Use of a Synthetic Peptide Epitope of Asp f 1, a Major Allergen or Antigen of Aspergillus fumigatus, for Improved Immunodiagnostics of Allergic Bronchopulmonary Aspergillosis

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Allergic bronchopulmonary aspergillosis (ABPA) is an immunologically complex allergic disorder caused by the fungal pathogen Aspergillus fumigatus. Elevated levels of total immunoglobulin E (IgE), specific IgE, and IgG antibodies in sera are important immunodiagnostic criteria for ABPA. International reference standards or standardized immunodiagnostic assays are not available due to a lack of well-defined diagnostic antigens. The present study was carried out to identify and evaluate the immunodiagnostic relevance of synthetic epitope peptides of Asp f 1, a major allergen, antigen, or cytotoxin of A. fumigatus. Five overlapping peptides were synthesized from the N terminus of Asp f 1, one of the potential immunodominant regions predicted by algorithmic programs. The 11-amino-acid synthetic peptide (P1) significantly inhibited both IgG binding (89.10% ± 4.45%) and IgE binding (77.32% ± 3.38%) of the standardized diagnostic antigen (SDA) (a well-defined pool of diagnostically relevant allergens and antigens of A. fumigatus). With a panel of sera of ABPA patients, allergic patients with skin test negativity to A. fumigatus, and healthy individuals, P1 showed a higher diagnostic efficiency than SDA (specific IgG, 100%; specific IgE, 98.3%). The diagnostic efficiency of P1 could be attributed to the presence of homologous epitopes in various immunodominant allergens or antigens of A. fumigatus. The ability of P1 to induce histamine release from sensitized mast cells and a Th2 type of cytokine profile in peripheral blood mononuclear cells of ABPA patients suggests its potential for use in intradermal testing. P1 could be further explored for development of a standardized, specific, and sensitive immunodiagnostic test for aspergillosis.
specific IgE antibodies in 85% of allergic aspergillosis patients and the absence of its homologous proteins in other fungi (3). Asp f 1-specific IgE antibodies appeared during the early phase of ABPA (15, 32). Identification of immunodominant regions of Asp f 1 may facilitate the development of specific and standard peptide-based diagnoses. Some of the epitopes of Asp f 1 have been identified by using T-cell clones and peripheral blood mononuclear cells (PBMCs) of *A. fumigatus*-sensitized patients, but they have not been evaluated for their diagnostic relevance (10, 18).

In the present study, the identification of probable immunodominant regions of Asp f 1 has been attempted using epitope prediction algorithms. Most of the algorithms predicted the N-terminal region of Asp f 1 as the potential immunodominant region. Five overlapping peptides from the N-terminal region were synthesized, purified, and analyzed by inhibition enzyme-linked immunosorbent assay (ELISA). P1 showed significant inhibition of IgE and IgG antibody binding of standardized diagnostic antigen (SDA; a well-defined pool of diagnostically relevant allergens and antigens of *A. fumigatus*). P1 was further examined for its serodiagnostic potential using a panel of sera from 20 clinically confirmed ABPA patients, 20 controls (allergic patients with skin test negativity to *A. fumigatus*), and 25 healthy individuals. The ability of P1 to induce histamine release from sensitized mast cells of ABPA patients and a Th2 type of cytokine profile in PBMCs of ABPA patients suggests its potential for use in intradermal testing.

### MATERIALS AND METHODS

SDA. *A. fumigatus* strain 285, isolated from the sputum of an ABPA patient visiting Vallabh Bhai Patel Chest Institute, Delhi, India) was grown in a synthetic broth (t-asparagine medium) for 3 weeks at 37°C in a stationary culture (4, 5). The filtrate obtained after separating the mycelium was dialyzed extensively against deionized water. The dialysate was subjected to ammonium sulfate precipitation (80% [wt/vol]) and lyophilized to get the protein-enriched antigenic fraction. The fraction obtained was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis (>30 proteins in the range of 12 to 100 kDa). The immunoreactivity of the fraction was analyzed by immunodiffusion, ELISA, and Western blotting (comprising most of the reported immunodominant allergens and antigens of *A. fumigatus* [see Fig. 4, lanes 2 and 4]). This fraction is routinely used in our laboratory for quantitating specific IgG and IgE antibodies in serum samples from patients with suspected allergic aspergillosis (>3,200 serum samples have been screened so far, including confirmed and suspected cases of aspergillosis, allergic patients, and healthy individuals) and is referred to as SDA in the present study.

