

Detection of IgG and IgE antibodies to *Aspergillus fumigatus* in human sera by immunogold assay

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Abstract

An immunogold assay (IGA) was developed to detect IgG and IgE antibodies to *Aspergillus fumigatus*. Sixteen sera from patients with allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, and normal controls were studied. All sera were also evaluated for antibodies against *A. fumigatus* by biotin-avidin linked enzyme immunosorbent assay (BALISA) and by agar gel double diffusion method. *A. fumigatus* specific IgG and IgE antibodies could be detected by IGA in all the patients' sera but not in the sera of normal controls. Both IgG and IgE antibodies to *A. fumigatus* could be demonstrated in all the sera by BALISA and normal controls showed only low levels of these antibodies. There was a positive correlation between the degree of reactivity detected by IGA, the BALISA titer and the precipitins by agar gel diffusion. It can be concluded that IGA is a reliable, sensitive and simple method capable of detecting both IgG and IgE antibodies against *A. fumigatus* in patient serum.

Introduction

Allergic bronchopulmonary aspergillosis (ABPA) and aspergilloma (fungus ball) constitute two major diseases caused by *Aspergillus fumigatus* [1, 2]. The diagnosis of these diseases is dependent on demonstration of specific IgG and IgE antibodies in patients' sera when the clinical features of the disease are present. The immunological methods most frequently used for diagnosis of these diseases are agar gel double diffusion (DD) and enzyme-linked immunosorbent assay (ELISA) [6, 11]. DD which is the

most widely used method detects only IgG and IgM but not IgE antibodies and requires relatively long incubation times before the results can be obtained. ELISA is more rapid and sensitive than DD in detecting both IgG and IgE antibodies; however special equipments are required for the technique. In addition, these methods often give variable and inconsistent results due to lack of standardized antigens [3, 9]. In recent years several semipurified and purified antigens have been isolated which give reproducible results, but the procedures involved in their preparation are cumbersome and the yield of such purified anti-

gens is often low [10, 12–14]. We made an attempt to develop a simple and dependable method to detect antibodies against *A. fumigatus* in patient serum, and which can be carried out with minimum equipment using whole organisms in order to reduce the variability of the antigens.

Affinity purified antibodies conjugated with colloidal gold have been used successfully in recent years to visualize a variety of antibodies and/or antigens [15, 16]. The usefulness of this method in detecting serum antibodies to microbial antigens, however, has not been investigated. In the present study we have developed and evaluated an immunogold assay (IGA) to detect *A. fumigatus* specific IgG and IgE antibodies in the sera of patients with ABPA and aspergilloma. The results compared favorably with other assays indicating the usefulness of IGA in demonstrating specific IgG and IgE antibodies to *A. fumigatus* in human sera. IGA is faster than DD and can detect both IgG and IgE to *A. fumigatus*. In addition, it is more simple than ELISA, can be performed in a routine clinical laboratory and is able to discriminate patients from controls.

Materials and methods

Human sera

Sixteen serum samples were selected for the study. These included five samples each of ABPA and aspergilloma and six samples from healthy subjects. The diagnosis of *A. fumigatus*-induced diseases was based on standard clinical and immunological criteria [19]. Sera from normal subjects were from laboratory personnel with no apparent evidence of any respiratory disease. All samples were stored in aliquots at -20°C and thawed for use as needed.

Determination of total serum IgE

A fluorescence allergosorbent test (FAST; 3M Diagnostic Systems, Santa Clara, CA) was used for the determination of total serum IgE. The

threshold level of detection of IgE with this test is 24 ng/ml.

Aspergillus fumigatus antigen

Antigen for gel diffusion and immunosorbent assays: *A. fumigatus* was grown at 37°C in a synthetic broth and the culture filtrate obtained from 3–4 weeks growth was freeze dried after extensive dialysis against distilled water at 4°C [9]. The quality of the antigen was ascertained by its immunochemical characteristic in comparison with in-house antigen standards with known reactivity using the sera of patients and controls [12].

