Aspergillus fumigatus-Specific Antibodies in Allergic Bronchopulmonary Aspergillosis and Aspergilloma: Evidence for a Polyclonal Antibody Response

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Patients with the Aspergillus-induced diseases allergic bronchopulmonary aspergillosis (ABPA), aspergilloma (fungus ball), and Aspergillus skin test-positive asthma were differentiated immunologically by radioimmunoassay based on their total immunoglobulin E (IgE) and Aspergillus fumigatus-specific IgE levels. In this study, a new, highly sensitive biotin-avidin-linked immunosorbent assay was used to evaluate A. fumigatus-specific antibodies of all immunoglobulin classes. Studied populations included 13 patients with ABPA, 12 with aspergilloma, 9 with A. fumigatus skin test-positive asthma, and 9 normal individuals without asthma. A. fumigatus-specific antibodies of all classes were elevated in patients with ABPA, variably elevated in those with aspergilloma, and lowest in the other two groups. This assay demonstrated significantly higher specific IgE antibody levels in the ABPA group over those of the other groups even with 1:1,000 dilutions of the sera. This study demonstrated that ABPA is a disease characterized by a polyclonal antibody response to Aspergillus antigen and not just a response to IgE and IgG antibody classes. The measurement of other antibody classes, particularly IgD and IgA, could enhance the immunodiagnosis of ABPA. The biotin-avidin-linked immunosorbent assay was found to be a highly sensitive assay that can be a clinically useful alternative to radioimmunoassay in the measurement of A. fumigatus-specific antibodies.

The high levels of total immunoglobulin E (IgE) and Aspergillus fumigatus-specific IgE and IgG found in patients with allergic bronchopulmonary aspergillosis (ABPA) have been used to differentiate these patients from those with aspergilloma or A. fumigatus skin test-positive asthma and from normal individuals (6, 17, 19, 24). Although aspergilloma can be diagnosed by nonimmunological methods, fungal-specific antibodies can be detected early in the disease and also help in determining the fungal species involved. However, because comparative studies of A. fumigatus antibody isotypes in Aspergillus-induced diseases had not been carried out previously, it was not known whether the antibody response in ABPA was polyclonal or whether it involved only a few immunoglobulin classes. Hence, we undertook this study to determine whether other A. fumigatus-specific immunoglobulin levels would be useful in differentiating patients with A. fumigatus-induced diseases.

The enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) have been used successfully to determine A. fumigatus-specific IgG and IgE levels in the sera of patients with ABPA and aspergilloma (19, 24). Because of relatively high antibody concentrations, the conventional ELISA has been shown to be useful in the quantitation of antigen-specific IgG (6, 19). However, because the conventional ELISA lacks sufficient sensitivity, this method cannot reliably determine the relatively low levels of antigen-specific IgE (19). To overcome the lack of assay sensitivity, it is common to use serum specimens, either undiluted or in modest dilutions, such as 1:5 or 1:10 (6, 24). The use of such high concentrations of patient serum is wasteful of potentially scarce specimens and can result in nonspecific errors in the assay. Therefore, the RIA, a more sensitive assay, has often been used to determine antigen-specific IgE levels (24). There are disadvantages to the RIA method, however, including the limited storage life of the radioisotope, the length of time required to complete the assay, the need for expensive radiation counters, the hazards of exposure to radioactivity, and the difficulties in disposal of the radioactive wastes.

To overcome these disadvantages, we have used a modified ELISA technique, the biotin-avidin-linked immunosorbent assay (BALISA), in this study of specific Aspergillus antibodies in human disease. The BALISA has a sensitivity comparable to that of the RIA but does not use radioisotopes. The method is based on the high-affinity binding (K_a=10^{-15}/M) between the vitamin biotin and the hen egg protein avidin. Each molecule of avidin binds four molecules of biotin. Both avidin and biotin can be covalently coupled to other molecules such as proteins and polysaccharides. Incorporating the biotin-avidin linkage into an assay system results in markedly enhanced sensitivity (7, 8, 20). BALISA has been used previously to determine low levels of keratin (13), polypeptide hormones (7), specific DNA (20), Candida antigens (1), antistreptococcal antibodies (15), hepatitis antibodies (10), and total allergen-specific IgE (16).