**Human sera.** The sera of ABPA patients were obtained from clinically confirmed cases (satisfying the criteria of Rosenberg et al.,) and control sera were obtained from *A. fumigatus* skin test-negative allergic patients registered at Vallabh Bhai Patel Chest Institute (26). The normal sera were obtained from healthy donors without an indication of pulmonary disease. The study was approved by the institute’s Human Ethics Committee, and the serum samples were taken with the written consent of the subjects.

**Purified Asp f 1 and MAb against Asp f 1.** Asp f 1 was purified from the SDA as described in an earlier communication (25). Monoclonal antibody (MAb) raised against Asp f 1, MAb 4A6 (ammonium sulfate precipitated), was a kind gift from L. Karl Arruda, Department of Clinical Allergy and Immunology, University of Virginia.

**Identification of immunodominant regions.** Ten algorithmic programs were used to identify the immunodominant regions of Asp f 1 (the protein sequence of Asp f 1 used was AAB22442 of the National Center for Biotechnology Information). They were Hopp and Woods (hydrophilicity), Fraga global scale (hydrophilicity), Kyte and Doolittle (hydrophathy), Novotny large sphere (accessibility), Welling (antigenicity), Parker (hydrophilicity; retention times in reverse-phase high-performance liquid chromatography), Janin (accessibility), bulk hydrophobic scale (hydrophobocity), Fauchere and Pilska (hydrophobicity), and Hopp scale (accessibility) (9, 35). Rothbard and Taylor’s predictions for T-cell epitopes and prediction of amphipathic helices were used manually to identify the potential T-cell epitopic domains in Asp f 1 (27).

### Synthesis of overlapping peptides.**

The N-terminal region of the immunodominant regions predicted by all 10 algorithmic programs and also showed the presence of potential T-cell epitopes in two manual predictions. Five overlapping peptides (Table 1) from this region were synthesized by solid-phase peptide synthesis using standard 9-fluorenylmethoxycarbonyl chemistry and purified by reverse-phase high-performance liquid chromatography on an analytical CE-18 column (Applied BioSystems). The synthetic peptides were characterized by fast atom bombardment-mass spectrometry (Jeu M360). (The sequences of the peptides used in the present study have been patented under the following numbers: Indian patent no. 184440, 189314, 189176, 751/Del/98, and 754/Del/98; U.S. patent no. 6262231; and European patent no. EP110768.)

### Diagnostic relevance of synthetic peptides.**

(i) Immunoreactivities of peptides with MAb.** The immunoreactivities of the synthetic peptides (P1 to P5) and Asp f 1 with the MAb raised against Asp f 1 were assayed by dot blotting. In brief, 1 μg of peptide or Asp f 1 in 1 in 1μl of phosphate-buffered saline (PBS) was applied to a Hybond C nitrocellulose membrane (Amersham Life sciences, Little Chalfont, United Kingdom). Five micrograms of *A. fumigatus* 3-week culture filtrate was used as a positive control, while 1 μg of purified bovine serum albumin was used as a negative control (since the culture filtrate is a mixture of a number of allergens and antigens, five times more was used than for the purified proteins). The dot blot was further processed according to the method for immunoblotting described in an earlier communication using MAb (1 mg/ml) and anti-mouse IgG peroxidase (1:1,000 dilution in PBS) (4).

