

Purified Recombinant *A. fumigatus* Allergens Induce Different Responses in Mice

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***Aspergillus fumigatus* an opportunistic fungus is associated with a number of diseases in humans. Allergy resulting from exposure to the *A. fumigatus* allergens has been recognized frequently. The damage caused by the disease is very striking in patients with atopy and those with cystic fibrosis. Avoidance to exposure is not feasible because *A. fumigatus* spores are ubiquitously distributed in the environment. Hence, immunotherapeutic regimens in severe forms of *A. fumigatus* allergy may have a high potential. However, before such forms of therapy can be envisaged, it is essential to understand the immunopathogenesis. In the present study, we investigated the role of purified *A. fumigatus* allergens in the development of allergic asthma in mice. We have used four major recombinant *A. fumigatus* allergens in the murine model. Mice exposed to Asp f 1, f 3, and f 4 showed inflammatory changes in the lungs and airway hyperreactivity. The immune responses, including elevated serum IgE, enhanced eosinophils, recruitment in the peripheral blood and lungs, and expression of regulatory cytokines, are characteristic of a Th2 response. Asp f 6 demonstrated only a reduced response in these animals. The results suggest that the pathology induced by crude *A. fumigatus* extract results from the cumulative effects of the allergens and the individual responses varied considerably with different purified antigens.** © 2001 Academic Press

Key Words: *Aspergillus fumigatus*; recombinant allergens; murine model; airway response; IgE; eosinophil; lung histology; allergic aspergillosis.

INTRODUCTION

Allergic bronchopulmonary aspergillosis (ABPA) is a disabling lung disease caused by *Aspergillus fumigatus* (*A. fumigatus*), a saprophytic mold considered the most important opportunistic fungal pathogen in humans (1–3). This disease can cause complications in atopic

individuals and patients with cystic fibrosis (3–5). The organism usually colonizes the damaged respiratory tract and results in clinical and pathological manifestations of ABPA (4). The clinical presentation includes mild bronchospasm progressing to an end stage pulmonary fibrosis. The patients invariably demonstrate specific serum IgE against *A. fumigatus*, enhanced peripheral blood and lung eosinophilia, and airway hyperreactivity to inhaled *A. fumigatus* allergens (3, 4). The immune response, thus, is predominately of a Th2 type (6). The various lymphoid cells, such as eosinophils, T-cells, mast cells, neutrophils, airway epithelial and vascular endothelial cells, smooth muscle cells, and their products, have been shown to play major roles singularly or in combination toward the initiation and perpetuation of the disease process (7–13). The roles of a number of cytokines and chemokines in the pathogenesis of ABPA have been elucidated. Understanding of the immunoregulatory events may contribute toward devising control measures and treatment of the disease (8, 14–18). Animal models have been developed to understand the pathogenesis of the disease and to investigate the various factors playing a role in the pathogenesis of ABPA (7–19).

The majority of the animal model studies of ABPA have been carried out in mice using crude *A. fumigatus* antigens or occasionally with intact organisms, particularly the spores (17–19). The results of these studies yielded valuable information on the roles played by various cytokines, eosinophils, IgE, and T-cell differentiation in the development of the disease (14, 15, 17–19). In recent years, a number of relevant allergens from *A. fumigatus* have been identified and the corresponding cDNAs have been cloned, sequenced, and expressed as recombinant proteins (20–29). Several of these recombinant allergens bound specifically to serum IgE from patients with ABPA and have been shown to be of value in the diagnostic discrimination between *A. fumigatus* allergy and ABPA (20, 26, 29).



However, none of these major allergens have been investigated in the murine model to understand their specific role in the pathogenesis of ABPA. The information gained from such studies may not only contribute to our understanding of the specific role of the individual allergens, but also help in devising measures to control the disease. By understanding the structure-function relationship of the allergens along with the knowledge of the immunopathogenesis of ABPA, it is possible to develop vaccines and immunotherapeutic agents (30). In the present study, we investigated four major allergens of *A. fumigatus*, namely, Asp f 1, Asp f 3, Asp f 4, and Asp f 6, in a mouse model of asthma and allergy and compared the responses in mice challenged with crude *A. fumigatus* extract. The results indicate considerable differences in the responses among mice exposed to the different allergens. Although all the allergens elicited a Th2 response in the animals, not all the allergens produced pulmonary airway response in the sensitized animals.

MATERIALS AND METHODS

Mice

Eight- to 10-week-old BALB/c mice purchased from Charles River Laboratories (Wilmington, MA) were used in the study. Animal studies were approved by the Institutional Animal Care and Use Committee.

A. fumigatus Antigen

The crude *A. fumigatus* antigen used was a mixture of culture filtrate and mycelial antigen (7, 10, 11). Culture filtrates and the mycelial extracts were prepared as described previously (3, 7, 31). In brief, the fungus was grown in a synthetic broth for 2–3 weeks at 37°C, and the medium was separated from the mycelium. The spent broth was filtered and dialyzed against deionized water at 4°C overnight, filter sterilized, and freeze-dried. For the mycelial antigen, *A. fumigatus* spores were inoculated into a synthetic broth and incubated at 37°C for 5 days on a rotary shaker at 100 rpm. The mycelial pellet was collected by filtration. The pellet was washed and suspended in sterile phosphate-buffered saline (PBS) before homogenization in a French press (31). The homogenized extract was dialyzed and concentrated to 1 mg protein/ml. Both culture filtrate and mycelial antigens were evaluated for their protein content by modified Lowry and by Bis Cinchoninic acid methods, SDS-PAGE profile, and IgE reactivity to positive and negative sera (20, 32, 33). Only antigen batches with reactivity profiles comparable to those of in-house standard antigens were used for the study.

