Immune Responses to Aspergillus fumigatus and Pseudomonas aeruginosa Antigens in Cystic Fibrosis and Allergic Bronchopulmonary Aspergillosis*

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Allergic bronchopulmonary aspergillosis (ABPA) in Cystic Fibrosis (CF) is well documented. *Aspergillus fumigatus* is the causative agent of ABPA, and *Pseudomonas aeruginosa* particularly the mucoid variety has been frequently isolated from the sputum of patients with CF. This study investigates the cellular and humoral immune response to both *A. fumigatus* and *P. aeruginosa* antigens in patients with CF and ABPA (CF/ABPA), CF only, and healthy controls. The *A. fumigatus* and *P. aeruginosa* antigen specific IgE and IgG in sera and peripheral blood mononuclear cell culture supernatants (PBMC sups), lymphoproliferation to antigens, and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) were measured. Results indicate significant elevated levels of *A. fumigatus* specific IgG (*A. fumigatus*-IgG) and *P. aeruginosa*-IgE in serum. Significant *P. aeruginosa*-IgG was measured in PBMC sups. The concanavalin A nonbinding *A. fumigatus* antigen, previously shown to induce specific T-cell responses in vitro in patients with ABPA, elicited significant lymphoproliferative response in a greater proportion of patients with CF/ABPA and not in CF or controls, underlining the importance of this antigen in the diagnosis of ABPA. In contrast, a greater proportion of the CF group responded to *P. aeruginosa* antigens compared with the controls and CF/ABPA. Hence, the CF and CF/ABPA groups respond to both *P. aeruginosa* and *A. fumigatus* antigens with the former group responding strongly to *P. aeruginosa* and the latter to *A. fumigatus* antigens. (Chest 1994; 106:513-19)

**Key words:** Allergic bronchopulmonary aspergillosis, Aspergillus antibodies; Cystic fibrosis; Pseudomonas antibodies

Cystic fibrosis (CF) is a fatal, autosomal recessive disorder in children and is associated with bronchiectasis, pancreatic insufficiency, and elevated sweat chloride. The CF gene on chromosome 7 encodes a protein identified as CF transmembrane conductance regulator, which regulates chloride ion transport in epithelial cell membranes. Pulmonary disease is almost universal in CF and causes over 90 percent of deaths. *Pseudomonas aeruginosa* is the commonest organism isolated in CF patients and is found in all deaths resulting from lung disease. The *P. aeruginosa* usually occurs in the unique mucoid form which creates a physical barrier to host defense mediated clearance.

Mears et al. first reported an association between allergic bronchopulmonary aspergillosis (ABPA) and cystic fibrosis (CF). The ABPA occurs with an incidence of 10 to 11 percent in patients with CF. Classic cases of ABPA are characterized by increased wheezing, fleeting pulmonary infiltrates, blood and sputum eosinophilia, elevated total IgE, and elevated IgE and IgG to *Aspergillus fumigatus*. Nearly one-half of CF patients have a positive immediate wheal and flare skin test to *A. fumigatus* and *A. fumigatus* specific IgE and IgG without evidence of ABPA. Aspergillus allergy appears coincident with *P. aeruginosa* colonization; and hence, it is difficult to distinguish the effects of either organisms. The diagnosis of ABPA, however, is clearly important from a clinical viewpoint because of its association with severe proximal bronchiectasis and a far more rapid decline in lung function in CF.

We have evaluated previously the T cell response to *A. fumigatus* antigens in ABPA and have shown that a concanavalin A (Con A) nonbinding *A. fumigatus* antigen is specific for ABPA. We have compared T-cell responses in patients with CF only, CF...
and ABPA, and healthy controls using both crude and purified A fumigatus antigens and antigens from both nonmucoid and mucoid variety of P aeruginosa. In addition, we have also studied the levels of IgE and IgG antibodies to both A fumigatus and P aeruginosa antigens. Data from this study show that the CF group respond strongly to P aeruginosa antigens while the CF/ABPA respond to A fumigatus antigens.

**METHODS**

**Subjects**

Three groups of subjects were included in this study. Patients with proven CF without ABPA (n=7) were selected from the regional cystic fibrosis center at the Medical College of Wisconsin. Patients with CF satisfying criteria for ABPA previously reported by (CF/ABPA, n=7), and healthy individuals (healthy control, n=10) without ABPA served as controls. Patients with cystic fibrosis were diagnosed as having ABPA based on deterioration of pulmonary functions with (1) immediate cutaneous reactivity to A fumigatus, (2) elevated total serum IgE, and (3) serum IgE and IgG antibodies to A fumigatus. Not all patients had blood eosinophilia or pulmonary infiltrates on roentgenograms or positive sputum culture of A fumigatus. All patients were treated with prednisone. Informed consent was obtained from all subjects.

