JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1983, p. 708–710
0095-1137/83/040708-03$02.00/0

Vol. 17, No. 4

Efficacy of Enzyme-Linked Immunosorbent Assay in Serodiagnosis of Aspergillosis

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Received 12 October 1982/Accepted 16 January 1983

Sera from 43 cases of aspergillosis, 73 cases of other bronchopulmonary diseases, and 50 healthy persons were examined for the presence of antibodies to *Aspergillus fumigatus*. Enzyme-linked immunosorbent assay proved to be more efficacious than the immunodiffusion test and counterimmunoelectrophoresis in the cases of allergic bronchopulmonary and invasive aspergillosis.

In the past few years, several novel methods have been introduced in the field of mycoserology (3). Of these, the enzyme-linked immunosorbent assay (ELISA), a test of younger generation, is a relatively simple but highly sensitive technique. There are, however, only a few sporadic reports concerning the usefulness of ELISA in the serodiagnosis of aspergillosis (1, 2, 8). The purpose of the present comparative study was to assess the efficacy of ELISA in demonstrating antibodies to *Aspergillus fumigatus* among patients with aspergillosis, especially when the commonly used methods such as the immunodiffusion test (IDT) and counterimmunoelectrophoresis (CIE) fail to yield a positive result.

An ammonium sulfate-precipitated protein-glycoprotein antigen was prepared from a strain of *A. fumigatus* (D142) as described previously (6). The dialyzed and freeze-dried antigen was reconstituted in appropriate buffer to give a desired concentration. A solution containing 5 mg of the antigen per ml was used in IDT and CIE, and the standard procedures were employed to carry out the tests (6, 7). ELISA was performed in disposable microtiter plates (cobalt-treated Dynatech Micro-ELISA, F-form, M 129B). The antigen was diluted in 0.05 M carbonate buffer, pH 9.6, to give a final concentration of 10 μg/ml, and a volume of 100 μl was put into each well. After overnight incubation at room temperature, the plates were washed with phosphate-buffered saline (7) containing 0.05% Tween 20 (PBS-T), pH 7.4, using a Dynatech Miniwash apparatus. The wells were then filled with 200 μl of PBS-T supplemented with 5% bovine serum, incubated at 37°C for 1 h, and washed three times with PBS-T. The plates were air dried and stored at 4°C until needed. Starting with 1:100, serial twofold dilutions of the positive and negative controls and the test sera were made in PBS-T directly in the plates. Leaving aside the first row of blanks, the wells were filled with 100 μl of the diluted sera. The plates were incubated for 1 h at 50°C and washed three times with PBS-T. During the initial exploratory study, we observed that by incubating the plates at 50°C instead of at 37°C or room temperature (at this and the subsequent steps) the test could be completed within a shorter period without affecting its sensitivity or specificity. A 1:3,000 dilution of affinity chromatography isolated anti-human IgG from goat, conjugated to alkaline phosphatase (Tago, Burlingame, Calif.), was prepared in 0.05 M Tris-hydrochloride buffer containing 1% bovine serum, pH 7.4. The optimal dilution of the conjugate and the antigen was determined through a checkerboard titration. Each well was filled with 100 μl of the diluted conjugate, and the plates were reincubated at 50°C for 1 h. After washing five times with PBS-T, 100 μl of a freshly prepared solution of the substrate (*p*-nitrophenyl phosphate, disodium, Sigma 104 phosphatase substrate; Sigma Chemical Co., St. Louis, Mo.) solution at 1 mg/ml in 0.016 M carbonate buffer containing 1 mM $MgCl_2$ was added into each well. The plates were further incubated for 45 min at 50°C, and the enzyme reaction was then stopped by adding 50 μl of 1 M NaOH solution. The color was first examined visually and recorded. Absorbance of the mixture was measured in a photometer (Titertek Multiskan) at 405 nm against the blanks. The results were considered as positive only when the absorbance was at least 2.1 times the mean of the negative serum used as reference (4, 5). A serum sample showing an absorbance of 0.3 or more at 1:800 or higher dilutions was considered positive. This criterion effectively discriminated between the sera from the unequivocally diagnosed cases of aspergillosis with a low level of antibodies and those from patients with no indication of infection by *Aspergillus* species.

TABLE 1. Comparative efficacies of ELISA, IDT, and CIE in the detection of antibodies to *A. fumigatus* in the sera of aspergillosis patients[a]

Diagnosis	No. of sera examined	No. positive for antibodies to *A. fumigatus* in:		
		ELISA	IDT	CIE
Bronchopulmonary aspergilloma	17	17	17	17
ABPA	18	18	14	13
Invasive aspergillosis	8	6	3	4
Systemic candidiasis	4	1	0	0
Miscellaneous bronchopulmonary diseases[b]	69	3	2	1
Apparently healthy donors	50	1	0	0

[a] Sera from other donors served as controls.