Recombinant *Aspergillus* Allergens

For developing the murine model we used recombinant Asp f 1, f 3, f 4, and f 6. Asp f 1 is a ribotoxin, Asp f 3 is a 16-kDa protein with homology to peroxisomal protein of *Candida boidinii*, and Asp f 6 corresponds to manganese-dependent superoxide dismutase (22, 24–26, 28). Asp f 4 has no homology with any known protein. Asp f 1, f 3, f 4, and f 6 were cloned from a cDNA library of *A. fumigatus* displayed on phage surfaces as described before (25). As *A. fumigatus* cDNA library was constructed in phagemid JuFo and displayed on the surface of filamentous phage M13 (34). The library was selectively enriched for phage displaying IgE binding molecules by biopanning in microtiter plates coated with serum IgE from individuals allergic to *A. fumigatus*. The selected clones binding to human IgE were sequenced using the dideoxynucleotide chain-termination method (35). After homology search the full genes were amplified by PCR, purified, and ligated into the high-level expression vector p [His]₆-DHFR (26) before being transferred to electrocompetent *Escherichia coli* cells. The constructs were verified by nucleotide sequence determinations and used to produce hexahistidine-tagged recombinant proteins as described previously (36). The fusion proteins were purified in a single step by Ni²⁺ chelate affinity chromatography. The purity, molecular size, and the reactivity with positive sera were ascertained by SDS-PAGE, Western blot, and ELISA (20).

Sensitization of Mice

Animals were lightly anesthetized using Metofane aerosol (Methoxyflurane, Pitman-Moore, Mundelein, IL) and 50 µg of *A. fumigatus* protein antigen in 50 µl of PBS was instilled in the nostrils using a sterile pipettor tip as described before (7, 8). The exposure was carried out twice weekly for 4 weeks. Animals were bled 1 week after the eighth exposure and the sera analyzed for *A. fumigatus*-specific antibodies. When a significant increase in antibody was detected by ELISA (fivefold over baseline), animals were boosted with an additional intranasal exposure with *A. fumigatus* antigens. Mice were sacrificed 5 days after the last antigen exposure and their sera studied for *A. fumigatus*-specific antibodies. The lungs were fixed in Formalin and subjected to histological evaluation. The peripheral blood samples were studied for eosinophils as described below. Five to seven animals were studied in most of the experiments, while 9 to 12 animals were used for airway reactivity in a methacholine challenge test, as previously reported (37).

Serum IgE

The total serum IgE was evaluated using a monoclonal antibody-based ELISA, as described previously (8, 14). The serum IgE level was expressed as ng/ml after comparing with mouse IgE standards (Pharmingen, San Diego, CA).

Aspergillus-Specific Serum Antibodies

Antibody isotypes belonging to IgG₁, IgG_{2a}, IgG_{2b}, and IgG₃ against *A. fumigatus* were studied by ELISA using the serum samples from different groups of mice. The method is essentially the same as reported previously (8, 14, 31). In brief, *A. fumigatus* allergens were coated onto the wells of microtiter plates (Immulon II, Fisher Scientific, Itasca, IL) at a concentration of 1 µg/ml. Serum dilutions (1:50 to 1:800) made in PBS-Tween 20 were added and incubated for 3 h at room temperature. The plates were washed and biotinylated isotype-specific anti-mouse antibody was added and again incubated. This was followed by washing the wells and addition of streptavidin-conjugated horseradish peroxidase. After incubation and washing, the color was developed with orthophenylene diamine (OPD) and the optical density determined using an ELISA reader (14). Some of the sera were studied using four to five doubling dilutions of the sera and the ELISA log titer was calculated as described before (38). Since no major difference was noticed, the data are presented as net optical density values.

Eosinophils

The peripheral blood eosinophils of all mice were evaluated before sensitization, just before the final dose, and at the time of euthanasia. Blood from tail vein was collected in 10 units of heparin/ml and stained with Discombe's fluid and the eosinophils were enumerated in a hemocytometer as described previously (8, 14).

Lung Histology

Lungs of the animals were inflated with 10% neutral buffered Formalin to prevent atelectasis. The specimens were fixed in 10% buffered Formalin (7, 14, 31). The tissues were processed and embedded in paraffin. The sections were cut at 5 µm thickness and stained by hematoxylin and eosin. The inflammatory responses, particularly infiltration of eosinophils and other inflammatory cells in the lungs of the different groups of animals, were studied and graded by microscopic examination as "no," "low," "moderate," and "severe" responses. The presence of eosinophils was graded as "+"

for 1–3 (minimal), "++" for 4–10 (few), "+++" for 11–30 (moderate), and "++++" for over 30 (numerous) in the high-power field of the lung section. The pathological reactions were read blinded, the results were recorded semiquantitatively, and the results compared (7, 31).

