Modified Indirect Fluorescent-Antibody Test for Aspergillosis

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Diffuse fluorescence in an indirect fluorescent-antibody test for antibody to *Aspergillus fumigatus* was eliminated by using germlings, rather than hyphae, as antigen. The modified method yielded diagnostic titers $\geq 1:32$ in 24 of 25 known cases, was highly specific, and showed good correlation with clinical status and results of immunodiffusion. This technique, which uses the germling antigen, inactivation of test serum at $59 \pm 1^\circ\text{C}$, and anti-immunoglobulin G conjugate, is a sensitive, specific, and reliable quantitative indicator of aspergillosis and is useful in following the course of the disease.

Serological tests have achieved general acceptance as important aids in the diagnosis of aspergillosis (6, 8). The most widely used and most extensively evaluated of these tests is immunodiffusion (ID), or double diffusion in agar gel (3).

In the mycology laboratories of the Division of Laboratories and Research ID is the routine procedure, but we continue to investigate improved or alternative techniques, including counterimmunoelectrophoresis (CIE) (7), complement fixation, and indirect fluorescent antibody (IFA).

The ID test is highly specific for aspergillosis, but its sensitivity varies: 100% in aspergilloma, about 70% in allergic bronchopulmonary aspergillosis (ABA), and lower in invasive aspergillosis, as described by M. A. Gordon (*in Handbook in Immunology*, CRC Handbook Series in Clinical Laboratory Science, in press). The deficiencies of the ID test are mainly inadequate sensitivity in some forms of aspergillosis and that it cannot easily be quantitated, although the number of precipitin bands often indicates the type of clinical involvement. In a recent prospective study of invasive aspergillosis (11), ID was found to be specific but somewhat deficient in sensitivity.

CIE has, in our hands, proven more sensitive but less specific than ID; it may therefore be useful for screening. The complement fixation test allows quantitation, but its sensitivity in aspergillosis has been questioned, and there are divergent reports on its specificity (8, 9, 12).

The IFA test is a sensitive, quantitative technique for serological determination of aspergillosis. Drouhet (4) found that patients' serum titers ranged from 1:40 to 1:320, whereas normal serum titers were 1:10 or 1:20. However, IFA titers were not well correlated with the number of precipitin arcs. Warnock (13) re-

ported that although most normal persons demonstrated low-level immunoglobulin G (IgG) antibodies to *Aspergillus fumigatus* by IFA, patients with either ABA or aspergilloma showed higher titers ($\geq 1:80$) than either the normal individuals or patients with other mycoses (1:10 or 1:20). Warnock and Eldred (14), investigating 15 patients with either ABA or aspergilloma, found significant IFA titers of IgG but not IgA or IgM antibodies in all sera. Precipitating antibodies in ID were also IgG, and there was good correlation between the positive and negative results of the two tests.

The most serious disadvantage of the IFA test has been nonspecific reactions arising from the methods of antigen preparation. Drouhet (4) used a suspension of *A. fumigatus* mycelial fragments as antigen for the IFA test; Warnock (13) used Riddell-type slide cultures. In our hands both methods have resulted in nonspecific fluorescent staining of the hyphae, with a potential for yielding false-positive results. Experience with the slide culture method for preparation of *A. fumigatus* antigens and with Al-Doory and Gordon's fluorescent staining of other mycelial organisms (1) had indicated to us that specific fluorescence tends to concentrate in the younger portions of the mycelium, particularly in the hyphal growing tips. Consequently, we investigated the effect of using germinating conidia of *A. fumigatus* as antigen for the IFA test.

We report here a modified IFA test for aspergillosis, correlation of IFA and ID results, and discussion of the value of this method for serological diagnosis of aspergillosis.

MATERIALS AND METHODS

ID test for aspergillosis. The antigen, aspergillin, was prepared by culturing *A. fumigatus* for 2 months at 37°C in stationary 500-ml flasks, each

containing 100 ml of Sabouraud liquid medium (Difco). We grew four strains of this species (DLR 28B, 29C, 30B, and 30C) separately and combined the final products. After incubation, the flasks were shaken vigorously to break up and wet the mycelial mats. The contents were filtered through Whatman paper no. 40, and the filtrate was sterilized by a Seitz-type filter, mixed with twice its volume of cold acetone, and centrifuged at 2,500 rpm for 25 min. The sediment was freed of acetone by evaporation and then dissolved in a volume of 0.85% NaCl solution equal to half the original volume of the crude filtrate.