**A fumigatus Antigens**

Four different antigens-culture filtrate, mycelial extract, Con A nonbinding and Con A binding antigens, obtained from A fumigatus were used as described earlier.11 The antigens have been characterized as to their protein profile in polyacrylamide gels and for their immunologic reactivity.12-15

**P aeruginosa Antigens**

Both noncapsulated (noncaps) and capsulated (caps) types of P aeruginosa were isolated from the sputa of patients with CF. The cultures were grown in synthetic medium for 24 hours at 37°C. The broth was then centrifuged at 10,000 revolutions per min (rpm) for 15 min. The bacteria were resuspended in PBS and homogenized using a French press at 10,000 psi. The homogenate was then centrifuged at 10,000 rpm for 30 min and the supernatant was dialyzed and lyophilized.

**Antigen Specific Lymphocyte Proliferation**

Proliferative assays were performed in 96 well flat bottom microtiter plates in medium alone and in presence of final concentrations of soluble A fumigatus or P aeruginosa antigens ranging from 100 μg/ml to 2 mg/ml to determine the optimal dose. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by the method of Boyum.66 Cells (2x10^5/well) were cultured in RPMI 1640 supplemented with 2 mmol/L glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10 percent fetal bovine serum (HyClone, Utah) for 7 days, at 37°C in a humidified atmosphere containing 5 percent CO2. The cultures were pulsed with 0.5 μCi/well of tritiated thymidine (specific activity, 6.7 Ci/mmol, New England Nuclear, Mass) for the final 18 hours of culture, harvested on glass fiber filter, and the radioactivity counted on a beta scintillation counter (Pack Beta, LKB, Sweden). Results were expressed as stimulation index (SI) as SI=cpm(antigen stimulated cultures)/cpm(medium alone cultures).

**In Vitro Immunoglobulin Synthesis**

For in vitro immunoglobulin E (IgE) synthesis PBMC (2X10^6 cells/well) were cultured in medium alone or A fumigatus and P aeruginosa antigens for 7 days in 24 well-cluster plates as described previously.11 Supernatants were collected and made cell free by centrifugation at 1,000 g for 10 min and stored at -70°C before being tested for total IgE by BALISA described below.

**Antigen Specific IgE and IgG**

The BALISA as a sensitive and reliable quantitative measure of anti-A fumigatus specific IgG and IgE antibodies in the sera of patients has been described previously.17 A modification of this method was used to measure Pseudomonas specific antibodies. Briefly, a mixture of culture filtrate and mycelial extract of A fumigatus antigens in PBS was used to coat each well of a microtiter plate (Dynatech Laboratories, Alexandria, Va) and incubated overnight at 4°C. The P aeruginosa coated to 1:100 dilution for both caps and noncaps antigens. The wells were washed once with phosphate buffered saline solution containing 0.05 percent Tween 20 (PBS-T). The plates were stored at -70°C until used. The wells were blocked by incubation at room temperature (RT) with 200 μl of PBS-T containing 1 percent bovine serum albumin (BSA-PBS-T). The wells were washed thrice with PBS-T.

All dilutions of the serum in the assay were made with the blocking solution. The samples (1:50 diluted serum for IgE, 1:1,000 for IgG or undiluted supernatants) were added to wells and plate incubated at RT for 3 hours. To detect IgE, this step was followed up by goat antihuman IgE (Sigma, St. Louis, Mo) at a 1:1,000 dilution for 1 hour. Subsequent steps included addition of 1:5,000 diluted biotinylated rabbit antigoat IgG (Zymed Laboratories, San Francisco, Calif) and 1:20,000 diluted streptavidin peroxidase (Sigma, St. Louis) with incubations of 1 hour each. For IgG, biotinylated goat antihuman IgG was added followed by streptavidin peroxidase.

The wells were washed thrice as above after each incubation. Finally, 200 μl of the substrate (0.015% H2O2 and 0.06 percent O-phenylenediamine) in citrate buffer (0.1M, pH 4.5) was added to each well. The color reaction was stopped with 4N H2SO4 (50 μl) and the color was read in an automatic ELISA plate reader (Dynatech, Chantilly, Va) using 490-nm filter.

**Leukotriene B4 Production**

Peripheral blood mononuclear cells from four CF, three CF/ABPA, and five healthy controls selected randomly were obtained as described above. Cells (1X10^6/ml) were incubated with calcium ionophore (A 23187, Calbiochem, La Jolla, Calif) for 30 min at 37°C, and supernatants were assayed for leukotriene B4 (LTB4) directly using a radioimmunoassay (RIA) as described earlier.18 The RIA used (Amersham, Arlington Heights, Ill) was sensitive to 1.6 pg/ml and had the following cross-reactivities: LTC4 and LTD4, <0.05%; 5-, 12-, and 15-HETE, <0.05%; and 20-OH-LTB4, 0.4%.