MATERIALS AND METHODS

Subjects. The study included 13 patients with ABPA, 12 with aspergilloma, 9 asthmatics with immediate wheal and flare skin reactions to Aspergillus antigen, and 9 normal controls. The patients with ABPA met the criteria for this disease described by Rosenberg et al. (17) and were seen either at Northwestern University School of Medicine or at The Medical College of Wisconsin Affiliated Hospitals. The aspergilloma patients met clinical, radiological, histopathological, and microbiological criteria for the disease as outlined by Varkey and Rose (22). Asthmatics with immediate wheal and flare skin reactivity to Aspergillus antigen and
with no features of ABPA and also nonallergic nonasthmatics served as control subjects.

**Antigen.** The antigenic material used in this study was a culture filtrate preparation obtained from *A. fumigatus* Ag507 as previously described (11). The material was lyophilized and stored at -70°C until just before use.

**Antibodies assayed.** Immunoglobulin classes IgG, IgE, IgD, IgA, and IgM were assayed by BALISA.

**BALISA.** The BALISA method is a modification of the conventional ELISA described by Voller et al. (23) and Kurup (10a) (Fig. 1). Briefly, 100 μl of *A. fumigatus* antigen (1 μg/ml) diluted in carbonate buffer (pH 9.6) was added to each well of a microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.) and incubated for 4 h at 37°C. The wells were emptied and washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween). The plate was stored at -70°C until used.

One hundred microliters of blocking solution (1% bovine serum albumin in PBS-Tween) was added to each well, and the plate was incubated at 37°C for 30 min. The wells were then washed three times with PBS-Tween. All dilutions of the antibodies used in the assay were made with the blocking solution.

Next, 100 μl of patient sera (1:1,000) was added to each well, and the plate was incubated at 37°C for 30 min. The wells were washed as above with PBS-Tween. One hundred microliters of a 1:1,000 dilution of goat anti-human IgG, IgE, IgD, IgA, or IgM (Sigma Chemical Co., St. Louis, Mo.) was then added to each well. The plate was incubated at 37°C for 30 min and washed as above. Subsequent steps included avidin and biotinylated antibodies (available as the Vectastain ABC Kit; Vector Laboratories, Burlingame, Calif.) as outlined by Hsu et al. (8). One hundred microliters of a 1:1,000 dilution of normal rabbit serum (in bovine serum albumin–PBS-Tween) was added to each well, incubated at 37°C for 30 min, and washed. One hundred microliters of a 1:200 dilution of biotinylated rabbit anti-goat immunoglobulin antibody was then added to each well, incubated at 37°C for 30 min, and washed. The next reagent was made by mixing an avidin solution (1:200 dilution) with an equal volume of horseradish peroxidase-conjugated biotin solution (1:200 dilution). One hundred microliters of the resulting solution was added to each well, incubated for 30 min at 37°C, and washed. Finally, 200 μl of a peroxide-activated orthophenylenediamine (Sigma) solution (0.06% orthophenylenediamine, 0.015% H₂O₂ in 1 M sodium citrate (pH 4.5) was added to each well. The plate was incubated at 37°C for 15 min, and the reaction was stopped by the addition of 25 μl of 8 N H₂SO₄. The A₄₀₅ of each well was then recorded on a micro-ELISA reader (Fisher Scientific Co., Pittsburgh, Pa.). Optimum reagent concentrations were determined from checkerboard titration studies, which were carried out with different concentrations of serum (1/100 to 1/10,000). Representative sera from each study group were used in these studies. Blank values were obtained for each assay by the substitution of diluent for serum. Each A₄₀₅ net value reported here is the difference between the test value and the corresponding blank value.

