rev* 1:51-60, 1988.
10. Hearn J. The role of aspergillus in the pathogenesis of aspergillosis. *Clin Microb Rev* 1:61-70, 1988.
11. Kurup SP. The role of aspergillus in the pathogenesis of aspergillosis. *Clin Microb Rev* 1:71-80, 1988.
12. Kurup SP. The role of aspergillus in the pathogenesis of aspergillosis. *Clin Microb Rev* 1:81-90, 1988.
13. Kurup SP. The role of aspergillus in the pathogenesis of aspergillosis. *Clin Microb Rev* 1:91-100, 1988.
14. Fratacci MD. The role of aspergillus in the pathogenesis of aspergillosis. *Clin Microb Rev* 1:101-110, 1988.
15. Kurup SP. The role of aspergillus in the pathogenesis of aspergillosis. *Clin Microb Rev* 1:111-120, 1988.

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2. Bardana EJ Jr. The clinical spectrum of aspergillosis. Part 2. Classification and description of saprophytic, allergic and invasive variants of human diseases. *CRC Crit Rev Clin Lab Sci* 13:85-159, 1980.
3. Kurup VP, Kumar A. Immunodiagnosis of Aspergillosis. *Clin Microbiol Rev* 4:439-456, 1991.
4. Longbottom JL. Antigens/allergens of *Aspergillus fumigatus*. Identification of antigenic components reacting with IgG and IgE antibodies of patients with allergic bronchopulmonary aspergillosis. *Clin Immunol Immunopathol* 53:354-362, 1983.
5. Greene R. The pulmonary aspergillosis: three distinct entities of spectrum of disease. *Radiology* 140:527-530, 1981.
6. Brummund W, Resnick A, Fink JN, Kurup VP. *Aspergillus fumigatus* specific antibodies in allergic bronchopulmonary aspergillosis and aspergilloma. Evidence for a polyclonal response. *J Clin Microbiol* 25:5-9, 1987.
7. Fink JN. Allergic bronchopulmonary aspergillosis. *Hospital Practice* 23:105-128, 1988.
8. Kurup VP. Enzyme-linked immunosorbent assay in the detection of specific antibodies against *Aspergillus* in patient sera. *Zentral Bakteriell Mikrobiol Hyg [A]* 261:509-516, 1986.
9. Wilson EV, Hearn VM, Mackenzie DWR. Evaluation of a test to detect circulating *Aspergillus fumigatus* antigen in a survey of immunocompromised patients with proven or suspected invasive disease. *J Med Vet Mycol* 25:365-37, 1987.
10. Hearn VM, Wilson EV, Latge JP, Mackenzie DWR. Immunochemical studies of *Aspergillus fumigatus* mycelial antigens by polyacrylamide gel electrophoresis and Western blotting techniques. *J Gen Microbiol* 136:1525-1535, 1990.
11. Kurup VP, Fink JN, Scribner GH, Falk MJ. Antigenic variability of *Aspergillus fumigatus* strains. *Microbios* 19:191-204, 1978.
12. Kurup VP, John KV, Resnick A, Fink JN. A partially purified glycoprotein antigen from *Aspergillus fumigatus*. *Int Arch Allergy Appl Immunol* 79:263-269, 1986.
13. Kurup VP, Resnick A, Scribner GH, Gunasekaran M, Fink JN. Enzyme profile and immunochemical characterization of *Aspergillus fumigatus* antigens. *J Allergy Clin Immunol* 78:1166-1173, 1986.
14. Frattamico PM, Long WK, Buckley HR. Production and characterization of monoclonal antibodies to a 58-Kilodalton antigen of *Aspergillus fumigatus*. *Infect Immun* 59:316-22, 1991.
15. Kurup VP. Production and characterization of a murine monoclonal antibody to *Aspergillus fumigatus* antigen having IgG- and IgE-binding activity. *Int Arch Allergy Appl Immunol* 86:400-406, 1988.
16. Kurup VP. Murine monoclonal antibodies binding to the specific antigens of *Aspergillus fumigatus* associated with allergic bronchopulmonary aspergillosis. *J Clin Lab Analysis* 3:116-121, 1989.
17. Kurup VP, Elms N, Fink JN. Characterization of a monoclonal antibody against Concanavalin A binding antigen of *Aspergillus fumigatus*. *Hybridoma* 10:387-393, 1991.
18. Ste-Marie L, Senechal S, Boushira M, Garzon S, Strykowski H, Pedneault L, de Repentigny, L. Production and characterization of monoclonal antibodies to cell wall antigens of *Aspergillus fumigatus*. *Infect Immun* 58:2105-2114, 1990.
19. Bardana EJ Jr, McClatchy JK, Farr RS, Minden P. The primary interaction of antibody to components of aspergilli. II. Antibodies in sera from normal persons and from patients with aspergillosis. *J Allergy Clin Immunol* 50:222-234, 1972.
20. Kurup VP, Ramasamy M, Greenberger PA, Fink JN. Isolation and characterization of a relevant *Aspergillus fumigatus* antigen with IgG and IgE binding activity. *Int Arch Allergy Appl Immunol* 86:176-182, 1988.
21. Goding JW (ed). *Monoclonal antibodies: Principles and Practices*. Academic Press, 1983, pp. 5-97.
22. Kurup VP. Detection of relevant farmer's lung antigens by using immunoblots of two-dimensional electrophoresis. *Diagn Clin Immunol* 5:25-29, 1987.
23. De Magaldi SW, Mackenzie DWR. Specificity of antigens from pathogenic *Aspergillus* species. I. Studies with ELISA and immunofluorescence. *Sabouraudia J Med Vet Mycol* 22:381-394, 1984.
24. Kim SJ, Chaparas SD. Characterization of antigens from *Aspergillus fumigatus*. *Am Rev Respir Dis* 118:547-551, 1978.
25. Piechura JE, Kurup VP, Fink JN, Calvanico NJ. Antigens of *Aspergillus fumigatus*. III Comparative immunochemical analyses of clinically relevant aspergilli and related fungal taxa. *Clin Exp Immunol* 59:716-724, 1985.
26. Sepulveda R, Longbottom JL, Pepys J. Enzyme linked immunosorbent assay (ELISA) for IgG and IgE antibodies to protein and polysaccharide antigens of *Aspergillus fumigatus*. *Clin Allergy* 9:359-371, 1979.
27. Kurup VP, Greenberger PA, Fink JN. 1989. Antibody response to low-molecular-weight antigens of *Aspergillus fumigatus* in allergic bronchopulmonary aspergillosis. *J Clin Microbiol* 27:1312-1316, 1989.
28. Murali PS, Kurup VP, Greenberger PA, Fink JN. Concanavalin A Nonbinding *Aspergillus fumigatus* antigen: a major immunogen in allergic bronchopulmonary aspergillosis. *J Clin Lab Med* 119:377-384, 1992. □