[b] Tuberculosis, bronchial asthma, lung cancer, etc. Secondary mycotic complications were suspected in these cases, and *A. fumigatus* was isolated in culture from nine of them.

A total of 166 samples of serum obtained from the same number of individuals were investigated (Table 1). These included 17 cases of aspergilloma, 18 of allergic bronchopulmonary aspergillosis (ABPA), and 8 cases of invasive aspergillosis. Sera from 4 cases of systemic candidiasis, 69 cases of miscellaneous bronchopulmonary diseases (tuberculosis, bronchial asthma, lung cancer, chronic bronchitis, etc.), including 9 patients whose sputa yielded *A. fumigatus* in culture, and 50 apparently healthy donors were examined with a view to assessing the specificity of the test.

As illustrated in Fig. 1, a concentration of 10 μg/ml of the antigen and a 1:3,000 dilution of the conjugate most effectively discriminated between positive and negative sera at a dilution of 1:800 in ELISA. At this dilution, the absorbance at 405 nm of the positive control serum was 1.26 (standard deviation, 0.17), and that of the negative control serum was 0.16 (standard deviation, 0.04). The comparative efficacy of ELISA in the detection of antibodies specific to *A. fumigatus* is summarized in Table 1. Serum samples from all the investigated cases of aspergilloma and ABPA gave a positive result in ELISA. It is noteworthy that sera from four of the ABPA patients were negative in IDT, CIE, or both. Sera from a majority of aspergilloma patients and from 50% of ABPA patients gave a positive result in ELISA even at a dilution of 1:12,800 or more. Of the eight cases of invasive aspergillosis, serum samples from six were positive for antibodies to *A. fumigatus* in ELISA against only four cases in IDT and CIE. The two patients in whom antibodies could not be demonstrated by any of the methods had severely impaired immunity and suffered from disseminated aspergillosis (confirmed histopathologically); a test for the presence of circulating antigen in their sera was not done. Only 3 of the 69 serum samples obtained from cases of miscellaneous bronchopulmonary diseases were positive at a dilution of 1:800 in ELISA. One of them was later diagnosed as a case of Farmer's lung and the precise etiology in the remaining two cases could not be established. A similar titer (1:800) was observed in a serum sample from an apparently healthy donor and another serum from one of the cases of systemic candidiasis. There was no other evidence suggestive of aspergillosis in these two individuals, and *A. fumigatus* was never isolated in culture.

Not much data are available concerning the usefulness of ELISA in the serodiagnosis of aspergillosis. Our observations are to some extent comparable to those of Holmberg et al. (2), who also used an ammonium sulfate-precipitated protein-glycoprotein antigen from *A. fumigatus*. They, however, employed macro-ELISA in the test tubes, which requires relatively large amounts of reagents. Of the other reports, Greenberger and Patterson (1) investigated sera only from the cases of ABPA and Sepulveda et al. (8) and have studied cases of aspergilloma and ABPA but not of invasive aspergillosis. Nevertheless, their findings as well as the data included in the present study amply support the efficacy of ELISA in the serodiagnosis of asper-

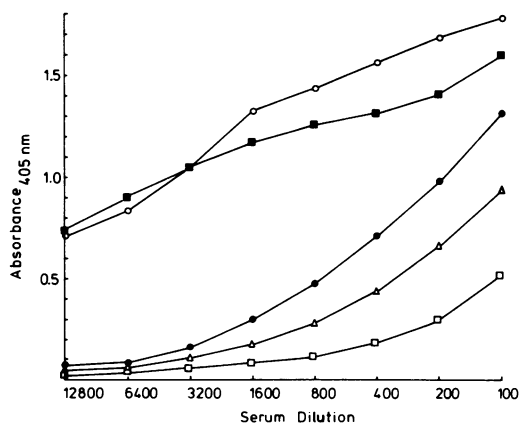

FIG. 1. Absorbance values at 405 nm obtained at various dilutions of the sera from positive (■) and negative (□) controls and from one case each of aspergilloma (○), ABPA (●), and invasive aspergillosis (△). The alkaline phosphatase-conjugated anti-human IgG was diluted 1:3,000, and the microtiter plates were precoated with 10 μg of protein-glycoprotein antigen per ml.

gillosis. Its advantage over the conventional techniques of IDT and CIE is clearly evident in cases of ABPA and invasive aspergillosis.

We acknowledge the technical assistance of W. Altmann and G. Heins.

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