Original articles

Comparison of antibody measurements against *Aspergillus fumigatus* by means of double-diffusion and enzyme-linked immunosorbent assay (ELISA)

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IgG antibody titers against Aspergillus fumigatus from different sera were measured by means of a standardized ELISA and compared with precipitates measured by double diffusion (D.D.). There was a significant correlation between the number of precipitates and the ELISA IgG titers in the 758 sera examined. However, in several individual sera within this group, large deviations between these two immunologic parameters were found. Further analysis indicated that ELISA detects antibodies against nonprecipitating antigenic components in addition to the antibodies detected by D.D. Furthermore, not all precipitating antigenic components appear to play a role in the detection of antibodies against *A. fumigatus* by ELISA. Patients with aspergillosis largely show increased titers of IgG antibody by ELISA even when the results of D.D. are negative, except those of patients with Aspergillus-provoked asthma which fall within a normal range. (J ALLERGY CLIN IMMUNOL 72:255-261, 1983.)

The D.D. test according to Ouchterlony is used as a routine diagnostic tool in detecting precipitating antibodies against *Aspergillus fumigatus*. In patients with different forms of aspergillosis, in patients with atopy for *Aspergillus* (positive skin tests), and also to a lesser extent in patients who are notatopic for *Aspergillus*, precipitating antibodies have been demonstrated. Measurement of antibodies against *A. fumigatus* by means of ELISA as described by Hommel et al. is a better parameter for the quantification of antibodies. The use of this method in patients with different forms of aspergillosis was recently demonstrated.

In our laboratory the results of antibody quantification against *A. fumigatus* by ELISA in patients' sera were in accordance with those of Sepulveda et al., showing increased titers of IgG antibodies against *A. fumigatus* in patients with aspergillosis. However, discrepancies were observed between antibody titer of individual sera and a corresponding precipitating pattern.

Also in the study of Mantyjärvi et al., the results of antibody detection against *A. fumigatus* by ELISA and D.D. in patients with farmer's lung disease showed differences. These observations suggested that the antigenic components are differently involved in the two assays. In order to study the quantitative relationship between the two immunologic assays, a comparative study of precipitating properties and corresponding ELISA titers against *A. fumigatus* was...
performed in a group of sera. The number of precipitation lines only indirectly reflects the quantity of antibodies, since (1) a second quantitative aspect of the D.D., the amount of precipitate present in one precipitation line, cannot be quantified and (2) different antibodies directed against the same antigenic component are not differentiated. This second quantitative aspect of the D.D. was studied visually for individual sera.

Our preliminary studies and those of Mantyjärvi also indicate that elevated titers of IgG antibodies against *A. fumigatus* may be found in groups of patients without well-defined forms of aspergillosis. Therefore the prevalence of antibodies against *A. fumigatus* measured by D.D. and ELISA was measured in a group of patients (n = 758) not limited by a known form of aspergillosis. The clinical relevance of the assays is discussed by examining the serologic data from a limited number of patients with aspergillosis (n = 51) who possess characteristics of the disease.

**MATERIALS AND METHODS**

**Antigenic extract**

Both D.D. and ELISA measurements were performed with one batch of metabolic antigen (No. 0057) of *A. fumigatus* obtained from a 2% glucose Sabouraud culture (35 days culture, pH of the medium 8.3) as described previously. Throughout this study experiments and reading of precipitating lines were carried out by the same technician.

**D.D. assay**

The D.D. was performed according to the method of Ouchterlony, with the use of an agarose gel with a depth of 4 mm, a central well of 10 mm diameter, peripheral wells of 3.5 mm, and an edge-to-edge distance of 4 mm. After application of the antigen (100 mg/ml *A. fumigatus*) and sera, the plates were incubated for 72 hr at room temperature in a small plastic box in order to keep humidity constant. Thereafter, the agarose gels were extensively washed, dried, and stained with Coomassie brilliant blue (Serva, Heidelberg, West Germany). Nonspecific precipitation caused by C-reactive protein was prevented by addition of citrate buffer in the gel. The dried and stained gels were stored and fixed on glass plates (5 by 5 cm) in order to compare visually the total amount of precipitate of individual sera.

The macromethod of D.D. using antigen concentrations of 100 mg/ml was used because of its great sensitivity in the detection of precipitating antibodies. Because of this sensitivity, 1 to 3 weak precipitates can also be found in sera of normal individuals. Therefore, in clinical practice, results were recorded as meaningful (positive) when the number of precipitates exceeded three.

**ELISA assay**

IgG antibody titers against *A. fumigatus* were determined by ELISA with microtiter plates as described by Vollert et al., and a double-antibody assay according to the method described by Sepulveda et al. with some modifications.