Pulmonary Airway Responses

Lung mechanics measurements were made in sensitized mice 5 days after the last antigen treatment and in unsensitized controls as reported before (7, 37). In the first series of experiments, 12 mice sensitized with crude antigen were compared with 12 unsensitized mice. Subsequently, the responses of mice sensitized with *A. fumigatus* allergens Asp f 1, Asp f 3, Asp f 4, and Asp f 6 were studied in groups of 9 or 10 animals. Their responses were compared to those of another 10 unsensitized mice interspersed randomly among the four groups of *A. fumigatus*-sensitized mice. The animals were anesthetized with an intraperitoneal (ip) injection of a solution of ketamine and xylazine (40 mg/kg each). After tracheotomy, the animals were given a maintenance dose of pentobarbital sodium (20 mg/kg ip) and placed in a Plexiglas body plethysmograph chamber, as previously described (7, 37). A saline-filled PE 50 tube (i.d. 0.58 mm) with side holes was positioned in the esophagus and connected through a port in the wall of the chamber to a pressure transducer for measurement of pleural pressure. The 18-gauge thin-walled metal tracheotomy tube was connected to a port in the wall of the chamber using polyethylene tubing PE 190 (i.d. 1.19 mm), allowing the animal to spontaneously breathe room air. A screen pneumotachometer connected through the wall of the plethysmograph and a Validyne (Validyne Engineering, Northridge, CA) differential pressure transducer were used to measure flow in and out of the chamber. The pneumotachometer output signal was linear over the range of flows tested (0–7 ml/s) and the frequency response of the system was such that amplitude decreased by less than 10% up to a frequency of 12 Hz. The breathing frequency of the mice studied did not exceed 3.5 breaths per second.

The signals from the pressure transducer and the pneumotachometer were processed using a Grass polygraph (Model 7) recorder (Astro-Med, Inc., West Warwick, RI). The flow signal was integrated using a Grass polygraph integrator (Model 7P10) to measure corresponding changes in lung volume. Pressure, flow, and volume signal outputs were digitized and stored on a computer using an analog-to-digital data acquisition system (CODAS-Dataq Instruments, Inc., Akron, OH).

The digitized data were analyzed for dynamic pulmonary compliance (C_{dyn}), pulmonary resistance (R_L), tidal volume, respiratory frequency, and minute venti-

lation from 6–10 consecutive breaths at each recording event. C_{dyn} and R_L were calculated from pleural pressure, airflow, and volume data using the method of Amdur and Mead as previously described (39). To correct for the resistance of the tracheal cannula, the pressure–flow curve relationship for the cannula alone was measured, and the cannula resistance was subtracted from the total resistance measured with the animal in place to determine the pulmonary resistance.

Nonspecific airway reactivity was assessed by a methacholine challenge test. For this, the mouse was placed in the body plethysmograph, and initial baseline measurements were made. Methacholine (Sigma Chemicals, St. Louis, MO) was injected ip in cumulative doses contained in 0.1 ml of sterile water, starting at 1 μ g. The next dose, given 3 min later, was 2 μ g, such that the total cumulative dose was 3 μ g. The successive doses were injected at 3-min intervals such that the successive cumulative doses were 1, 3, 10, 30, 100, 300, 1000, and 3000 μ g. Data from 6–10 breaths were recorded every 30 s. The length of the interval between injections was based on previous observations that the peak response occurs within the first 3 min after challenge.

Statistical Analysis

The mean serum antibody levels total serum IgE and eosinophil numbers in mice challenged with different allergens were compared with crude antigen exposed and using unpaired two-tailed nonparametric analysis. The values of R_L and C_{dyn} between animals treated with crude antigen and treated with purified allergens were compared using Student's unpaired *t* test (SigmaStat, SPSS Science, Chicago, IL). For the comparison, $P < 0.05$ was considered statistically significant.

RESULTS

IgE Response in Mice Exposed to Aspergillus Antigens

The total serum IgE levels in mice exposed to crude *A. fumigatus* extract and recombinant *A. fumigatus* allergens are shown in Fig. 1. The crude *A. fumigatus* extract-sensitized mice showed a substantial increase in the total serum IgE level ($> 16,000$ ng/ml of serum) compared to normals and presensitization levels. The IgE response levels in mice sensitized with recombinant allergens were much lower (Fig. 1).

Aspergillus-Specific IgG Subclass Antibodies

The antibodies against various recombinant allergens and crude *A. fumigatus* extract are shown in Fig. 2. There was no major difference in the IgG₁ antibody levels in the sera of the different groups of mice against

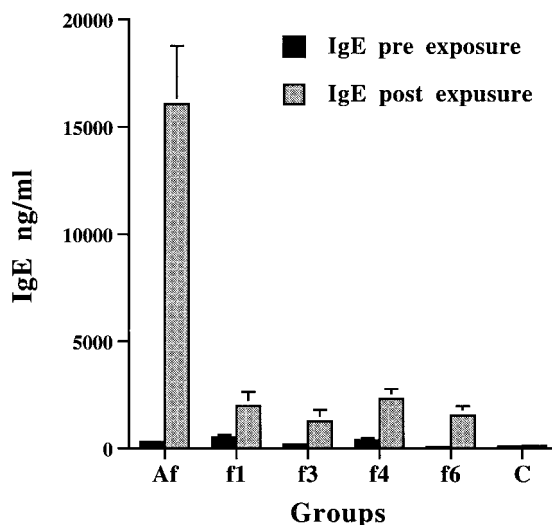


FIG. 1. Total serum IgE response in the sera of mice before (Pre) and after (Post) challenge with various *A. fumigatus* allergens. The various mice groups are those challenged with *A. fumigatus* crude (Af) Asp f 1 (f1), Asp f 3 (f3), Asp f 4 (f4), and Asp f 6 (f6). Control animals (C) represent the last group. For details see Materials and Methods.