Antigen for immunogold assay (IGA)

The young hyphae of *A. fumigatus* was used as antigens in IGA. Slide cultures of *A. fumigatus* were prepared by a modified method of Riddel *et al.* [18] as described before [8]. Briefly, Petri dishes containing a set of two slides pre-coated with 0.1% agarose on a bent glass rod with filter papers at the bottom were sterilized. Pre-coated slides were used to facilitate better adherence of hyphae and to prevent its detachment during the immunostaining procedures. Sabouraud's agar (1.5 to 2.0 mm thick) in a Petri dish was cut into blocks (40×10 mm). One block each was aseptically transferred to the center of a slide. Spores from a 3 day old culture of *A. fumigatus* on Sabouraud's agar was harvested in 5 ml sterile physiological saline. Using a sterile cotton applicator, all 4 sides of the agar block on the slide was inoculated with the suspension. Another pre-coated slide was placed on the top of the inoculated agar block. The filter papers were moistened with sterile distilled water to provide humidity for growth. After 36 h incubation at 37°C , when sufficient mycelial growth had occurred, agar blocks were removed and both slides were air dried and fixed in methanol for 30 min after which the slides were stored at 4°C and could be used for at least 4 weeks without any loss of antigenicity.

Agar gel double diffusion (DD)

A microtemplate modification of immunodiffusion method was used [12, 20]. Briefly, plastic templates with holes for antigens and sera were overlaid on agarose poured on pre-coated glass slides. The holes were filled with 20 μ l of the antigens/sera after the agarose was solidified. The slides were kept in a moist chamber for 72 h after which the templates were removed, the gels were washed and the slides were stained with Coomassie blue R 250. The reactions were graded as '–' (negative) and '+' (positive) depending on the presence of precipitin arcs and number of arcs.

Enzyme immunoassay

The BALISA used in the present study has been described previously [7]. Briefly, wells of polyvinyl microtiter plates (Nunc-Immunoplate, Denmark) were coated with 100 μ l of culture filtrate antigen (1 μ g/ml) in carbonate buffer (pH 9.6) by incubating for 3 h at 37 °C and then overnight at 4 °C. The wells were washed 3 times with phosphate buffered saline containing 0.05% (v/v) Tween 20 (PBST). Any free binding sites in the wells were blocked by treating with 1% bovine serum albumin (BSA) in PBST for 1 h at 37 °C. The wells were treated with 100 μ l of serum dilutions (1:100, 1:1000) for 1 h at 37 °C, goat antihuman IgG or IgE (1:1000, Sigma, St. Louis, MO), biotinylated rabbit antigoat IgG (1:5000, Zymed, San Francisco, CA) and streptavidin peroxidase (1:40000, Sigma, St. Louis, MO) each for 30 min at 37 °C. The wells were washed 3 times with PBST in between the addition of reagents. After washing, 200 μ l of chromogenic substrate (0.06% Orthophenylene diamine and 0.015% H₂O₂ in citrate buffer, 0.1 M, pH 4.5) was added and incubated for 15 min. The reaction was stopped by the addition of 20 μ l of H₂SO₄ and the absorbance was measured at 490 nm using an ELISA reader (Mini Reader, Dynatech, Chantilly, VA). Appropriate controls were included in each plate.