Wells of the microtiter plates (AR plates; Greiner) were sensitized with 250 µl of metabolic antigen (1 mg/ml antigen, 0.1M NaHCO₃, pH 9.6) and incubated overnight at 4°C. After washing four times with 250 µl of PBS buffer (0.1M, pH 7.4) containing 0.05% polysorbate 20 (Tween 20; ICI Americas, Inc., Wilmington, Del.) and 0.2% gelatin (PTG buffer) with the use of the Dynatech washing equipment, the plates were incubated 4 hr at room temperature containing the same washing buffer in order to decrease aspecific binding in the succeeding antibody binding steps. In order to measure IgG antibodies against the immobilized antigen, the following successive incubation steps were performed:

1. Dilute patients' and control sera (250 µl) 1:250 in PTGB buffer and add in duplicate to the wells; incubate for 60 min at 37°C; wash four times with PTGB buffer.
2. Incubate with 250 µl of RaHulG (Nordic Immunology) diluted 1:500 in PTGB buffer for 60 min at 37°C; wash four times as described above.
3. Incubate with 250 µl of enzyme conjugate (GoRaIgG-alkaline phosphatase; Miles Laboratories) diluted 1:1000 in PTGB buffer for 60 min at 37°C; wash four times.
4. Incubate for 30 min at 20°C with substrate (250 µl of p-nitrophenylphosphate, 1 mg/ml, in 10% diethanolamine buffer and 0.5 mM MgCl₂, pH 9.8).

The reaction was stopped by the addition of 50 µl of 3N NaOH. The absorbance at 405 nm was measured on a Beckman spectrophotometer (Model 25) after dilution of the samples with distilled water (1:9). In order to compare ELISA titers from different microtiter plates, positive and negative standards were set up in every microtiter plate. The negative-pool serum was a pool of 10 sera that had been shown to be negative in precipitating antibodies (zero precipitation lines), and its titer (A₀) was assumed to be due to aspecific binding of serum proteins. The positive-control serum was one showing eight precipitates by D.D.

The serum concentration dose-response curve of this positive serum with fourfold dilution steps (Fig. 1) showed...
a maximal binding of antibodies between 1 and 0.25 μl of serum and reached the value of the negative control (\(A_y\)) when the serum was diluted 1:1000.

The absorbance value found with 1 μl of serum is defined as \(A_x\). The titers of the individual sera \((A_x)\) were expressed as percentages of the titer of the positive control serum by the following equation:

\[
\% A_x = \frac{A_x - A_y}{A_x} \times 100\%
\]

A group of 50 sera of normal individuals, obtained from the Blood Transfusion Service of the University Hospital, showed a mean titer of 8.1% ± 11.7. None of these sera showed elevated numbers of precipitates by D.D. Therefore titers of individual sera exceeding 31.5% (mean ± 2 S.D.), as indicated in Fig. 1, were regarded as positive. This value corresponds with a dilution of the positive control serum of more than 1:200 as compared with the standard assay conditions (1 μl of serum per 250 μl of assay buffer).

**Sera**

The 758 sera studied were from patients who visited the outpatient department and/or were hospitalized and who were for any clinical reason suspected of a possible involvement of *Aspergillus*. Sera included in this study were from subjects with either reversible or irreversible obstruction, patients suspected of different forms of extrinsic al-lergic alveolitis, and nonasthmatic subjects with different forms of lung pathology such as cystic fibrosis, sarcoidosis, bronchiectasis and/or cavities (including aspergilloma), and bullae, making them more susceptible for *Aspergillus*.

The sera of the patients who revisited the outpatient department at several occasions were repeatedly measured, and these data are included in this comparative study. A limited number of the sera \((n = 51)\) were from patients with known histories of aspergillosis. The clinical significance of the serologic data of this group was investigated by comparing the titers of antibodies found in separate groups of patients classified into four categories: (1) patients with aspergilloma (sera 20; subjects 13); (2) patients with ABPA (sera 15; subjects 8) by criteria derived from United Kingdom and United States studies\(^2,14\); (3) patients with *Aspergillus*-provoked asthma (sera 4; subjects 4); and (4) an AC group (sera 12; subjects 7). The last group consisted of patients with a variety of pulmonary disorders of longer duration (e.g., bronchiectasis, emphysematous bullae, status after lobectomy, pre-existing tuberculosis), who sometimes showed the fungus in the expectorations, had some precipitation lines, and sometimes showed a positive skin test or RAST.

It is important to note that the IgG ELISA titers were not used in the aforementioned criteria for diagnosis of the patient. The original diagnosis of a patient was maintained in this study also when criteria changed because of the
dynamics of the ongoing process or because of corticosteroid medications.