**Statistical Analysis**

The data was analyzed using Student’s t test (two-tailed, independent samples). A P value of less than 0.05 was considered statistically significant.

**RESULTS**

**Antigens**

The A fumigatus antigens-culture filtrate (AF 5302), mycelial extract (MK6), and Con A nonbinding were diluted to a stock concentration of 1 mg/ml, 200 μg/ml, and 6 mg/ml of total protein respectively. The Con A binding antigen was diluted to a concentration of 500 μg/ml total weight. The protein content in the P aeruginosa antigens was determined to
be 920 μg/ml for capsulated and 1.4 mg/ml for noncapsulated. In the sodium dodecyl sulfate-polyacrylamide gel, the crude *A. fumigatus* antigens show multiple bands (MW range, 14-150kDa), whereas the Con A-nonbinding antigen has four low molecular-weight proteins (MW range, 18-30kDa), whereas no bands were stainable with Coomassie blue in the Con A bound antigen. The noncapsulated *P. aeruginosa* antigens show multiple protein bands with a wide range of molecular weight like the crude *A. fumiga*

### Table 1—Lymphocyte Response to Aspergillus and Pseudomonas Antigens in Healthy Controls, Cystic Fibrosis, and Patients With Cystic Fibrosis and ABPA

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Stimulation Index*</th>
<th>No. (%) of subjects showing significant response†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Healthy Control</td>
<td>CF/ABPA</td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture filtrate</td>
<td>2.00 ± 0.3</td>
<td>3.92 ± 1.2</td>
</tr>
<tr>
<td>Mycelial extract</td>
<td>1.47 ± 0.2</td>
<td>1.46 ± 0.5</td>
</tr>
<tr>
<td>Con A nonbinding</td>
<td>1.08 ± 0.18</td>
<td>1.46 ± 0.5</td>
</tr>
<tr>
<td>Con A binding</td>
<td>1.77 ± 0.6</td>
<td>6.6 ± 2.5</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsulated</td>
<td>2.23 ± 0.42</td>
<td>5.7 ± 3.0</td>
</tr>
<tr>
<td>Noncapsulated</td>
<td>1.95 ± 0.53</td>
<td>5.7 ± 2.7</td>
</tr>
</tbody>
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*Mean ± SE.
†Individual response > Mean ± 2 SD of response to respective antigen by healthy controls is considered significant.

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**Aspergillus fumigatus antigens**

**Ps. aeruginosa antigens**

**Figure 1.** Proliferative responses of PBMCs from healthy controls, patients with CF, and patients with CF and ABPA to Af antigens (Af 5302, MK6, Con A nonbound and Con A bound) and Ps antigens (Cap and Noncap). Stimulation indices (SI=counts per minute in culture with antigen/count per minute in culture with medium alone) are shown for each individual. Horizontal broken lines and corresponding numbers indicate mean+2SD values (SI) of response to respective antigen by healthy control subjects.
the CF/ABPA group. Furthermore, the number of responders in the CF group was higher.

A fumigatus and P aeruginosa Specific Antibodies in Serum

As no universal standard for quantitation of A fumigatus or P aeruginosa specific antibodies is available, we are reporting the data in optical density (OD) values. To eliminate variability all samples were tested by ELISA for a particular antibody on the same day under identical conditions. Both CF and CF/ABPA groups had significant levels of A fumigatus specific IgG in sera compared with controls (Table 2). The groups also had significant increases in IgE antibodies to P aeruginosa antigens. Statistically higher levels of IgE specific to capsulated than to noncapsulated antigen was shown (Table 2). The P aeruginosa specific IgG was not significantly elevated in the patient groups.

Specific Antibodies in Culture Supernatants

In contrast to P aeruginosa specific antibody levels in serum, the specific IgG in 7-day PBMC culture fluids from the patient groups were elevated compared with controls. Patients with CF produced higher levels of antibodies to P aeruginosa antigens than the CF/ABPA group (Table 3). A fumigatus specific antibodies were negligible in all groups tested.
(data not shown).

**LTB4 in Culture Supernatants**

The PBMC from CF/ABPA (2,367 ± 145 pg/ml) group produced significant levels of LTB4 compared with both CF (1,225 ± 347) and control (1,560 ± 280) groups.