**Statistical analysis.** A comparison of study groups for each variable was accomplished with one-way analysis of variance, and the least-significant difference multiple comparisons test (21) was used to analyze differences between subject group means. In cases for which all subject groups showed a wide range of standard deviation, student's *t* test was also used in an effort to achieve better statistical precision. A probability level (*P*) of 0.05 or smaller was used for judging statistical significance.

**RESULTS**

Results of the study are summarized in Tables 1 through 3. The mean *Aspergillus*-specific antibody levels in the population groups were given as net A₄₀₅ (Table 1). Mean *A. fumigatus*-specific antibody levels of all immunoglobulin classes were significantly higher in ABPA than in the other three groups (Table 1). In the case of aspergilloma, only mean *A. fumigatus*-specific IgG levels were elevated relative to the asthmatic and normal control groups. Individual *A.*
**TABLE 1. Levels of antibody classes in population groups as determined by BALISA**

<table>
<thead>
<tr>
<th>Subject group (n)</th>
<th>Variable</th>
<th>IgG</th>
<th>IgE</th>
<th>IgD</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABPA (13)</td>
<td>Mean</td>
<td>1.128</td>
<td>0.400</td>
<td>0.323</td>
<td>0.587</td>
<td>0.403</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.412</td>
<td>0.311</td>
<td>0.278</td>
<td>0.391</td>
<td>0.337</td>
</tr>
<tr>
<td>Aspergilloma (12)</td>
<td>Mean</td>
<td>0.845</td>
<td>0.126</td>
<td>0.095</td>
<td>0.300</td>
<td>0.121</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.315</td>
<td>0.067</td>
<td>0.072</td>
<td>0.168</td>
<td>0.074</td>
</tr>
<tr>
<td>Skin test positive for asthma (9)</td>
<td>Mean</td>
<td>0.129</td>
<td>0.040</td>
<td>0.015</td>
<td>0.122</td>
<td>0.051</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.061</td>
<td>0.041</td>
<td>0.015</td>
<td>0.032</td>
<td>0.041</td>
</tr>
<tr>
<td>Normal (9)</td>
<td>Mean</td>
<td>0.140</td>
<td>0.029</td>
<td>0.013</td>
<td>0.111</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>0.096</td>
<td>0.033</td>
<td>0.022</td>
<td>0.050</td>
<td>0.066</td>
</tr>
</tbody>
</table>

The A. fumigatus-specific IgG values for A_{405} ranged from 0.475 to 1.676 for ABPA; from 0.392 to 1.193 for aspergilloma; from 0.034 to 0.222 for Aspergillus skin test-positive asthma; and from 0.015 to 0.233 for normal controls. Individual A. fumigatus-specific IgE values ranged from 0.021 to 0.990 for ABPA and from 0.013 to 0.266 for aspergilloma, whereas A. fumigatus-specific IgD values ranged from 0.060 to 0.922 for ABPA and from 0.010 to 0.279 for aspergilloma. A. fumigatus-specific IgA values ranged from 0.236 to 1.448 for ABPA and from 0.170 to 0.781 for aspergilloma. The corresponding IgM values for A_{405} ranged from 0.055 to 1.137 for ABPA and from 0.032 to 0.311 in aspergillum. No specific differences were detected between the immunoglobulin levels of the asthmatic and normal subjects, with the exception of slight nonsignificant elevations in the specific IgE levels of a few asthmatics.

**DISCUSSION**

In this study, A. fumigatus-specific antibodies belonging to all of the immunoglobulin classes were measured in the sera of patients with ABPA, aspergilloma, and skin test-positive asthma and in the sera of normal subjects. Mean antibody concentrations of all immunoglobulin classes were significantly greater for ABPA than for all other groups and slightly higher than for aspergilloma. Differences between ABPA subjects and either the skin test-positive asthma patients or normal controls were significant at a $P < 0.001$ for all antibody classes, whereas differences between ABPA and aspergilloma populations were significant at a $P < 0.05$ for A. fumigatus-specific IgG and at a $P < 0.005$ for A. fumigatus-specific IgA, IgD, and IgM (Table 2).

