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Key Words

Allergic aspergillosis
Aspergillus antigen
 Cytokines
 Eosinophils
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Introduction

Bronchopulmonary mycoses are immunological lung diseases occurring in susceptible individuals exposed to certain fungi [1, 2]. Of the several fungi, *Aspergillus fumigatus* (Af) is by far the most predominant organism associated with the disorder known as allergic bronchopulmonary aspergillosis (ABPA) [3]. The immune responses characteristic of this disease include elevated total serum IgE and antigen-specific IgG and IgE levels, peripheral blood and lung eosinophilia, and antigen-induced lymphocyte trans-

Abstract

Objective: Allergic bronchopulmonary aspergillosis, a disabling hypersensitivity lung disease, results from inhalation of *Aspergillus fumigatus* antigens present in contaminated environments. A murine model has been developed to understand the immune mechanism involved in allergic bronchopulmonary aspergillosis. We have investigated the immunoregulatory role of different physical forms of *A. fumigatus* antigens, such as *A. fumigatus* spores, soluble antigens, and soluble antigen coupled inert particles, in the model. **Methods:** BALB/c mice were exposed to soluble *A. fumigatus* antigens, spores, or inert particles of comparable size to the spores coupled with *A. fumigatus* soluble antigens. Antibody and eosinophil response, pulmonary pathology, and cytokine expressions were studied. **Results:** Peripheral blood eosinophilia and pulmonary inflammation with influx of eosinophils into the lung was detected more in animals exposed to particulate antigens than in those exposed to soluble antigen. However, the total serum IgE and *Aspergillus*-specific IgG levels showed only a slight increase in the former groups as opposed to elevated levels in animals exposed to soluble antigen. The cytokine expression in in vitro antigen stimulated spleen cells showed a typical Th2 pattern in all antigen-exposed animals. IL-5 mRNA could be detected in the spleen cells cultured with antigen from all groups of antigen-exposed animals. **Conclusion:** Particulate *A. fumigatus* antigens induced eosinophilia in mice prior to the elevation of serum IgE levels. This pattern of IgE and eosinophilia is reversed with the soluble antigen exposure in this model.

formation [3–5]. If intervention is delayed, the disease may progress to central bronchiectasis, leading to pulmonary dysfunction and death. The immune mechanism(s) involved in the pathogenesis of ABPA is not understood, and this knowledge is essential for control by appropriate intervention and treatment.

In order to understand the immune mechanism(s) involved in ABPA, several animal models have been developed; most of the significant characteristics of human ABPA have been reproduced in murine models [6–9]. We have developed a model of ABPA by exposing BALB/c

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mice to soluble antigens of Af [6]. Both intraperitoneal and intranasal administration of Af antigens resulted in elevated IgE and peripheral blood and lung eosinophilia [6, 10]. It was noted that intranasal instillation of particulate antigen enhanced eosinophilia in these mice [6].

The role of various cytokines, particularly IL-4, IL-5, and IFN- γ , in regulating IgE and eosinophilia in the murine model of aspergillosis has also been studied [11, 12]. However, in these studies only soluble antigens from Af were used. As the natural history of the disease is assumed to be through inhalation of Af spores from the environment, we have used Af spores in the current study. Since the physical nature of the antigen may exert some effect on the immune response, we have studied the response to particulate antigens in the form of Af-soluble antigen-coated beads which have identical sizes to that of spores. The results indicate enhanced IgE production after exposure of mice to soluble *Aspergillus* antigen, while early eosinophilia was more pronounced in animals challenged with particulate *Aspergillus* antigen such as spores and inert particles coated with *Aspergillus* antigen.

Materials and Methods

Mice

10- to 12-week-old female BALB/c mice were obtained from Sasco Laboratory (Omaha, Nebr., USA) and housed in our Veterinary Medical Unit. The animals were allowed food and water ad libitum. Animal studies were carried out according to the guidelines of the National Institute of Health, and the protocol was approved by the Institutional Animal Studies Committee.

