

Ultrastructural demonstration of specific IgG and IgE antibodies binding to *Aspergillus fumigatus* from patients with aspergillosis

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Aspergillus-induced diseases usually demonstrate elevated circulating antibodies belonging to different isotypes. The antigens currently used to detect antibodies are crude culture filtrate and mycelial extracts of A. fumigatus (Af). Most Af-associated diseases result from the inhalation of the spores of the organisms present in the environment. However, it is not known whether specific circulating antibodies directed only against spore or mycelia of Af exist in the sera of patients with Af-induced diseases. With colloidal gold we have investigated thin sections of spores and hyphae of Af for their reactivity with Af-specific IgG and IgE antibodies. The results indicate that both spores and hyphae reacted identically with IgG and IgE antibodies from patients. None of the sera from normal control subjects reacted in this system, although low levels of antibodies were detected in the sera by ELISA. Sera from both patients with allergic bronchopulmonary aspergillosis or aspergilloma reacted with cell envelope antigens, whereas sera from patients with invasive aspergillosis also bound to cell sap. This method therefore demonstrates localization of antigens binding to different isotypes in the sera from different clinical forms of aspergillosis and may be useful in purifying specific antigens for immunodiagnosis. (J ALLERGY CLIN IMMUNOL 1991;87:683-8.)

Af-induced diseases are characterized by high levels of specific antibodies in the sera of affected patients, a finding which has been used in the differential diagnosis of the disease.¹ Although elevation of all specific anti-Af isotypes have been detected in most of the *Aspergillus*-induced diseases, there is considerable difference in the predominance of certain immunoglobulin isotypes, a difference which has been effectively used in specific diagnosis.^{2, 3} For example, in *Aspergillus*-induced asthma, immediate wheal-and-flare skin reactivity to Af antigen is detected, indi-

Abbreviations used

Af:	<i>Aspergillus fumigatus</i>
PBS:	Phosphate-buffered saline
ABPA:	Allergic bronchopulmonary aspergillosis
BALISA:	Biotin avidin-linked immunosorbent assay
IEM:	Immunoelectron microscopy
IGS:	Immunogold staining
TBSA:	Tris-buffered saline containing 0.1% bovine serum albumin

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cating the presence of high levels of specific IgE antibodies, whereas other antibody isotypes specific to Af are not detectable.⁴ In aspergilloma (fungus ball) and hypersensitivity pneumonitis, high levels of most antibody isotypes except IgE have been detected.¹⁻³ In contrast, in ABPA, the predominant antibody isotypes against Af are IgG, IgE, and IgA, and their detection has been useful in the diagnosis.⁵ The antigens used in the detection of the serum antibodies are mostly extracts of culture filtrates or mycelia of Af. However, antigen preparations vary in their immunologic and chemical characteristics,⁶ and patients may demonstrate diverse immune response when they are exposed to such components of fungi as conidia

TABLE I. Localization of specific antigens in spores and hyphae of *Aspergillus* determined by colloidal gold binding

Subjects No.	Diagnosis	Colloidal gold binding to				Agar gel diffusion	Antibodies by BALISA		Total IgE (ng/ml)
		Conidia		Hyphae			IgG	IgE	
		IgG	IgE	IgG	IgE				
1	ABPA	+	+	+	+	+	1.313*	0.367	13,084
2	ABPA	+	+	+	+	+	1.304	0.370	11,369
3	Aspergilloma	+	---	+	---	+	0.222	0.033	279
4	Aspergilloma	+	---	+	---	+	0.361	0.100	†
5	Invasive as- pergillosis	+	---	+	---	+	0.372	0.306	---
6	Invasive as- pergillosis	+	---	+	---	+	0.718	0.394	---
7	Normal	---	---	---	---	---	0.164	0.036	12
8	Normal	---	---	---	---	---	0.063	0.020	65

*Optical density values when serum dilutions 1:100 for IgE and 1:1000 for IgG were used.

†Not detected.

(spores), mycelial fragments, and soluble antigens. The present study was designed to determine whether there are differences in the antigenic components of conidia, germinating conidia, and hyphae as detected by their reactivity to circulating antibodies in patients with different clinical forms of aspergillosis. Specific binding of antibodies were evaluated with a colloidal gold probe on ultrathin sections of *Af*, conidia, and hyphae.