each of the immunizing *A. fumigatus* proteins (Fig. 2A). The anti-IgG_{2a} antibody, on the other hand, showed some differences in the different groups of mice challenged with the allergens. Animals exposed to crude *A. fumigatus* extract demonstrated the least IgG_{2a} response, while those exposed to Asp f 1 and Asp f 4 showed markedly enhanced responses. Crude extract and Asp f 6, showed lower levels of IgG_{2b} in the sera of immunized mice. However, Asp f 1 and f 4 showed elevated levels of IgG_{2b} in the sera of sensitized mice compared to the other allergens. Except Asp-f-4-exposed mice, all others showed only very low levels of specific IgG₃ antibody in the sera.

Eosinophils

The eosinophils in the peripheral blood of animals belonging to the various groups of mice challenged with *A. fumigatus* allergens are shown in Fig. 3. The maximum number of blood eosinophils was detected in mice challenged with crude *A. fumigatus* extract. All the animals sensitized with recombinant allergens showed fewer eosinophils in their peripheral blood than those with crude antigen. Although not statistically significant, recombinant allergen Asp f 3 showed slightly enhanced eosinophil numbers in the peripheral blood, while the groups challenged with Asp f 1, Asp f 4, and Asp f 6 showed reduced eosinophil counts in the peripheral blood (Fig. 3).

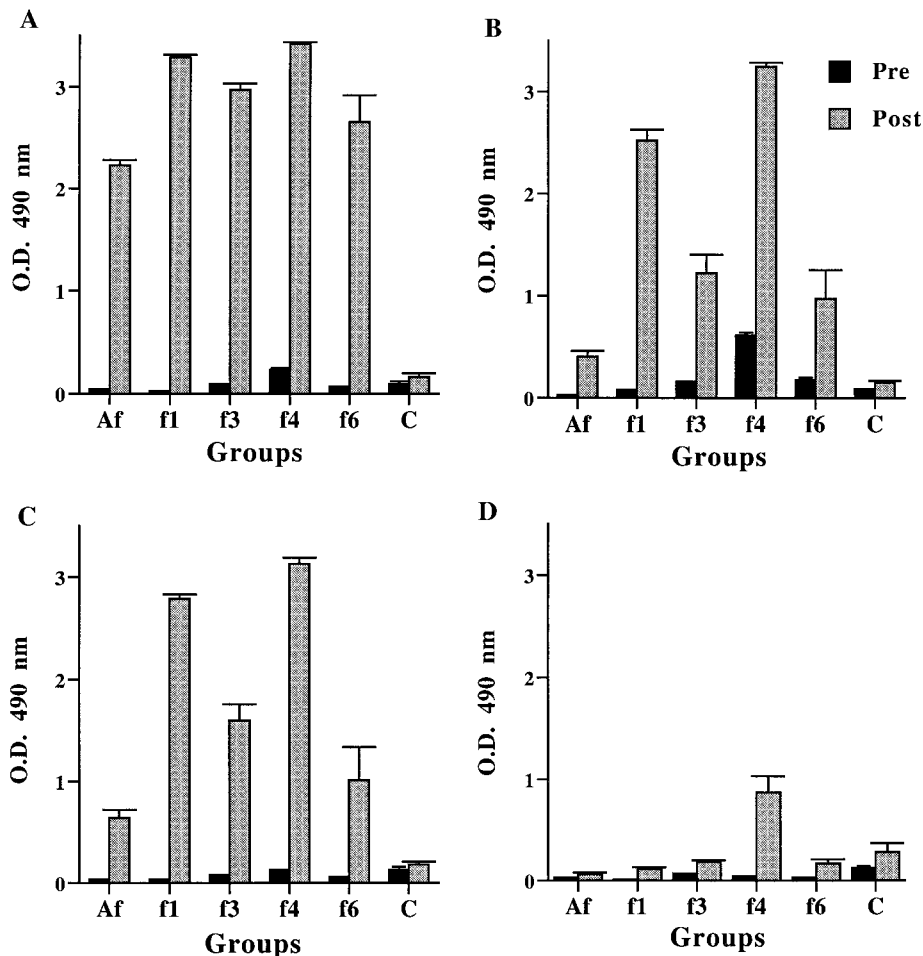


FIG. 2. *Aspergillus*-specific IgG₁ (A), IgG_{2a} (B), IgG_{2b} (C), and IgG₃ (D) are shown. The groups are the same as those in Fig. 1. The ELISA values are represented as optical density at 490_{nm}. For details see Materials and Methods.