The test procedure included large central wells for test and control sera and both large and small peripheral wells for the antigen, as recommended by Proctor (10). Test plates were held at room temperature.

Protocol A: Mass production of IFA slides. For mass production of IFA slides, follow the steps outlined below.

(i) Culture *A. fumigatus* (DLR 30A) on Sabouraud dextrose agar slants at 27°C for 2 to 3 weeks.

(ii) Harvest the spores by carefully flooding the culture with about 8 ml of sterile distilled water containing 1 drop of Tween 80. Twirl each tube slowly to dislodge spores only. Transfer heavy spore suspension (McFarland nephelometer no. 4) to a sterile test tube and allow it to settle for 5 to 10 min. Then transfer the supernatant to a sterile tube and examine a drop microscopically for the presence of conidia and the absence of other structures.

(iii) Pipette 1 ml of this spore suspension into each of six to eight 100-ml tissue culture flasks (75-cm² surface growth area) containing 15 ml of sterile Sabouraud dextrose broth. Mix gently, and incubate the flasks horizontally at 42°C for 6 to 8.5 h. (Incubation may be interrupted at any point by storage at 4°C.) Examine, with an inverted microscope, after 6 h for completion of germination. Germination is complete when a large majority of spores have become swollen and when most have formed germ tubes—varying from just a bulge to several times the diameter of the swollen spore—but before branching has begun (Fig. 1). Germination must be stopped at or before this stage either by adding Formalin (2%, final concentration) or by storing at about 4°C, followed by adding Formalin. Fresh, ungerminated spores fluoresce very weakly or not at all. Nonspecific fluorescence, i.e., fluorescence of hyphae in the apparent absence of specific aspergillus antibody, appears with the onset of branching.

(iv) Allow the Formalin-treated suspension to stand overnight at room temperature.

(v) Filter through a 0.45-μm membrane filter (Nalgene filter unit, disposable). Suspend germings in 10 ml of sterile 0.85% NaCl solution, and filter again. Then suspend the germings in 0.1% Formalin-treated saline solution (1.5 to 2.0 ml).

(vi) Sonically treat the suspension for 15 s in an MSE (Measuring and Scientific Equipment, Ltd., London) apparatus at maximum amplitude (about 7 to 8 μm, peak to peak) to disperse any clumps. Repeat this treatment if microscopic examination shows clumping.

(vii) Adjust the suspension, with 0.1% Formalin-

treated saline at pH 7.6, to a density of 75 to 100 spores per low-power microscope field. This suspension can be stored at 4°C up to 4 weeks.

(viii) With a diamond-point pencil, heavily inscribe two 1-cm circles on an ordinary 1-mm-thick glass slide. Sterilize the slide by soaking it in 95% ethanol and then flaming.

(ix) Spread two bacteriological loopfuls of germinated spore antigen within each circle. Place the slide in a dust-free chamber, and allow it to air-dry at room temperature for 1 h.

(x) Fix the antigen for 3 min in a solution of 95% ethanol (60 ml), chloroform (30 ml), and Formalin (10 ml). Place the slide in 95% ethanol for 4 min, rinse by dipping in distilled water, and air-dry.

(xi) Slides can be stored at -20°C for at least 30 days.

Protocol B: Preparation of individual antigen slides. Prepare slides individually or in small numbers as follows.

(i) Culture and harvest as in protocol A, steps i and ii.

(ii) Prepare a light spore suspension (about 10 to 15 spores per low-power field) with a turbidity less than that of the no. 1 McFarland nephelometer standard. This can be stored at 4°C for up to 2 weeks.

(iii) Prepare slides as in protocol A, step viii.

(iv) Place a small drop of Sabouraud dextrose broth within each inscribed circle.

(v) Add a loopful of the spore suspension in each circle and mix carefully within the circle.