MATERIAL AND METHODS

Human sera

Eight serum samples were selected for the study. These included two samples each from patients with ABPA, aspergilloma, and invasive aspergillosis, and two serum samples from normal control subjects. The diagnosis of *Aspergillus*-induced diseases was based on standard clinical and immunologic criteria.^{1, 2, 7} Sera from normal subjects were from laboratory personnel with no apparent evidence of disease. All samples were stored in aliquots at -70°C and thawed for use as needed.

Preparation of *Af* for IEM

Spores from a 4-day-old culture of *Af* on Sabouraud's agar were harvested in 15 ml of sterile physiologic saline. These spores were washed several times and resuspended in 2 ml of a fixative solution (4% paraformaldehyde and 0.1% glutaraldehyde). *Af* spores were grown at 37°C in a synthetic broth for 4 and 72 hours and were washed and fixed as above. The fixed growth was then washed three times in PBS (pH 7.4), after which the pellet of cells was fixed in osmium tetroxide, dehydrated in alcohol, and embedded in Spurr (Polysciences, Inc., Warrington, Pa.). Ultrathin sections were cut on the Ultratome III, LKB,

Bromma, Sweden, and grids with thin sections were used in immunochemical stainings.

IGS

Colloidal gold having a size of 15 nm was prepared according to the method of Frens.⁷ Rabbit antigoat IgG (affinity-purified heavy chain specific) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and labeled with colloidal gold, according to the method described by Geoghegan and Ackerman.⁸ The stabilized antibody-labeled colloidal gold was then stored at 4°C for up to 6 months and discarded when clumping was detected.

The staining procedure was performed in wells of polyvinyl microtiter plates (Nunc Immunoplate, Roskilde, Denmark). The grids were gently transferred into the reagents to ensure that the specimens were always face up. The grids were then incubated for 30 minutes in PBS (pH 7.4) containing 5% nonfat dry milk (dry milk, Carnation Co., Los Angeles, Calif.), 0.05% (vol/vol) Tween 20, and 0.1% sodium azide (PBS-milk-Tween). After microplate wells were washed three times for 2 minutes each with Tris-buffered saline (pH 8.2) containing 0.1% (wt/vol) BSA and 0.1% sodium azide (TBSA), 200 μl of serum dilutions were added to the microplate wells. All the dilutions of test reagents were prepared in PBS-milk-Tween. The *Af*-specific IgG antibodies were detected at 1:1000 dilutions of sera, whereas a 1:100 dilution was used to detect specific IgE antibodies. After incubating for 1 hour at room temperature, the grids were washed three times for 2 minutes each in TBSA and then incubated in 200 μl of goat antihuman IgG (1:100, Sigma Chemical Co.) or IgE (1:100, Sigma Chemical Co.). After a 1-hour incubation at room temperature, the grids were treated for 1 hour with a 1:25 dilution of rabbit antigoat IgG conjugated to colloidal gold particles prepared as described above. The grids were washed again in TBSA, followed by two washings each in PBS and in

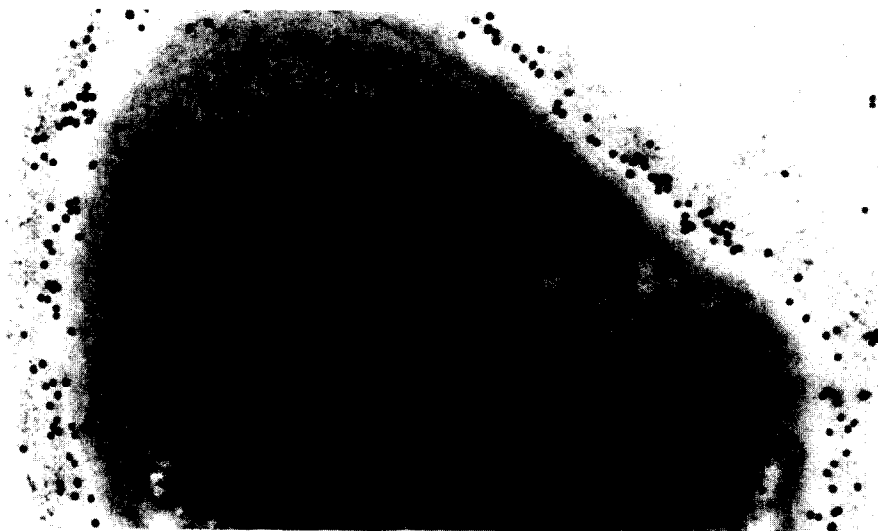


FIG. 1. Ultrathin sections of *Af* spore examined by IEM. The section was incubated with a serum from a patient with ABPA followed by goat antihuman IgG and rabbit antig goat IgG conjugated with colloidal gold. (Original magnification $\times 20,000$.)