RESULTS

During this study 758 sera were tested. In the D.D. assay 199 sera (26.2%) did not show any precipitates, 504 sera (66.4%) formed 1 to 3 precipitates, and 55 sera (7.4%) showed precipitates ranging in number from 4 to 11 (Fig. 2).

Measurement of ELISA antibody titers showed 631 sera (83.2%) negative for IgG antibodies against A. fumigatus and 127 sera (16.8%) positive.

In Fig. 2 the number of precipitation lines of the individual sera is plotted against the mean values of the corresponding IgG ELISA titers. Although the number of precipitation lines is only a rough indication of an amount of precipitate, a good correlation is found between the numbers of precipitates and the mean of the corresponding IgG ELISA titers. Furthermore, linear regression analysis revealed a corre-

lation between the number of precipitation lines of individual sera (n = 758) and the corresponding IgG ELISA titers (r = 0.5705). However, as can be seen from the large S.D. values (Fig. 2), discrepancies were also found between these two parameters with individual sera, indicating that the quantity of precipitation lines and corresponding ELISA titers of individual sera often are not related to one another.

In Table 1 the numbers of sera with normal (≤31.5%) or increased (>31.5%) ELISA titers and corresponding numbers of precipitation lines are summarized. Eighty-eight sera were detected as having few precipitins (0 to 3 precipitation lines) but increased ELISA titers. By contrast, a smaller group of sera (n = 16) was found with 4 to 6 (weak) precipitins that were negative by ELISA.

In Fig. 3 some individual sera are demonstrated, ordered according to increasing ELISA titer that, in addition, demonstrated that increasing titers of ELISA IgG antibodies are found in sera with decreasing numbers and total amounts of precipitate (sera 3 to 5). From these discrepant observations it is concluded that antibodies against A. fumigatus are measured by ELISA that are not detected by D.D., and, on the contrary, some antibodies detected by D.D. are only partially measured or not measured at all by ELISA. The ELISA IgG antibody assay especially detects antibodies against antigenic components in addition to the D.D. assay (n = 88). Similar deviations of antibody titers and precipitates were also observed with the sera of patients with aspergillosis, present in this group of sera (see Table 1, numbers in brackets).

In Fig. 4, A to C, the ELISA IgG titers and corresponding number of precipitation lines of individual sera are shown for the sera from patients with aspergillosis, ABPA, Aspergillus-provoked asthma, and the AC group. As can be seen in Fig. 4, A, sera of patients with aspergillosis exclusively show both highly elevated titers of ELISA IgG titers and increased numbers of precipitation lines. In this figure, one observation is shown of a patient with a longstanding history of aspergillosis, but this patient had coughed up his fungus ball spontaneously about 1 yr before (5 weak precipitates, ELISA titer 0%). Although increased titers of both antibody assays are found with the sera of patients with aspergillosis, sera are also found with only a minor number of precipitates (4 to 6) that still show highly elevated ELISA IgG titers (see also Fig. 3).

In accordance with earlier observations, the patients of our study with ABPA showed fewer precipitating antibodies (Fig. 4, B), some sera with 2 to 3 (minor) precipitates, and one serum with no precipitates at all. By contrast, all sera were positive for
FIG. 3. D.D. patterns and the corresponding ELISA IgG titers against A. fumigatus of sera from patients with aspergillosis (first serum from an AC patient with cystic fibrosis; sera 2 to 5 from patients with aspergillosis).

TABLE I. Number of sera with normal (≤31.5%) or increased (>31.5%) ELISA titers and corresponding number of precipitation lines

<table>
<thead>
<tr>
<th>ELISA titer (%)</th>
<th>Number of precipitation lines</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>&gt;31.5</td>
<td>9</td>
</tr>
<tr>
<td>≤31.5</td>
<td>190</td>
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*Numbers in parentheses are the number of sera that could be ascribed to patients with aspergillosis.

ELISA IgG antibody, indicating that measurement of ELISA IgG titers adds information on antibodies against A. fumigatus in addition to the information obtained from D.D. that is diagnostically important. The observations with sera of one individual patient who was regularly seen during this period because of treatment of the ABPA (by corticosteroid and antifungal therapy) has been indicated separately (Fig. 4, B, connected dots), showing a decrease in both immunologic parameters during the course of the therapy. The sera from all the patients in the AC group showed an increased number of precipitation lines (8) and/or increased titers of ELISA IgG antibodies (10). Again large discrepancies can be observed between the two immunologic parameters. The sera of the four patients with Aspergillus-provoked asthma show low titers of ELISA IgG (<33%) and 4 to 5 (weak) precipitates. One serum was negative in both assays.