(ii) Inhibition ELISA.** To evaluate the diagnostic relevance of the synthetic peptides, the pooled sera of 10 ABPA patients (diluted 1:100) were preincubated with various peptides (P1 to P5; concentrations ranged from 50 ng to 1 μg). The preincubated sera were centrifuged at 10,000 rpm for 5 min to remove any insoluble complexes. The immunoreactivities (specific IgE and IgG binding) of the preincubated sera with SDA (1 μg/well) were analyzed by indirect ELISA as described in an earlier communication (6). Briefly, microtiter plates were coated with SDA (1 μg/well) in 100 mM sodium bicarbonate (pH 9.2) overnight at 4°C. The plates were washed with PBS containing 0.05% Tween 20 and blocked with PBS containing 1% bovine serum albumin for 1 h at 37°C. Serum samples (preincubated with 100 ng of various peptides) were diluted 1:100 (for IgG) and 1:25 (for IgE) in PBS with 0.05% Tween 20 and 1% bovine serum albumin. After 2 h of incubation with the diluted serum samples, the plates were washed and incubated with 100 μl of peroxidase-conjugated anti-human IgG (for IgG) or anti-human IgE (for IgE) (appropriately diluted in PBS with 1% bovine serum albumin) for 3 h at room temperature. The plates were washed and incubated with substrate solution containing H2O2 (1 μM/l) and orthophenylenediamine dihydrochloride (1 mg/ml) for 20 min at room temperature. The optical densities at 490 nm were measured with an ELISA reader (Nunc Immunoreader). Serodiagnostic efficiency of P1. The binding of P1 with specific IgE and IgG antibodies in the sera of 20 clinically confirmed ABPA patients, 20 controls, and 20 healthy individuals was compared with that of SDA (1 μg/well) by indirect ELISA as described in an earlier communication (6). The optimum amount of P1 titrated by ELISA was observed to be 80 ng/well in a dose-response curve. A 16-aminoc-terminal sequence from gp120 of HIV (QINMQQKVKGMAYAP) was used as a control peptide during the studies.

### Table 1. Overlapping synthetic peptides from the N-terminal region of Asp f 1 and histamine release

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence (amino acid residue no.)</th>
<th>Histamine released (mean ± SD) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>LNPKTNKWEKD (10–20)</td>
<td>478 ± 23</td>
</tr>
<tr>
<td>P2</td>
<td>INOOLNPK (6–14)</td>
<td>329 ± 19</td>
</tr>
<tr>
<td>P3</td>
<td>INOOLPKTNKWEKD (6–20)</td>
<td>369 ± 18</td>
</tr>
<tr>
<td>P4</td>
<td>TNKWEKD (14–20)</td>
<td>356 ± 17</td>
</tr>
<tr>
<td>P5</td>
<td>LNPKTNKWEKD (10–22)</td>
<td>278 ± 13</td>
</tr>
<tr>
<td>SDA</td>
<td>Well-defined pool of allergens/antigens of <em>A. fumigatus</em></td>
<td>726 ± 26</td>
</tr>
</tbody>
</table>

*The values are averages of histamine release stimulated by the peptides (10 μg/well) and SDA (10 μg/well) from sensitized basophils of three clinically confirmed ABPA patients by a whole-blood assay. For each patient, incubation with the peptide or SDA was carried out in triplicate. The SD for the values were within ±5%.

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Data analysis. The cutoff for positive scores was calculated for SDA and P1 from the mean absorbance value obtained for the 20 healthy individuals plus 3 standard deviations (SD). The following definitions were used to calculate the corresponding diagnostic parameters: true-positive values (tp), sera from patients with clinically confirmed ABPA showing positive readings; false-negative values (fn), sera from patients with clinically confirmed ABPA showing negative readings; false-positive values (fp), sera from controls and healthy individuals showing positive readings; true-negative values (tn), sera from controls and healthy individuals showing negative readings; sensitivity, tp × 100/(tp + fn); specificity, tn × 100/(tn + fp); and diagnostic efficiency, (tp + tn)/100/(tp + fn + fp + tn + fn). All values in the study are expressed as the mean ± SD of triplicate values for each sample. The immunoreactivities of P1 and SDA were compared by the one-population analysis of variance test in the MicroCal Origin version 3.0 statistical package.