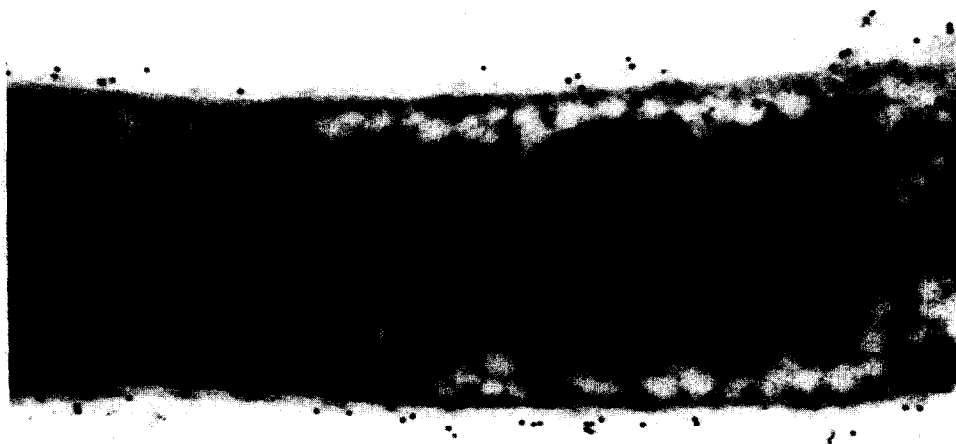


FIG. 2. IEM of *Af* hypha incubated with the serum from the same patient with ABPA as in Fig. 1, treated with antihuman IgG illustrating gold particles on the cell wall. (Original magnification $\times 20,000$.)

double-distilled water and air-dried, and the specimens were examined with an RCA EMU4 electron microscope (RCA Corp., Cherry Hill, N.J.) at 50 kV.

Specificity of IGS

The specificity of IGS technique was tested by use of appropriate negative and positive controls in each run as well as by absorbing the antibody with other antigens associated with hypersensitivity lung diseases. Soluble antigens from *Penicillium* sp (10 mg/ml), *Candida albicans* (100 mg/ml), *Faenia rectivirgula* (5 mg/ml), and *Af* (10 mg/ml) were used to absorb specific or cross-reacting antibodies by incubating the patient's sera and antigen at room temperature for 1 hour. After incubation, the serum samples were centrifuged, and the supernatant was used

to study colloidal gold binding compared to unabsorbed sera.

Total serum IgE

The total serum IgE was measured by a fluorescence allergosorbent test (FAST; 3M Diagnostic Systems, Santa Clara, Calif.).⁹ *Af*-specific IgG and IgE antibodies were detected by BALISA,^{3, 10} and specific precipitins were studied by the agar gel double-diffusion method.^{6, 11}

RESULTS

Colloidal gold binding to *Af* spores and hyphae in comparison with other antibody assays are presented in Table I. The results of the colloidal gold assay



FIG. 3. IEM of germinating spore illustrating accumulation of gold particles; sections were treated as in Fig. 1. (Original magnification $\times 13,990$.)

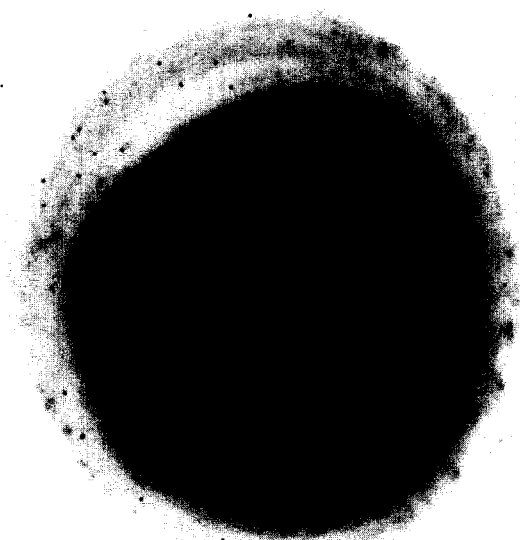


FIG. 4. *Af* spore incubated with a serum from a patient with ABPA and treated with goat antihuman IgE and rabbit antigoat IgG conjugated with colloidal gold. (Original magnification $\times 20,000$.)