Immunogold assay (IGA)

Areas of distinct hyphal growth were circularly delineated on the reverse side of the slides with a diamond pencil. Three well separated areas were selected on each slide. The slides were rinsed in two 15 min changes of Tris buffer (pH 8.2) containing 0.1% (w/v) BSA (TBSA) and 20 mM sodium azide. After drying in air, the slides were transferred into a humid chamber. Rubber rings (6.5 mm in internal diameter and 1.2 mm thick) which were used to retain the reagents in marked areas, were rinsed in several changes of distilled water, blotted dry, and positioned on the previously marked areas of fungus growth. 40 μ l of serum dilutions were pipetted into the areas enclosed by the rubber rings. The presence of *A. fumigatus* IgG antibodies was tested using 1:100, 1:500 dilutions (in TBSA) of sera from healthy subjects. Specific IgE antibodies were evaluated with 1:2, 1:5 and 1:10 dilutions (in TBSA) of sera of patients and healthy subjects. After incubating 2 h in the humid chamber at 22 °C, the rubber rings were removed, slides were rinsed in TBSA and dried as before. The air dried slides were transferred back to the humid chamber and the rings repositioned. After that, 40 μ l of goat antihuman IgG or IgE (Sigma, St. Louis, MO) diluted to 1:80 was poured into the marked areas. After 2 h incubation, the rubber rings were removed and the slides were washed as described earlier. The slides were then treated with rabbit antigoat IgG (Sigma, St. Louis, MO) conjugated with colloidal gold, at a dilution of 1:80 and 1:40 in order to detect IgG and IgE respectively. The slides were left overnight in a humid chamber at 4 °C, were then washed again in TBSA, passed through changes of 75% absolute alcohol and xylol and mounted. The dilutions of serum and the reagents were chosen after repeated experiments with positive and negative sera. Affinity purified rabbit antigoat IgG was labelled with colloidal gold according to the procedure described by Geoghegan and Ackerman [4]. The preparations were examined by light and phase contrast microscopy. Appropriate controls were included with each experiment.

Grading of the reaction was recorded independently by three observers. A positive reaction under light microscope was evidenced by a brownish color on the surface of the hyphae due to binding of gold particles to the *A. fumigatus* hyphae. The results were recorded as negative when the hyphae appeared normal without any gold particles binding to their surface. The results were confirmed by phase contrast microscopy since a positive reaction consisted of hyphae stained reddish brown in color. This could be distinguished from the hyaline shining appearance of hyphae in the negative reaction. The reaction was graded from '–' to '+++' for negative to strongly positive. The highest dilution which consistently gave at least a '+' reaction was recorded as the titer of antibody in their serum. The questionable reactions were designated as $\pm$.

Results

The results of the study are shown in Table 1. Sera from all 10 patients showed precipitins

against *A. fumigatus* by agar gel double diffusion whereas control sera were all negative. *A. fumigatus* specific IgG and IgE antibodies were detected by BALISA in all patients and normal controls. However, the titers of IgG and IgE antibodies were consistently higher in the sera of patients than in controls. The level of IgG antibodies in one patient with aspergilloma was slightly lower than that of one normal control.

The IGA technique detected *A. fumigatus* specific IgG (Figs. 1 and 2) in all patients' sera; 7 sera showed a titer of 1:1000 while 2 showed a titer of 1:500. All control sera except one gave a negative reaction (Fig. 3 and 4). Specific IgE antibodies to *A. fumigatus* were detected by IGA in all patients, 7 gave a positive reaction at 1:10 dilution of serum, 1 at 1:5 and 2 at 1:2 dilution. The sera of all healthy persons were negative for specific IgE when tested at these dilutions. The degree of reactivity showed positive correlation with the BALISA results. The total serum IgE ranged from 96 to 29499 ng/ml (mean 12402 ng/ml) in patients and from 12 to 224 ng/ml (mean 101 ng/ml) in controls. Two of

Table 1. Results of Immunogold Assay Compared with DD and BALISA for Detecting IgG and IgE Antibodies to *A. fumigatus*.