Cytokine profile induced by P1 in PBMCs. PBMCs (from three ABPA patients, three controls, and three healthy individuals) were fractionated, and 2 × 10⁶ cells/well were incubated with SDA (10 μg/ml) and P1 (10 μg/ml) for 6 days in RPMI 1640 medium with 10% complement-inactivated fetal calf serum. A 16-amino-acid sequence from gpl20 of HIV (10 μg/ml) was used as a control peptide during the studies (see above). The supernatant medium was collected for cytokine analysis, and the proliferation response was measured colorimetrically. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (to a final concentration of 0.5 mg/ml) was added to each well for 30 min, followed by extraction of the dye from the cells by acidified isopropanol. The absorbance was measured at 590 nm (23). Cytokine assays were carried out in supernatants of PBMCs by ELISA kits for human gamma interferon (IFN-γ), IL-2, IL-4, IL-6, and IL-10 (Pharmingen, San Diego, Calif.) according to the manufacturer’s instructions.

In vitro histamine release assay. To evaluate histamine release induced by various peptides (10 μg) and SDA (10 μg) from the sensitized basophils of ABPA patients (present in 100 μl of 1:7-diluted whole blood), a histamine immunoassay kit (Immunotech, Marseille, France) was used according to the protocol for whole-blood assay. A standard curve was drawn with histamine standards, and the corresponding histamine release by various peptides and SDA was calculated from the standard curve.

Purification of P1-specific antibodies. In order to obtain P1 bound to the solid support, the peptide P1 was synthesized by solid-phase synthesis using a 9-aminomethylcarbonyl chemistry protocol in a separate experiment. After the completion of synthesis, the alpha-aminoo-protecting group and the side chain-protecting groups were selectively removed, leaving the peptide bound to the solid support. An aliquot of the support-bound peptide was cleaved, and the peptide thus obtained was confirmed by fast atom bombardment-mass spectrophotometry. For purification of P1-specific antibodies, P1 bound to the solid support was incubated with pooled sera from 10 ABPA patients (diluted 1:1 with 1 M phosphate buffer, pH 9.0, at 4°C). The bound antibodies (P1-specific antibodies) were eluted with 0.1 M citric acid buffer (pH 3.0) at 4°C, neutralized with 1.5 M Tris-HCl (pH 7.5), and dialyzed against PBS.

Binding of SDA with P1-specific antibodies. SDA was electrophoresed by SDS-PAGE (12% acrylamide) and electroblotted onto a nitrocellulose membrane. The membrane was blocked with 3% defatted milk in PBS, washed, and further incubated with pooled sera of ABPA patients (diluted 1:100 in PBS) or P1-specific antibodies (0.5 mg/ml) for 2 h at 37°C. The washed membranes were incubated with anti-human IgG peroxidase (diluted 1:1000 in PBS) and anti-human IgE peroxidase (diluted 1:5,000 in PBS) separately for 2 h at 37°C. The immunoblots were washed and developed with diamobenzidine (12.5 μg) and H₂O₂ (25 μl) in 25 ml of 0.25 M sodium acetate buffer, pH 6.0.

RESULTS

The N-terminal region of Asp f I was one of the immuno-dominant regions predicted by all 10 algorithmic programs. The region showed the presence of an amphipatic helix and potential T-cell epitopes according to the criteria of Rothbard and Taylor (27). Five overlapping peptides (Table 1) from this region were synthesized and purified. All five peptides reacted with the MAb raised against Asp f I on the dot blot, indicating the epitopic nature of the peptides (result not shown). The diagnostic relevance of these peptides is evident from an inhibition ELISA that showed significant inhibition of specific IgE (34 to 77%) and IgG (67 to 89%) antibody binding of SDA by the peptides (Fig. 1). Peptide P1 exhibited maximum inhibition of the binding of SDA to both specific IgE antibodies (77.32% ± 3.38%) and specific IgG antibodies (89.1% ± 4.45%) and was selected for further analysis of its immunological and antigenic properties.