In the study by Wang et al. (24) with an RIA, a statistically significant elevation in A. fumigatus-specific IgE and IgG levels was found relative to those of the A. fumigatus skin test-positive and normal control groups. In the study by Forman et al. (5), which used polystyrene tube RIA, A. fumigatus-specific IgE was elevated in two of five patients with ABPA but in no individuals with aspergilloma or in the control groups. In that study, the specific IgG concentrations were elevated in four of five ABPA patients, compared with only low to moderate levels in the aspergilloma and control subjects.

Total IgD levels in sera have been reported to be elevated in 40 to 50% of the patients with ABPA (3, 18), but there have been no reports of A. fumigatus-specific IgD elevations. Because all ABPA patients (including two patients with A. fumigatus-specific IgE levels) had elevated A. fumigatus-specific IgD levels, this antibody might prove clinically useful in the immunological documentation of these patients. Moreover, one of the ABPA patients with normal A. fumigatus-specific IgE levels had an elevated A. fumigatus-specific IgA level. Therefore, specific IgA antibody levels could also help enhance the immunodiagnosis of ABPA. A. fumigatus-specific IgM levels were elevated in only 8 of 13 (62%) patients with ABPA. Fewer ABPA patients in our study show elevation of this immunoglobulin when its levels are compared with those of specific antibodies of other immunoglobulin classes. The reasons for this difference are not clear but may relate to the time lapsed after exposure to the Aspergillus antigen. If A. fumigatus-specific IgM levels could be correlated with the A. fumigatus antigen exposure (recent or previous), the measurement of this antibody would be quite useful clinically as well.

Differentiation between the ABPA and other patient groups could best be made by comparing A. fumigatus-specific IgE and IgG levels (Table 3). The mean concentration of these antibodies for ABPA was at least three times that for aspergilloma, the disease with the next highest level of antibodies. For ABPA, the specific IgA level was twice as high as for aspergilloma. Although the mean A. fumigatus-specific IgG and IgM levels were significantly higher for ABPA than for aspergilloma, serum precipitins were generally stronger for aspergilloma than for ABPA. Previous studies from our laboratory (12) and that of others (9) involving indirect immunofluorescence, hemagglutination, and the ELISA indicated that the number and strength of precipitin arcs produced in agar gel diffusion do not correlate well with reactivity in these serological methods. This lack of correlation may be due to differences in the selectivity of component binding to the solid phase. Antibody specificity or

**TABLE 2. Statistical significance of intergroup antibody levels**

<table>
<thead>
<tr>
<th>Groups compared</th>
<th>Significance ($P &lt; $) by immunoglobulin group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>ABPA and aspergilloma</td>
<td>0.005</td>
</tr>
<tr>
<td>ABPA and skin test positive for asthma</td>
<td>0.001</td>
</tr>
<tr>
<td>ABPA and normal</td>
<td>0.001</td>
</tr>
<tr>
<td>Aspergilloma and skin test positive for asthma</td>
<td>0.001</td>
</tr>
<tr>
<td>Aspergilloma and normal</td>
<td>0.001</td>
</tr>
<tr>
<td>Skin test positive for asthma and normal</td>
<td>NS</td>
</tr>
</tbody>
</table>

* NS, Not significant by both least-significant difference multiple comparison test and Student’s t test.

**TABLE 3. Positive test results by BALISA**

<table>
<thead>
<tr>
<th>Population (n)</th>
<th>No. (%) of subjects positive* for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
</tr>
<tr>
<td>ABPA (13)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Aspergilloma (12)</td>
<td>12 (100)</td>
</tr>
<tr>
<td>Skin test-positive for asthma (9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Normal (9)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Denotes antibody level that is beyond 2 standard deviations of the mean of the normal population.
competition, or epitope-specific antibody interaction (or all of these factors).