Patient No.	Diagnosis	Immunogold		Double Diffusion	BALISA		Total IgE ng/ml
		IgG	IgE		IgG OD**	IgE OD	
1	ABPA	1:1000*	1:10	+	1.187	0.318	29499
2	ABPA	1:1000	1:10	+	1.024	0.337	28303
3	ABPA	1:500	1:10	+	0.785	0.211	555
4	ABPA	1:1000	1:10	+	1.191	0.776	23092
5	ABPA	1:1000	1:5	+	1.304	0.370	11369
6	Aspergilloma	1:100	1:2	+	0.361	0.100	96
7	Aspergilloma	1:1000	1:10	+	1.143	0.319	326
8	Aspergilloma	1:1000	1:10	+	1.086	0.388	15240
9	Aspergilloma	1:500	1:2	+	0.222	0.033	279
10	Aspergilloma	1:1000	1:10	+	0.983	0.267	15264
11	Normal	1:50***	–	–	0.164	0.036	12
12	Normal	–	–	–	0.250	0.133	109
13	Normal	–	–	–	0.165	0.056	95
14	Normal	–	–	–	0.063	0.020	65
15	Normal	–	–	–	0.259	0.103	224
16	Normal	–	–	–	ND	ND	ND

* Highest serum dilution giving a definite positive reaction.

** Optical density read at 490 nm

*** Questionably positive when tested at 1:50 dilution.

ND = Not done.



Fig. 1. A slide culture of *Aspergillus fumigatus* showing positive reaction by immunogold assay (IGA) using ABPA patient serum ($\times 400$).

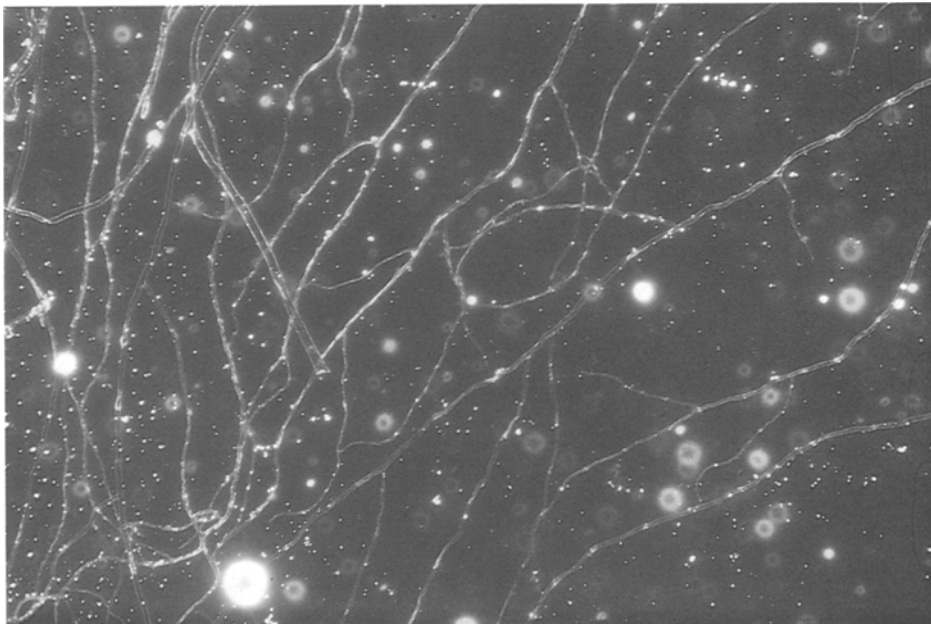


Fig. 2. A slide culture of *Aspergillus fumigatus* showing positive reaction by IGA using positive control serum under a phase contrast microscope ($\times 125$).



Fig. 3. A slide culture of *Aspergillus fumigatus* showing negative reaction by IGA using normal control serum ($\times 125$).