In order to explore the diagnostic potential of P1, we examined its binding to specific IgE and IgG antibodies in sera of ABPA patients, controls, and healthy individuals by indirect ELISA (Fig. 2). All ABPA sera showed significant IgE and IgG binding to P1 in comparison to those of controls and healthy individuals. The mean absorbance value of P1 binding with specific IgE antibodies in the sera of ABPA patients was 5.907- and 7.807-fold higher than for the controls and healthy individuals, respectively. The mean absorbance value of P1 binding with specific IgG antibodies in the sera of ABPA patients was 15.30- and 10.22-fold higher than for the controls and healthy individuals, respectively. There was no significant difference between the absorbance values for P1 and SDA with respect to their binding with specific IgE and IgG antibodies in the sera of ABPA patients (P < 0.05). The reactivity of P1 with the IgE and IgG antibodies in the sera of controls was significantly lower than that of SDA, suggesting a higher specificity of P1 than SDA (P < 0.05). The ELISA absorbance values (mean ± SD) for A. fumigatus-specific IgG binding of control peptide with the sera of 20 ABPA patients and with the sera of 20 controls were 0.078 ± 0.007 and 0.051 ± 0.011, respectively. The ELISA values (mean ± SD) for A. fumigatus-specific IgE binding of control peptide with the sera of 20 ABPA patients and with the sera of 20 controls were 0.058 ± 0.009 and 0.037 ± 0.004, respectively. The diagnostic efficiencies of P1 and SDA to detect specific IgG and IgE antibodies in the sera are compared in Table 2.

In order to investigate the utility of P1 for intradermal test-
ing, it was subjected to lymphoproliferation and histamine release assays. On incubation with P1 and SDA, the PBMCs of ABPA patients showed 5.132- and 7.217-fold proliferation, respectively. PBMCs of controls incubated with P1 and SDA showed 1.239- and 2.375-fold proliferation, respectively. PBMCs of healthy individuals incubated with P1 and SDA showed 1.072- and 1.517-fold proliferation, respectively. The cell supernatants from the lymphoproliferation assay were analyzed for IFN-γ/H9253, IL-2, IL-4, IL-6, and IL-10, and the results are shown in Fig. 3. The PBMCs of ABPA patients incubated with SDA showed decreased levels of IFN-γ but increased levels of IL-2, IL-4, IL-6, and IL-10 in comparison to those of healthy individuals. The PBMCs of ABPA patients incubated with P1 showed a cytokine profile similar to that of SDA, although at a lower magnitude. The P1-induced decrease in the IFN-γ level was 1.440-fold lower than that of SDA, while the increases in the levels of IL-2, IL-4, IL-6, and IL-10 induced by P1 were 1.365-, 1.034-, 4.024-, and 1.969-fold, respectively, less than those induced by SDA. PBMCs of ABPA patients and healthy individuals incubated with the control peptide did not show stimulation of IFN-γ, IL-2, IL-4, IL-6, and IL-10 cytokines.

P1 induced maximum histamine release from the sensitized basophils of ABPA patients in comparison to the other four peptides; however, the histamine release induced by P1 was 34.15% less than the histamine release induced by SDA (Table 1).
The absorbance values of P1 and SDA with sera of ABPA patients for both specific IgE and IgG antibodies were comparable and suggest that the epitope represented by P1 (sequentially or conformationally) may be present in multiple allergens and antigens of A. fumigatus. BLAST analysis of the amino acid sequence of P1 with the genome sequence of A. fumigatus (TBLASTN analysis of the peptide sequence with the whole genome assembly of the shotgun sequence of A. fumigatus at The Wellcome Trust Sanger Institute website [http://www.sanger.ac.uk]) showed that the complete sequence appeared only once (with a score of 65) but partially matched with 38 sequences in the whole genome (with a score range of 31 to 40). To investigate the presence of epitopes similar to P1 in allergens and antigens other than Asp f 1, P1-specific antibodies were purified from pooled sera of 10 ABPA patients. P1-specific IgG antibodies recognized six allergens and antigens (with molecular masses of 24, 29, 55, 66, 77, and 88 kDa) (Fig. 4, lane 5) besides Asp f 1 (18 kDa). P1-specific IgE antibodies recognized three allergens and antigens with masses of 27, 34, and 43 kDa besides Asp f 1 (Fig. 4, lane 3).