(vi) Place the inoculated slides in a moist chamber and incubate at 42°C for 6 to 8.5 h. Four slides can be incubated in a 150-mm petri dish, the cover of which holds a filter paper disk saturated with water. Incubation can be interrupted at any stage by refrigeration for a few days at about 4°C.

(vii) Examine the slides for proper germination as in protocol A, step iii.

(viii) Kill the germings by removing the filter paper disk and exposing the slides to 60 to 65°C heat for 1 h.

(ix) Perform steps x and xi in protocol A.

Protocol for IFA test. Perform the IFA test by following the steps below.

(i) Inactivate the test serum and control serum by heating for 30 min at 59 ± 1°C (5). Filter the serum, if necessary, to make sure it is clear.

(ii) Make eight serial twofold dilutions (1:8 to 1:1,024) of the test serum in volumes of 0.4 ml, using sterile 0.85% NaCl.

(iii) Remove the antigen slides from cold storage and restore them to room temperature.

(iv) Test two dilutions per slide if possible. Spread the diluted serum evenly within each circle, and incubate the slide in a moist chamber at room temperature (about 25°C) for 30 min.

(v) Dip slide in phosphate-buffered saline (PBS), pH 7.4, and wash for 5 min in each of two changes of PBS.

(vi) Gently and carefully blot the slide dry with filter paper. (Slides may also be air-dried; time required is 0.5 h. Proper blotting removes fewer than 10% of the germings.)

(vii) Place 1 drop of sheep anti-human IgG fluorescein conjugate (Burroughs Wellcome Co.), di-

FIG. 1. Stages in germination of conidia of *A. fumigatus*, in Sabouraud dextrose broth at 42°C. (a) Ungerminated; (b) preparatory swelling, with a few spores showing an apical protrusion; (c) early, or "schmoo"; (d) "tadpole"; (e) germ tube; and (f) branched germ tube. Conidia in stages b-e (5.5 to 8 h) are suitable for the IFA test. Methylene blue wet mounts. $\times 338$.

luted 1:4 with pH 7.6 PBS, on each antigen spot, and spread it carefully with an applicator stick.

(viii) Place the slides in a moist chamber at room temperature for 30 min.

(ix) Dip the slides in PBS, pH 7.4, and then place in two changes of PBS for 5 min each. Wash for 5 min in distilled water and blot gently with filter paper.

(x) Add a drop of buffered (pH 8.1) glycerol mounting fluid and a cover slip.

(xi) Read fluorescence on a scale of 0 to 4+, with 2+ or greater considered positive. The end point (titer) of each test serum is the highest dilution giving at least 2+ fluorescence.

RESULTS

This modified method produced specific, uniform fluorescent staining of the entire periphery

of the germling, including its subtending spore coat, as well as of swollen but ungerminated conidia, whereas nonswollen conidia did not fluoresce. Results were consistent and reproducible, and end points were generally sharp. Conidia swollen by means of hypotonic saline solution produced few germ tubes and stained poorly.

Correlation with clinical status. Of the 25 proven cases of aspergillosis in this series, sera from 24 yielded IFA titers of 1:32 to 1:512, distributed as shown in Table 1, with notably high titers in cases of ABA. Only four of these sera did not react in the ID test (two aspergilloma, two invasive), the rest yielding two, three, or four precipitin bands, except for four sera which gave only one band each (two aspergilloma, two

TABLE 1. *Distribution of IFA titers in 25 cases of aspergillosis*

Type of aspergillosis	No. of cases with IFA titer of:					
	16	32	64	128	256	512
ABA					1	2
Aspergilloma	1	6	4	3	3	
Invasive		1	3		1	

invasive). One of the aspergilloma sera, which gave only one precipitin band together with an IFA titer of 1:64, was from a patient whose later specimens showed as many as four precipitin bands and an IFA titer of 1:256. No particular correlation could be discerned, in general, between IFA titer and the number of ID bands.

Correlation with ID positivity or negativity.

Forty-six routine specimens were selected for IFA testing on the basis of ID or CIE positivity. Of the 20 ID-positive specimens, only four had IFA titers of $\leq 1:16$, while of the 26 ID-negative specimens, only one had an IFA titer of $>1:16$ (Table 2). It is uncertain, because of the lack of pertinent clinical information, whether this last specimen represents a false-positive diagnostic result.