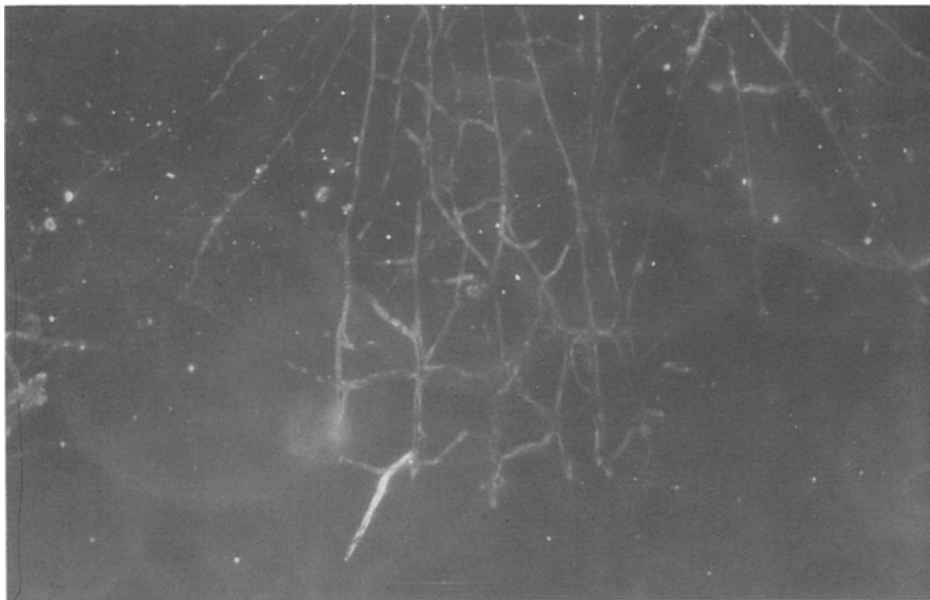


Fig. 4. A slide culture of *Aspergillus fumigatus* showing negative reaction by IGA using normal control serum (phase contrast) ($\times 125$).

the patients with aspergilloma had high total IgE values; it is not known whether they had concomitant ABPA.

Discussion

The present study has demonstrated that an immunogold assay (IGA) can be used to detect

A. fumigatus specific IgG and IgE antibodies in the sera of patients with *A. fumigatus*-induced diseases. One normal control had a questionable positive reaction for IgG at a 1:50 dilution. This person, a laboratory worker, has been frequently exposed to *A. fumigatus* antigens and the presence of antibodies in his serum may be due to the frequent stimulation of his immune system.

The IGA technique has several advantages

compared to other immunological methods: First, it is faster than DD, and unlike DD, IGA can also detect *A. fumigatus* specific IgE antibodies which is one of the essential criteria in the diagnosis of ABPA [2]. Furthermore, this method also has the added advantage that it can be utilized as a semi-quantitative assay to detect the levels of the antibodies.

Secondly, compared to enzyme- or radio-immunoassays (EIA and RIA) IGA is simple to be performed in a routine clinical laboratory without special equipments. In addition, as shown in the present study, IGA was able to discriminate patients from normal controls, whereas EIA and RIA often show detectable antibodies in controls due to the high sensitivity and less specificity of these techniques.

Thirdly, IGA has advantages over the indirect immunofluorescence (IF) method, where a fluorescent microscope is necessary and where auto-fluorescence of fungi may cause false positive results [8]. Similarly, the nonspecific reactivity of other staining methods such as immunoperoxidase (IP) are not found in immunogold staining [17]. Similar to IF- or IP-methods, IGA can also be successfully used in immunohistology. Furthermore, the sensitivity of IGA can be increased substantially with silver enhancement for comparable sensitivity with BALISA or RIA [16].

In conclusion, the IGA method offers a novel and simple tool to measure *A. fumigatus* specific IgG and IgE antibodies in patients' sera. The results of the present study indicate that IGA is more sensitive than DD and discriminates patients from the controls. Similar to the BALISA method, IGA detects both IgG and IgE antibodies against *A. fumigatus* in the sera of patients. In concordance with the results of DD, control sera failed to show detectable antibodies by IGA. Thus, immunogold staining can be successfully used to detect antibodies against specific antigen in patient serum faster, simpler and more reliably.

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