Histology

The histopathological features of the lungs in all different groups of mice sensitized with *A. fumigatus* allergens were broadly similar but differed in severity. The nonsensitized control mice showed normal lung histology (Fig. 4A). The lungs of sensitized mice showed significant interstitial inflammation, with peribronchiolar and perivascular infiltrates (Figs. 4B, 4C, 4E, and 4F). The inflammatory cells consisted primarily of small lymphocytes, with lesser numbers of plasma cells and epithelioid histiocytes. Neutrophils and eosinophils were absent or rarely detected. Lymphocytes were often seen in nodular aggregates with no germinal centers. Alveolar spaces contained a few macrophages and lymphocytes. The epithelium lining the bronchioles was surrounded by slightly hyperplastic dense chronic inflammatory cells. No goblet cells were detected in the respiratory epithelium. There were no mucus plugs identified in the airways (Fig. 4D). The

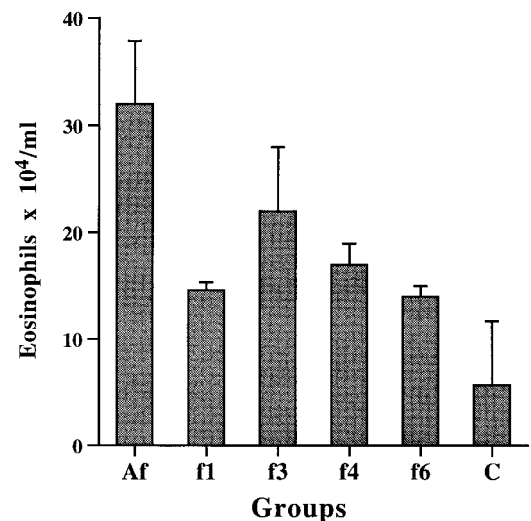


FIG. 3. Eosinophils in the peripheral blood of various groups of mice studied. The groups are the same as those in Fig. 1. For details see Materials and Methods.