**DISCUSSION**

Significant levels of Asp f 1-specific antibodies are present in a majority of ABPA patients in the early stages of the disease (3, 15, 32). The present study attempted to analyze the diagnostic relevance of the N-terminal region of Asp f 1 (6 to 22 amino acid residues), which has been predicted to be an important epitopic region by 12 algorithmic programs used in the present study and earlier studies (Table 3) (10, 22, 32). The N-terminal region has been shown to be important for the allergenicity of a number of other allergens, like hevein pre-protein, rPhl p 6, and Bet v4 (7, 33, 36).

All five peptides, P1, P2, P3, P4, and P5, reacted with MAb on dot blots and showed significant inhibition of IgE (34 to 77%) and IgG (67 to 89%) antibody binding of SDA, indicating that the N-terminal region of Asp f 1 is diagnostically

![Graph showing cytokine levels in the PBMCs of ABPA patients incubated with SDA and P1.](http://cvi.asm.org/)

**FIG. 3.** Cytokine levels in the PBMCs of ABPA patients incubated with SDA and P1. PBMCs of the ABPA patients and healthy individuals (Normals) were incubated with SDA, P1, and control peptide (Con Pep; HIV epitopic peptide) (each at a concentration of 10 μg/well). The supernatants were subjected to ELISA-based cytokine assays for human IFN-γ, IL-2, IL-4, IL-6, and IL-10. The values represent the mean values of three readings each from three ABPA patients and three healthy individuals, and the SD for each value was within a ±5% range.

![Image showing IgG and IgE antibody binding to electrophoresed allergens or antigens (SDA) on immunoblots (SDS–12% PAGE).](http://cvi.asm.org/)

**FIG. 4.** P1-specific IgG and IgE antibody binding to electrophoresed allergens or antigens (SDA) on immunoblots (SDS–12% PAGE). Lane 1, molecular mass markers; lane 2, IgE binding of SDA with pooled sera of ABPA patients; lane 3, IgE binding of SDA with P1-specific antibodies; lane 4, IgG binding of SDA with pooled sera of ABPA patients; lane 5, IgG binding of SDA with P1-specific antibodies.
TABLE 3. Identified T-cell and B-cell epitopes of Asp f 1 using human T-cell clones isolated from ABPA patients and synthetic peptides inducing proliferation of PBMCs of A. fumigatus-sensitized mice

<table>
<thead>
<tr>
<th>Locations of epitopes</th>
<th>Source used for validation</th>
<th>Nature of epitope</th>
<th>Th1/Th2 type</th>
<th>T-cell tolerance</th>
<th>Reference</th>
</tr>
</thead>
</table>

* a The amino acid residue number is given according to the protein sequence of Asp f 1 in AAB22442 of the National Center for Biotechnology Information.
magnitude than SDA, suggests that it could be further explored for intradermal skin testing. There have been numerous reports of diagnostically relevant native and recombinant antigens for serodiagnosis of aspergillosis (12, 14, 15, 16, 19, 25, 28, 30). The reported diagnostic sensitivity and specificity vary greatly among the different reports, even for similar antigen preparations. The higher diagnostic efficiency of P1 than SDA in ELISA may lead to the development of a sensitive and specific serodiagnostic assay for aspergillosis. Furthermore, P1 constitutes a standardized reagent that can be obtained by chemical synthesis in any laboratory. Another advantage of P1 is that it binds to ELISA plates by passive adsorption on the plastic surface and does not need conjugation to a carrier protein. However, the relevance of P1 for serodiagnosis of allergic aspergillosis patients other than ABPA patients remains to be examined. The present study has shown that P1 is diagnostically relevant for ABPA patients in the Indian population. Since there is a probability of heterogeneous recognition of an IgE epitope by sensitive patients from various ethnic groups, the peptide P1 needs to be further evaluated in other populations for universal diagnostic application (11).

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