demonstrate that antigens capable of reacting with specific antibodies in sera to *Af* are present in both the hyphae and spores (Figs. 1 to 5). The spores and hyphae reacted identically against specific antibodies, as evidenced by the distribution of colloidal gold particles bound to the sections. However, the young ger-

minating spores and short hyphae almost always demonstrated high concentration of colloidal gold particles (Fig. 3), indicating high concentrations of antigenic components in the young hyphae.

Sera from patients with ABPA demonstrated both specific IgG and IgE antibodies against cell wall and cell-membrane components (Figs. 1 to 4). A serum dilution of 1:100 detected IgE antibody by this method, whereas a 1:1000 dilution elicited reproducible results with IgG antibodies. All sera from patients with ABPA demonstrated binding of colloidal gold particles on the cell wall and cell membrane of both hyphae and spores. Occasional scattered gold particles could be detected in the cytoplasm of hyphae. However, gold particles accumulated in large numbers both on the cell envelope as well as in the cytoplasm of the growing tips of the hyphae (Fig. 3).

Sera from patients with aspergilloma also demonstrated frequent reactivity with cell envelope. Strong IgG reactivity at 1:1000 serum dilution was detected, whereas no IgE antibody binding to the hyphae or spores was noted. Sera from patients with invasive aspergillosis demonstrated colloidal gold particles on both the cell envelope and cytoplasm of *Aspergillus*, although comparatively fewer particles were observed in the cytoplasm. Even though *Af*-specific IgE antibody was detected in these sera by ELISA, IgE binding to spores or hyphae was not detected by the immunogold assay.



FIG. 5. *Af* hypha reacted with a serum from a patient with ABPA as in Fig. 4; gold particles appear in the cell wall of the hypha. (Original magnification $\times 10,250$.)

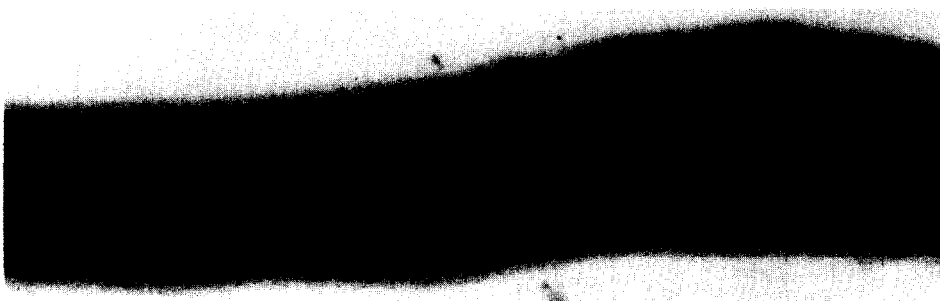


FIG. 6. IEM appearance of *Af* hypha stained with serum from a normal subject and antihuman IgG. (Original magnification $\times 13,990$.)

The specificity of the reactions was ascertained by an inhibition assay with both homologous and heterologous antigens. When patients' sera were absorbed with *Af* antigen, a dose-dependent inhibition of colloidal gold reaction was detected. In contrast, no reduction in the number of colloidal gold particles attached to the *Aspergillus* hyphae or spores was noted when the sera were absorbed with *Penicillium*, *Candida*, and *Faenia* antigens. None of the normal control sera bound IgG or IgE to *Af* spores or hyphae (Fig. 6).

DISCUSSION

The immunogold binding studies with thin sections of *Af* demonstrated that antigenic components bound to specific antibodies in the sera of patients with *Aspergillus*-induced diseases are identical in both spores and hyphae. The germinating spores and hyphal tip consistently demonstrated more gold particles both on the cell envelope and cytoplasm. Similar results were obtained when immunofluorescent assay was used.^{12, 13} The enhanced metabolism detected in the germinating spores and hyphal tips leading to pro-

tein synthesis may be attributable to the increased binding of colloidal gold particles.