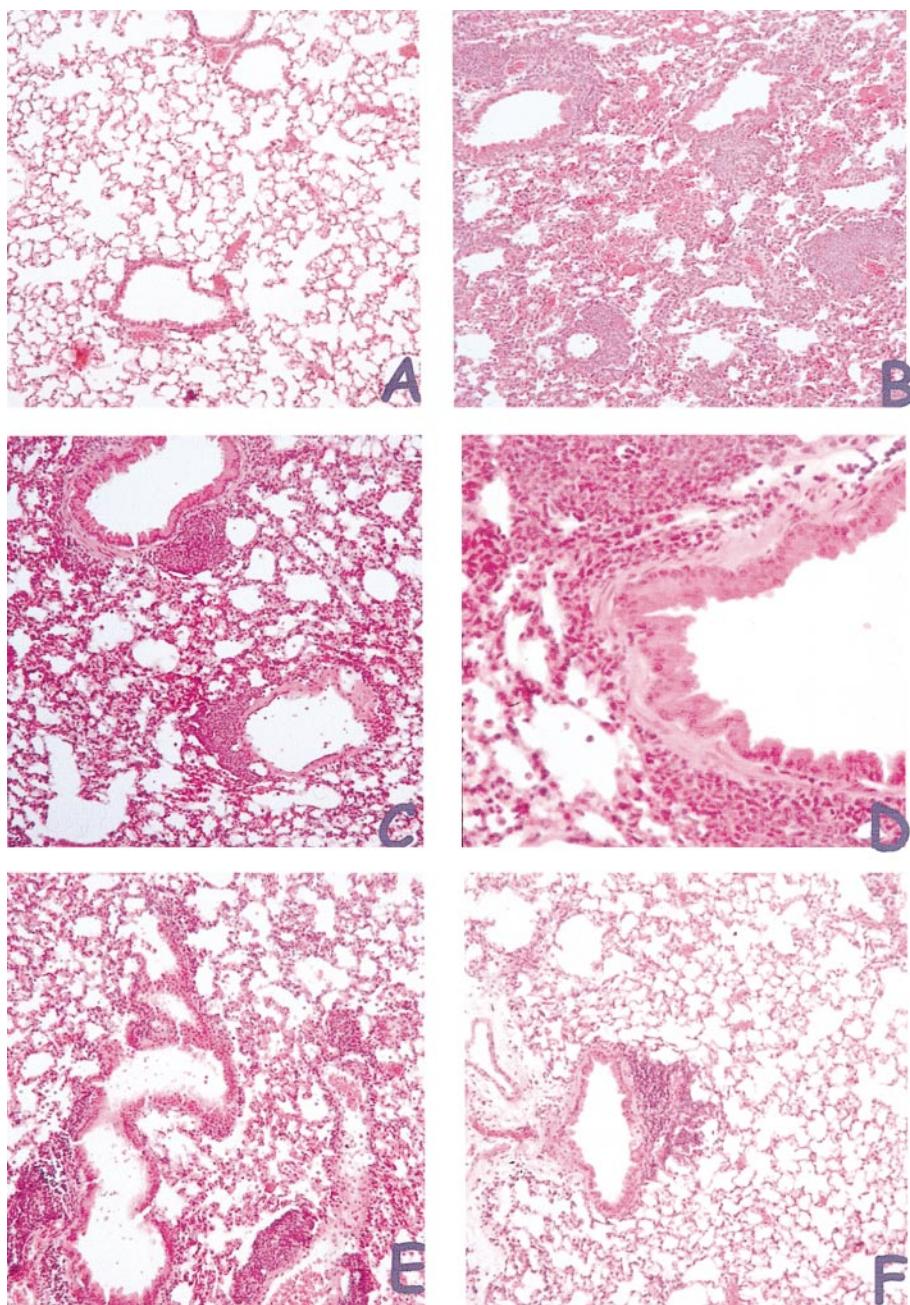


FIG. 4. Histology of the lungs of control and *A. fumigatus* allergen-exposed mice. (A) Lung of an unsensitized control mouse showing normal histology (original magnification, $\times 60$); (B) lung of Asp-f-1-exposed mouse showing moderate peribronchiolar and perivascular chronic inflammatory infiltrates, consisting primarily of small lymphocytes, which present as nodular aggregates (original magnification, $\times 60$); (C) lung of Asp-f-3-challenged mouse showing slightly fewer peribronchiolar and perivascular chronic inflammatory infiltrates than does the Asp f 1 mouse (original magnification, $\times 60$); (D) higher magnification of C showing peribronchiolar chronic inflammatory infiltrate in nodular lymphocytes aggregate. The airway is lined by hyperplastic respiratory epithelium containing no goblet cells. The airway lumen does not show mucus plugs (original magnification, $\times 160$); (E) lung of Asp-f-4-challenged mouse showing a degree of mild peribronchiolar and perivascular chronic inflammatory lymphocytic nodular infiltrate similar to that shown in C ($\times 60$); and (F) lung of Asp-f-6-exposed mouse showing only occasional mild peribronchiolar chronic inflammatory infiltrates (original magnification, $\times 60$). Five to seven animals were used in each group and representative figures from the groups are presented.

intervening pulmonary parenchyma was minimally or mildly involved, but frequently showed inflammatory cells. Occasionally interstitial fibrosis was also de-

tected. There was no evidence of acute necrotizing or lymphocytic vasculitis, nodular granulomas, or multinucleated histiocytic giant cell reactions.

The lungs of mice exposed to crude *A. fumigatus* extract showed chronic interstitial infiltration with lymphocytes, small numbers of plasma cells, epithelioid histiocytes, and neutrophils, and large numbers of eosinophils (Fig. 5). The bronchioles were surrounded by chronic inflammatory infiltrates and frequently lined by hyperplastic respiratory epithelium. The lung changes were also characterized by intra-alveolar inflammatory infiltration of macrophages in addition to the other cells mentioned above. In comparison the lungs of mice challenged with *A. fumigatus* crude extract, mice exposed to Asp f 1, Asp f 3, and Asp f 4 (Figs. 4B–4E and 5) showed only mild to moderate peribronchiolar and perivascular interstitial infiltration. Asp-f-1-challenged animals showed more severe inflammatory cells adjacent to the airways and blood vessels (Fig. 4B) than seen in mice challenged with Asp f 1, f 3, and f 4. The lungs of mice challenged with Asp f 6 invariably showed the least severe response, with only a few mild chronic infiltrates in the peribronchiolar and perivascular areas of the pulmonary interstitial tissues (Fig. 4F).

Airway Response

The baseline prechallenge value of R_L for 22 unsensitized control mice was 0.65 ± 0.07 (SE) cm H₂O/ml/s. The baseline value for C_{dyn} was 0.040 ± 0.004 ml/cm H₂O. There were no significant differences between controls for the crude and purified antigen groups (Figs. 6 and 7). Mice sensitized with crude antigen had a significantly higher baseline R_L and lower C_{dyn} than the unsensitized controls. Baseline values for mice sen-

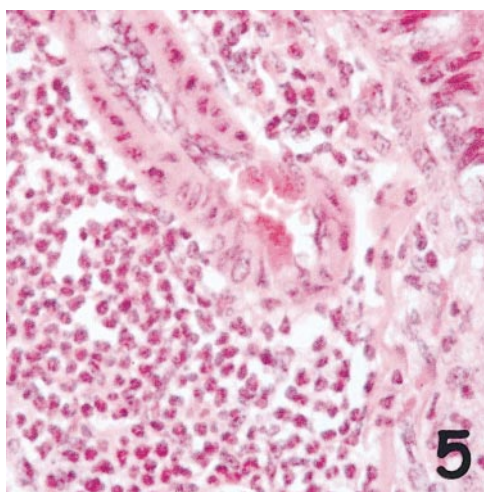


FIG. 5. The lung of a mouse exposed to crude *A. fumigatus* allergen showing severe peribronchiolar and perivascular infiltrate consisting of lymphocytes, plasma cells, histiocytes, and eosinophils. Hematoxylin and eosin staining; original magnification, $\times 400$. For more details see legend to Fig. 4.