In a separate set of 12 ID-positive sera, 10 were IFA-positive (1:32 to 1:256). One of the exceptions, with an IFA titer of 1:16, was from a patient whose second specimen, taken 3 weeks later, titrated 1:64. The other exception, for which no clinical history was available, had an IFA titer of $<1:8$.

Serial specimens. Serial specimens were obtained in nine cases of aspergillosis, of which six were diagnosed as aspergilloma, two as ABA, and one as invasive disease. In each case there was consistency in the consecutive IFA titers and in the number of ID bands. Either both of these values remained essentially constant, or they showed parallel changes through two to five specimens, reflecting the clinical course of the disease. For example, in a clinically stable case of aspergilloma with a possible invasive component, successive sera over 3 months showed IFA titers of 1:512, 1:256, and 1:256, and each yielded four ID bands. In a clinically deteriorating aspergilloma case followed for 4 months, the serial ID results were negative, one band, two bands, and four bands, with IFA titers of $<1:8$, 1:64, 1:128, and 1:256, respectively. In a third case of aspergilloma, which showed some clinical improvement over 9 months, five serial specimens yielded four, two, four, three, and one ID band and IFA titers of ND (not done), 1:128, 1:128, 1:64, and 1:16, respectively.

To summarize this series: of the nine cases,

seven were both ID positive and IFA positive (1:32 to 1:512) on the first specimen. Of the two original ID negatives, one was also IFA negative ($<1:8$) but later became both ID positive (four bands) and IFA positive (1:256). In the other case, successive specimens were ID negative but had IFA titers of 1:32 and 1:64, respectively. The latter case was one of necrotizing granulomatous aspergillus pneumonitis, which had been resected before the blood specimens were drawn. The specimens were taken during an ensuing development of maxillary sinus aspergillosis.

DISCUSSION

These results confirm the usefulness of the IFA test for diagnosis of aspergillosis and indicate that the quantitation it provides may be of value in following the course of the disease.

The IFA method with germings of *A. fumigatus*, anti-IgG conjugate, and heat-inactivated test serum yielded consistently reproducible results, which demonstrate that the test is both sensitive and specific as an indicator of aspergillosis. We found the specific fluorescence easy to read.

The sensitivity of this method is demonstrated by the strong correlation of positive IFA tests with clinical status and with ID results. We discerned no particular correlation between the number of precipitin bands for a given serum and its IFA titer, but few of the proven cases displayed fewer than two bands.

The specificity of the improved IFA procedure is indicated by the fact that of 26 ID-negative sera, only 1 had a positive IFA titer, and in this case a diagnosis of aspergillosis could not be ruled out. In one case of proven aspergillosis three successive serum specimens were positive for IFA but negative for ID.

In reviewing a total of 46 cases where sera showed IFA titers $\geq 1:32$, we found that a titer $\geq 1:64$ very probably indicated aspergillosis. A titer of 1:32 was usually associated with either known or probable aspergillosis, but lack of clinical data in the remaining cases leaves open the question of possible false positives. These 46 specimens were collected at various stages of the disease. If serial specimens were taken,

TABLE 2. *Correlation of IFA titers with ID results in 46 routine ID- or CIE-positive sera*

ID	No. of cases with IFA titer of:							
	<8	8	16	32	64	128	256	512
Positive	1	2	1	5	6	4		1
Negative	18	5	2		1			

beginning early in the course of the infection, IFA results would undoubtedly prove to be even more useful, as the serial results show.

We found the IFA test to be specific in all cases in which we had sufficient clinical information to evaluate it and must therefore disagree with a recent assessment of this test as being of "no apparent value. Equally reactive in patients with various forms of aspergillosis and normal controls" (2). The nonspecific reactions in these normal controls might possibly have been averted had germings, rather than mature hyphae, been used as the antigen.

We are currently engaged in further development and evaluation of CIE, which is more sensitive than ID, for aspergillosis screening of sera. We are also compiling a much larger series of cases for a more definitive comparative evaluation of ID, IFA, CIE, and complement fixation in the diagnosis and prognosis of various types of aspergillosis.

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