DISCUSSION

Comparison of ELISA titers of large groups of individual sera is hampered by the variable binding of antigenic components to different microtiter plates and differences in antigenic extracts used. In this study this difficulty was largely overcome by the introduction of a standard assay procedure using one batch of antigen and expression of individual titers in percentages of a positive control serum that was used in every assay procedure (see Methods). By means of this standardized procedure, the antibody titers of individual sera were measured in a group of patients with different forms of lung disease, including patients with aspergillosis. It was shown that, in general, increasing numbers of precipitation lines found by D.D. are significantly related with increasing ELISA IgG titers. However, it was also shown that the results of the two serologic assays may have considerable deviations from one another. The discrepancies between the two assays are two-directional, showing both (1) sera with slightly elevated numbers of precipitates by D.D. and negative ELISA IgG titers (n = 16) and (2) a larger group of sera (n = 88) with elevated ELISA IgG titer but negative or limited numbers of precipitates (1 to 3). Furthermore, individual sera were demonstrated with total amounts of precipitate that did not correlate with the corresponding ELISA IgG titers.

The discrepant observations can be explained by assuming that (1) by ELISA, antibodies can be detected against antigenic components that do not precipitate in D.D.* but are immobilized on the polysty-

*Note: During the XI International Congress of Allergology and Clinical Immunology (London, 1982), Longbottom showed by CRIE that A. fumigatus can form precipitates that cannot be visualized by normal staining procedures. The possibility that these antigenic components, by binding to polystyrene surfaces, will be an important cause of the observed discrepancies is likely but needs further study.
rene surface and (2) not every precipitating antigenic component is bound to the polystyrene surface, either because of limitations defined by the mechanism of binding or due to competition with stronger binding components.

Similar deviations between detection of precipitating antibodies and ELISA IgG titers were also described recently for *Alternaria* antigens² and thermophilic *Actinomyces*.¹² These observations and our study indicate that patients may show antibodies against nonprecipitating antigenic components⁶ whereas antibodies against the precipitating antigenic components present in the same extract are limited or even absent. In addition to this latter explanation, it also remains possible that a group of precipitating antigenic components are present in the extract in concentrations too low to cause a detectable precipitate in D.D. but are predominantly bound to the polystyrene surface.

In accordance with previous observations,¹⁰ patients with aspergilloma showed both highly elevated IgG titers by ELISA and increased numbers of precipitates with the *Aspergillus* extract (Fig. 4). Patients with ABPA or *Aspergillus* colonies also showed elevated ELISA titers⁶,¹⁰ but only some of them showed increased numbers of precipitates (Fig. 4, B). One patient (Fig. 4, B) was observed who showed that both ELISA titers and D.D. can be used to follow the immunologic response during therapy.

Our observations on sera with weak or no precipitating antibodies in patients with aspergillosis are in accordance with recently described patients with ABPA without detectable precipitating antibodies in their serum.¹⁵ The difficulty of detecting precipitating antibodies in patients with ABPA has already been noted⁴,¹⁰ and therefore the use of several antigenic extracts was introduced.⁵,¹⁰ The results of our study indicate that the failure to find antibodies against *A. fumigatus* by means of D.D. is not necessarily a result of the limitation of the antigenic extract (amount of antigenic components) but may be due to the limitation of the Ouchterlony technique, detecting only those components that are able to precipitate (and can be visualized (see footnote).

The small group of patients with *Aspergillus*-provoked asthma (Fig. 4, C) did not show elevated IgG titers by ELISA when compared with a group of normal individuals. This observation is in accordance with previous reports⁸ on patients with *Aspergillus*-provoked asthma in which titers were much lower than those of patients with ABPA.

With the introduction of the sensitive enzyme assay, a relative large group of sera (88/758) was found with limited numbers (1 to 3 weak precipitates) or absence of precipitates in the D.D. and with elevated ELISA IgG titers against *A. fumigatus*. Within this group 13 of the 88 sera were from patients with aspergillosis (Table I), indicating the diagnostic importance of antibody detection by ELISA in addition to that by D.D. However, a residual group of sera (77/
758) was also found with elevated ELISA IgG titers from patients not known to have aspergillosis. Cross-reactivity between antigens of *Aspergillus* and other microorganisms and house dust as was demonstrated by Bardana1 can possibly (in part) be an explanation for the elevated ELISA IgG titers in this latter group. However, information on either skin reactivity and/or IgE antibodies against *A. fumigatus* is incomplete in this latter group. Therefore the (clinical) significance of elevated IgG ELISA antibody titers in sera of patients with weak or absence of precipitating antibodies needs further study.

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REFERENCES