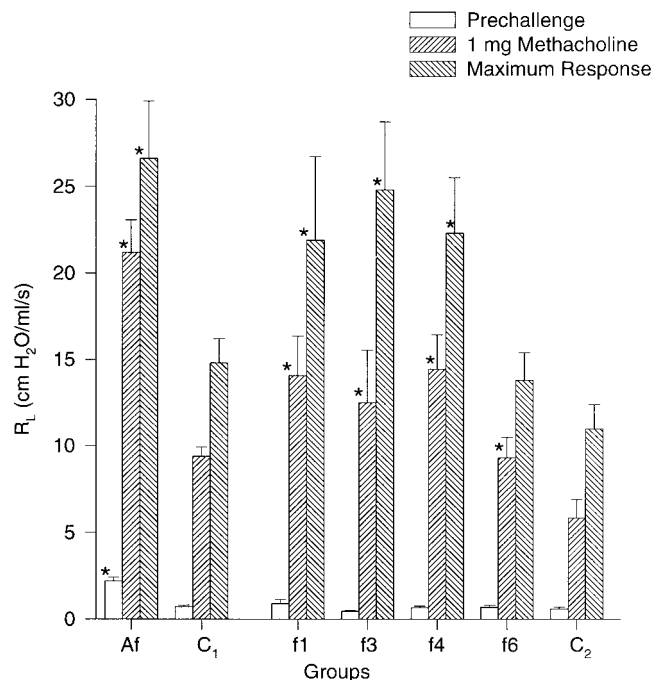


FIG. 6. Airway resistance (R_L) in mice before (prechallenge) and after challenge with methacholine. Both the maximum response and the response to a lung dose are shown. The various groups are those sensitized with *A. fumigatus* crude (Af), unsensitized controls used with *A. fumigatus* crude antigen (C₁), Asp f 1 (f1), Asp f 3 (f3), Asp f 4 (f4), and Asp f 6 (f6), and unsensitized controls (C₂) used with the recombinant allergens. (* R_L significantly greater than that of unsensitized control, $P < 0.05$, unpaired t test). Nine to 12 animals from each group were studied.

sitized with purified antigen were not significantly different from those for unsensitized mice.

A methacholine challenge procedure was used to determine if the airways of *A. fumigatus*-sensitized mice were more reactive than those of unsensitized control mice. Increasing doses of methacholine up to a maximum cumulative dose of 3 mg were given ip as described above. Only 45% of the mice sensitized with crude *A. fumigatus* extract survived to the highest challenge dose. The survival rate for the mice sensitized with purified antigen was 84%. Unsensitized mice had a 100% survival rate.

Airway responses to the 1-mg dose of methacholine were compared. This was the highest dose for which a complete data set was available. The results are shown in Fig. 6. All allergen-sensitized mice showed significantly increased reactivity compared to that for unsensitized mice. The R_L of the mice sensitized with crude extract was significantly higher than that of the groups sensitized with purified antigens. However, the prechallenge R_L value was also significantly higher. Because the maximum R_L response to methacholine challenge did not always coincide with the maximum dose, the maximum airway response irrespective of dose was

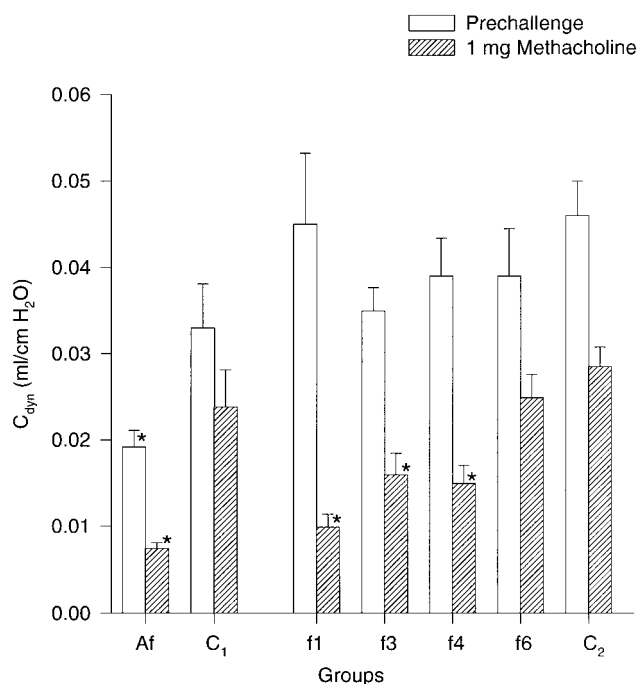


FIG. 7. Lung compliance (C_{dyn}) in mice before (prechallenge) and after challenge with 1 mg of methacholine. The various groups are those sensitized with *A. fumigatus* crude (Af), controls used with Af (C₁), Asp f 1 (f1), Asp f 3 (f3), Asp f 4 (f4), Asp f 6 (f6), and unsensitized controls (C₂) used with the recombinant allergens. (* C_{dyn} significantly less than that of unsensitized controls, $P < 0.05$, unpaired t test). Nine to 12 animals from each group were studied.

also compared between the groups. The results are shown in Fig. 6. Based on the maximum values obtained for R_L , mice sensitized to the crude *A. fumigatus* extract and to allergens Asp f 1, Asp f 3, and Asp f 4 also showed significantly increased reactivity compared to that for unsensitized mice. In contrast, allergen Asp f 6 did not increase airway responsiveness to methacholine challenge. The R_L of the mice sensitized with crude extract was not significantly higher than that of the groups sensitized with purified allergens that showed hyperreactivity.

The effect of methacholine challenge on C_{dyn} is shown in Fig. 7. Methacholine challenge resulted in a significant decrease in C_{dyn} for all groups. Mice sensitized to the crude *A. fumigatus* extract and to allergens Asp f 1, Asp f 3, and Asp f 4 all showed a significantly greater decrease in C_{dyn} than unsensitized mice. The decrease in C_{dyn} for the Asp-f-6-sensitized mice was not significantly different than that observed in the unsensitized control group.