**Discussion**

Patients with CF are unusually susceptible to recurrent and chronic bacterial colonization in the lower respiratory tract. There is a high prevalence of bronchopulmonary infection with mucoid *P. aeruginosa*. Several reports have also highlighted an increase in allergy skin prick test reactivity and increased levels of total and specific IgE antibodies in CF.10 Major atopic responses are to *A. fumigatus* a ubiquitous fungus, which is known to complicate CF with ABPA. As Aspergillus allergy appears coincident with *P. aeruginosa* it is difficult to distinguish the host immune response to the two organisms. In the present study, the immune response to both *P. aeruginosa* and *A. fumigatus* antigens in CF and CF/ABPA were studied to try and make a diagnostic distinction between these two responses. In our earlier study with ABPA, it was shown that the Con A nonbinding fraction of *A. fumigatus* was more specific to the disease.11 In the present study, the lymphocyte transformation response to *A. fumigatus* culture filtrate and mycelial extract antigen in both CF and CF/ABPA were no different from that of the control group. The *A. fumigatus* is a common fungus in the environment and the exposed population has been shown by us11 and others20,21 to respond rather uniformly to crude preparations from this fungus. The purified fractions of Con A nonbinding *A. fumigatus* antigens was more discriminatory between the test groups in this study. Half of the patients with CF and ABPA specifically responded to this antigen. The CF group did to a lesser extent and the healthy group did not. In an earlier report, we have noted similar findings where the Con A nonbinding *A. fumigatus* antigens could distinguish between ABPA and *A. fumigatus* skin prick test positive patients without ABPA. It is of great interest to observe that this semipurified protein fraction is more specific to ABPA as a disease by itself and as a complication in other diseases. Con A nonbinding antigen has been studied for its humoral responses22 and shown to contain antigenic and allergenic immunodominant epitopes.23 The CF group responded more specifically to the Con A binding group compared with the other groups.

While a greater proportion of the CF/ABPA group responded in *in vitro* culture to *A. fumigatus* antigens (except Con A binding) the CF group responded better to *P. aeruginosa* antigens. The underlying fungal infection seen in the former could give rise to such differences. Several *P. aeruginosa* components including proteases,24,25 slime layer antigens,26,27 and outer membrane proteins28 are capable of stimulating cell mediated immunity. Heat-killed *P. aeruginosa* was mitogenic for human lymphocytes and induced production of TNF-alpha and IL-1.29 Lymphocyte unresponsiveness to killed *P. aeruginosa* with progression of pulmonary disease, however, has been reported.30-32

The levels of LTB4 in CF/ABPA, a significant inflammatory mediator in chronic pseudomonal respiratory disease, was elevated consistent with the chronic production of LTB4 noted by others.33

The presence of Aspergillus specific antibodies in both CF and CF/ABPA group in the present study has been described previously. The *A. fumigatus* specific antibodies in CF/ABPA patients have also been shown by Knutsen et al34,35 They have suggested the possibility of a subclinical pulmonary inflammation due to *A. fumigatus*. The presence of these antibodies have been correlated with decreased lung function.36,37 Nikolazik et al10 have described CF patients with *A. fumigatus* specific IgE and IgG but no evidence of full blown ABPA. They have also shown that the major response is to a high molecular weight antigen of *A. fumigatus* selectively released when *A. fumigatus* is cocultured with *P. aeruginosa*.38

Increased levels of specific IgE to capsulated *P. aeruginosa* were detected over noncapsulated *P. aeruginosa* in both CF and CF/ABPA groups. The *P. aeruginosa* specific IgE has been reported before in patients with chronic *P. aeruginosa* infection.39 Contrary to other reports,40,41 we did not detect significant levels of *P. aeruginosa* specific IgG in the serum of patients over controls. This may be from differences in the coating antigens used in ELISA in the present study. Cordon et al42 have shown that IgG antibodies titers in sputum may be undetectable in early *P. aeruginosa* infection.

The *P. aeruginosa* specific antibody levels produced *in vitro* were the reverse of what was detected in serum from these patients. This leads us to speculate the presence of circulating immune complexes formed by the specific IgG antibodies making them unavailable in serum. The extent to which immune complexes involving *P. aeruginosa* antigens contribute to the pathogenesis of pulmonary damage in CF is still under consideration.43

The present study showed that the Con A nonbinding antigen specifically stimulated cells from patients with cystic fibrosis and ABPA while the *P. aeruginosa* antigens were more immunogenic to patients with cystic fibrosis alone. Also, both patient groups produce significant amounts of antibodies to
both *A. fumigatus* and *P. aeruginosa*. The role of these antibodies in the pathogenesis of the human disease remains to be studied. We have recently shown the protective role of *A. fumigatus* specific antibodies in a murine model of ABPA.44

In summary, the results from this study indicate that while these tests may allow one to rule-in the diagnosis of ABPA with CF, a negative test at this point is not sufficient to rule-out such a diagnosis.

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