This study demonstrated that the mean circulating A. fumigatus-specific IgG levels were significantly greater (P < 0.001) in patients with aspergillosis than in either the skin test-positive asthmatic or the normal control population, confirming previous findings (2, 4). Although mean values of A. fumigatus-specific IgA, IgD, and IgM were greater for aspergillosis patients than for the asthmatic and normal populations, this difference was not statistically significant because of a wide range of values found for the aspergillosis group. Thus, 9 of 12 (75%) aspergillosis patients had elevated A. fumigatus-specific IgA concentrations, and 8 of the 12 (67%) had elevated A. fumigatus-specific IgD concentrations. Only 1 of the 12 (8%) had elevated A. fumigatus-specific IgM levels, and 2 of 12 (16%) had A. fumigatus-specific IgE elevations.

No difference between the mean A. fumigatus-specific antibody levels of any class could be demonstrated by BALISA for patients with skin test-positive asthma versus our normal population. The only individual from these two groups with an elevated A. fumigatus-specific immunoglobulin level was an asthmatic patient who had mildly elevated A. fumigatus-specific IgE. In prior studies, few direct comparisons have been made between Aspergillus skin test-positive asthmatic and normal subjects. Sepulveda et al. (19) compared A. fumigatus-specific IgE for patients with ABPA and aspergillosis and normal controls but did not include asthmatics in their study. Greenberger and Patterson (6) compared A. fumigatus-specific IgE levels for ABPA patients and A. fumigatus skin test-positive asthmatics, but did not include normal controls in their study. Wang et al. (24) compared A. fumigatus-specific IgE and IgG levels for subjects with ABPA, A. fumigatus skin test-positive asthmatics, and normal controls by the polystyrene tube RIA and by the labeled-antigen radioimmunoassay. No statistically significant difference between A. fumigatus skin test-positive asthma and control results was found (24). In a limited study of A. fumigatus-specific IgE and IgG levels in ABPA, aspergillosis, and skin test-positive asthma patients and normal subjects, Forman et al. found no significant difference in these levels between the asthmatic and normal subjects by the polystyrene tube RIA (5).

ABPA can be differentiated from the skin test-positive asthmatic and normal control groups by A. fumigatus-specific IgG, IgD, and IgA levels by the BALISA (Table 3). This test was 85 to 100% sensitive in confirming ABPA and was specific (100%) for ABPA relative to the control groups. These results compared favorably with A. fumigatus-specific IgE sensitivity and specificity for ABPA, which were 85 and 89%, respectively. These results were obtained with a 1:1,000 dilution of patient serum samples, compared with the approximately 1/10 dilution in serum which is required for A. fumigatus-specific IgE determination by the conventional ELISA. With particular reference to A. fumigatus-specific IgG concentrations, which are a hallmark in aspergillosis (14), the BALISA method was useful in distinguishing patients from controls (100% sensitivity and specificity).

In summary, our finding here of elevation in all classes of antibodies in ABPA shows that ABPA is a disease characterized by a polyclonal antibody response to Aspergillus antigen and not just a response of IgE and IgG antibody classes. Although only the specific IgE levels appear to be useful in differentiating ABPA and aspergillosis, the other specific antibodies, particularly IgA and IgD, can all be used to differentiate ABPA from normal subjects and skin test-positive asthmatics. Measurement of these specific antibodies could enhance the immunodiagnosis of ABPA.

ACKNOWLEDGMENTS

We thank Roy Patterson and Paul Greenberger for supplying some of the sera used in this study, Gertrude Scribner for her technical assistance, and John Kalbfleisch for his assistance with statistical analyses.

This investigation was supported in part by Asthma and Allergic Diseases Center grant AI19104 from the National Institute of Allergy and Infectious Diseases and by the Veterans Administration.

LITERATURE CITED