DISCUSSION

Previous studies by our group as well as by other investigators using a crude mixture of *Aspergillus* ex-

tract and *A. fumigatus* spores demonstrated enhanced IgE and eosinophils in murine models of allergic aspergillosis (7–10, 13, 15, 17). These studies also demonstrated lung inflammation resulting from intranasal exposure to *A. fumigatus* antigens. The response to the allergens in the lungs has been characterized as peribronchiolar and perivascular accumulation of inflammatory cells, including lymphocytes, macrophages, eosinophils, and neutrophils. Airway hyperresponsiveness was also reported in these animal models (7, 9, 10, 17). Cytokine knockout mice, particularly IL-4, IL-5, IL-10, and IL-13 and those immunoglobulin deleted, such as IgE knockout and B-cell knockout mice exposed to *A. fumigatus* extract, demonstrated inflammatory response in the lungs and airway hyperreactivity, indicating that the pathogenesis in T-cell mediated (7–11). There was no major difference in the lung inflammatory responses in the IgE and immunoglobulin knockout mice and wild-type control mice (9, 11). However, the evidence indicates that *A. fumigatus*-induced lung reaction and airway responses are not IgE mediated (7–11). Furthermore, mice depleted of eosinophils with anti-IL-5 antibody and IL-5 knockout mice challenged with *A. fumigatus* again showed airway hyperreactivity and persistence of lung pathology (11–14). Thus, it is essential to elucidate the structure–function effects following exposure to the individual purified allergens to understand their role in the immunopathogenesis of ABPA. There was enhanced mucus secretion, goblet cell metaplasia, and epithelial shedding detected at different periods after exposure or after exposure to different antigens. This information may help in understanding the cumulative effect of allergens and their role in pathogenesis as a basis for developing measures to control the disease.

A. fumigatus extract has been shown to contain a number of allergens, toxins, and enzymes (39). It will be interesting to understand the role each single component plays in the overall response leading to allergy and asthma. Hence, the present study focused on investigating the effects of individual allergen exposure to immunological response, airway remodeling, the inflammatory process, and airway hyperreactivity. The antibody response and the eosinophil numbers in the peripheral blood indicate an enhanced Th2 response in the mice exposed to crude *A. fumigatus* extract. Mice exposed to *A. fumigatus* spores also showed a similar response (17). However, the four recombinant allergens showed fewer pronounced Th2 responses, as indicated by IgE levels in the sera and eosinophil numbers in the peripheral blood and pulmonary tissue. The baseline increase in R_L and decrease in C_{dyn} observed in mice sensitized with crude antigen were not found in mice sensitized with purified antigen. All the recombinant allergens except Asp f 6 were able to induce consistent airway hyperreactivity in mice exposed to the

respective allergens. Asp f 6 showed attenuated immune and inflammatory responses in mice compared to other allergen-challenged mice and no airway hyperreactivity based on the C_{dyn} and maximum R_L response to methacholine challenge.

The results presented indicate several interesting findings, which may have direct implications in the pathophysiology of ABPA. Asp f 6 has been shown to react with IgE antibody from fewer ABPA patients, and in parallel this allergen mounted decreased immune response and no airway reactivity in the murine model (7). The remaining three recombinant allergens induced a strong inflammatory response in the lungs and airway hyperreactivity comparable to that of mice exposed to *A. fumigatus* extract, in spite of the reduced IgE responses. Thus, the response observed in the present murine model is not IgE dependent, as was observed before (9–11). There are several other components of *A. fumigatus*, such as proteases, esterases, and toxins, that may also contribute to the cumulative effect (40). Hence, for understanding the pathogenesis of *A. fumigatus*-induced allergy these different fungal components and allergens may be studied alone and in combination in the murine model.

There have been only a few studies where purified allergens were used in the development of a murine model of allergy (41). Der-p-1-exposed mice developed eosinophil influx, IgE response, and airway inflammation. However, no airway response was studied in these animals. Other results are comparable to the results obtained in the present study with recombinant *A. fumigatus* allergen. *In vitro* studies using Der p 1 showed time-dependent breakage of tight junctions of canine kidney and human bronchial epithelial monolayers (42). Similar studies with *A. fumigatus* allergens may contribute to the mechanism of bronchial inflammation and airway responses.

The IgG₁ and IgG_{2a} isotype responses are indicators of the prevailing immune responses, as IgG₁ and IgE represent a Th2 response, while expression of IgG_{2a} is IFN- γ mediated and hence, a Th1 type response. A marked IgE and eosinophil response can be seen in patients during exacerbation phases of ABPA, while those in remission have fewer eosinophils and reduced IgE levels, indicating a Th2 response in the former state and a Th1 response in the latter (2, 4–6). Crude antigen produced higher levels of IgE and low levels of IgG_{2a} in mice, while recombinant allergens demonstrated enhanced levels of IgG_{2a} compared to mice exposed to *A. fumigatus* crude extract. However, in spite of the predominant Th1 response, there was significant airway resistance in these animals, suggesting the role of other contributing factors, including the cumulative effects of all the components present in the crude antigen in the pathogenesis of ABPA. At the present time, we are not certain of the regulatory events causing the

Th1 responses in these animals. Other factors produced by airway epithelial and vascular endothelial cells, by inflammatory and noninflammatory cells of the lungs, as well as the interaction between these factors need to be studied before understanding the pathogenesis.

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