The demonstration of both IgG and IgE antibodies reacting with the cell wall and envelope sites of both spores and hyphae were striking. Among the different patient groups studied, only sera from patients with ABPA demonstrated IgE binding activity. Both conidia and hyphae of *Aspergillus* demonstrated IgE antibody binding sites as determined by the sticking of colloidal gold particles. Although sera from patients with invasive aspergillosis had IgE levels similar to that of the patients with ABPA by ELISA, the immunogold assay consistently failed to detect IgE specific binding, whereas IgG binding was heavy compared to that of ELISA titers (Table I).

It is not known at this time whether the elevated ELISA titer was due to IgE or to a nonspecific reaction. It is also not clear that denaturation of some of the epitopes resulting from fixation might have prevented the binding of antibody leading to false-negative reaction. Sera from patients with aspergilloma and from control subjects failed to demonstrate any IgE antibody reactivity, and this finding is in

agreement with our previous findings when a 1:100 dilution of the sera was used in BALISA.¹⁴

Colloidal gold particles binding to conidia and hyphae of *Aspergillus* were detected at a serum dilution of 1:100 for IgE and 1:1000 for IgG. There was a slight increase in the gold particles detected when sera were used undiluted or at high concentrations. However, nonspecific reactivity was also detected at these concentrations of the sera. A dilution of 1:100 for IgE and 1:1000 for IgG was found to be most appropriate for colloidal gold IEM. This method may therefore provide additional information on the antigens associated with the various *Aspergillus*-induced diseases and, in turn, may help in isolating major antigens from *Af* useful for reliable immunodiagnosis.

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REFERENCES

1. Bardana EJ Jr. The clinical spectrum of aspergillosis. I. Epidemiology, pathogenicity, infection in animals, and immunology of *Aspergillus*. CRC Crit Rev Clin Lab Sci 1980;13:21-83.
2. Rosenberg M, Patterson R, Mintzer R, Cooper BJ, Roberts M, Harris KE. Clinical and immunologic criteria for the diagnosis of allergic bronchopulmonary aspergillosis. Ann Intern Med 1977;86:405-14.
3. Brummund W, Resnick A, Fink JN, Kurup VP. *Aspergillus fumigatus* specific antibodies in allergic bronchopulmonary aspergillosis and aspergilloma: evidence for a polyclonal antibody response. J Clin Microbiol 1987;25:5-9.
4. Sepulveda R, Longbottom JL, Pepys J. Enzyme-linked immunosorbent assay (ELISA) for IgG and IgE antibodies to protein and polysaccharide antigen of *Aspergillus fumigatus*. Clin Allergy 1979;9:359-71.
5. Greenberger PA, Patterson R. Allergic bronchopulmonary aspergillosis. Chest 1987;91S:165S-71S.
6. Kurup VP, Resnick A, Scribner GH, Gunasekaran M, Fink JN. Enzyme profile and immunochemical characterization of *Aspergillus fumigatus* antigens. J ALLERGY CLIN IMMUNOL 1986;78:1166-73.
7. Frens G. Controlled nucleation for the regulation of the particle size in monodisperse gold suspension. Nature 1983;241:20-4.
8. Geoghegan WD, Ackerman GA. Adsorption of horseradish peroxidase, ovomucoid, and anti-immunoglobulin of colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin, and goat antihuman immunoglobulin G on cell surfaces at the electron microscopic level: a new method theory and application. J Histochem 1977;25:1187-1200.
9. Gugnani HC, Reijula KE, Kurup VP, Fink JN. Detection of IgG and IgE antibodies to *Aspergillus fumigatus* in human sera by immunogold assay. Mycopathologia 1990;109:33-40.
10. Kurup VP, Resnick A, Kalbfleish J, Fink JN. Antibody isotype responses in *Aspergillus* induced diseases. J Lab Clin Med 1990;115:298-303.
11. Kurup VP, Fink JN, Scribner GA, Falk MJ. Antigenic variability of *Aspergillus fumigatus*. Microbios 1978;19:191-204.
12. Gordon MA, Lapa EW, Kane J. Modified indirect fluorescent antibody test for aspergillosis. J Clin Microbiol 1977;6:161-5.
13. Kurup VP, Fink JN. Evaluation of methods to detect antibodies against *Aspergillus fumigatus*. J Clin Pathol 1978;69:414-7.
14. Kurup VP, Ramasamy M, Greenberger PA, Fink JN. Isolation and characterization of relevant *A. fumigatus* antigen with IgG and IgE binding activity. Int Arch Allergy Appl Immunol 1988;86:176-82.