Af Antigen

The antigen used was a mixture of culture filtrate and mycelial extracts from Af. The method of antigen extraction has been described in detail previously [13, 14]. In brief, Af was grown as stationary culture in a synthetic broth (AOAC and Zapek broths 1:1 v/v with 1% glucose) for 2–3 weeks at 37°C, and the culture filtrate was dialyzed against deionized water and freeze-dried. Extract was prepared from the mycelium of Af grown at 37°C for 72 h in aerated cultures. The antigen was prepared by homogenizing the washed mycelium in a French press at 703.66 kg/cm² and dialyzing as above. Both culture filtrate and mycelial antigens were evaluated for their protein contents, sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles, and reactivities with patient and normal sera [14]. Antigens showing reactivity comparable to in-house standards only are used.

Antigen-Coated Beads

The particulate antigen used for intranasal exposure was prepared by coupling Af to polystyrene microbeads (3- μ m diameter polybeads; Polysciences, Warrington, Pa., USA) with glutaraldehyde according to the instructions of the manufacturer as previously described [6]. The amount of antigen bound to the surface of the beads could not be precisely quantitated because of the low protein content of the antigens.

The antigen-coated beads (1.68×10^8 /ml) gave an optical density of 0.802 A₄₅₀ on reaction with a 1:1,000 dilution of a patient serum in an enzyme-linked immunosorbent assay (ELISA) as compared with 0.259 with uncoated control beads treated identically. The coated beads also agglutinated a 1:100 dilution of serum containing 90 μ g/ml of Af-specific IgG1 from a mouse immunized with *Aspergillus* antigen.

Spores

Af spores were harvested from a 4-day-old culture of Af on Sabouraud's agar. The spores were washed several times in phosphate-buffered saline containing 0.05% Tween 20 to remove the medium components. The washed spores were counted in a hemacytometer, while viable counts were determined by the dilution plate method. Spore preparations with 95% or more viability were used to challenge the animals.

Immunization of Mice with Af Antigen

10- to 12-week-old female BALB/c mice were exposed intranasally to Af antigens twice a week for 8 weeks [6, 11]. The animals were then divided into three groups, and each group was exposed either to soluble antigen, antigen-coated beads, or spores. Sterile saline treated animals served as controls. The mice were immunized intranasally following light anesthesia with methoxyflurane (Metofane; Pitman-Moore, Mundelein, Ill., USA). Two hundred micrograms of Af antigen (about 50 μ g of protein) in 50 μ l of phosphate-buffered saline was instilled into the nostrils with the tip of a pipette. The animals were similarly challenged with 10×10^6 antigen-coated or plain beads or 1×10^6 spores. The animals were held in an upright position for 2 min to regain normal breathing. The mice were bled 1 week after the last antigen exposure, and the total serum IgE and Af-specific IgG antibody levels were determined by ELISA. The mice were finally challenged with the respective preparation and sacrificed 2 and 7 days after final exposure. The animals sacrificed after 7 days were studied for serum IgE, Af-specific IgG, peripheral blood eosinophils, bone marrow eosinophil peroxidase, cytokine mRNA, cytokine production by spleen cells, and histology of the lungs. The mice sacrificed after 2 days of Af exposure were tested for serum IgE, eosinophils, eosinophil peroxidase, and histology. Most significant parameters at this time point only were tested based on our previous studies [6, 11, 12].

Eosinophils in Peripheral Blood

Animals exposed to various Af antigens were bled from the tail vein, and the eosinophils were enumerated on a hemacytometer after staining with Discombe's fluid [6, 15]. The mean value of the eosinophil counts from 5 animals was utilized for comparisons.

Spleen Cell Cultures

Spleens from mice sacrificed 7 days after the last exposure to antigen were collected aseptically, separately cut into small pieces, and gently minced against a sterile No. 100 steel mesh screen with the piston of a sterile plastic syringe as previously described [10]. The suspended cells were washed in RPMI 1640 medium (Gibco, Grand Island, N.Y., USA) containing antibiotics.

Erythrocytes were lysed with Tris-buffered ammonium chloride, and the cells were washed in RPMI 1640 medium. The cells were counted on a hemacytometer, and 2×10^6 cells/ml were cultured in complete RPMI 1640 medium with 10% fetal bovine serum, glutamine, penicillin (100 U/ml), and streptomycin (100 μ g/ml), as described previously [10]. Purity and viability of lymphocytes were con-

sistently greater than 90%. The cells were cultured with 5 µg of concanavalin A or 10 µg of soluble antigen per milliliter in a 24-well plate. After 60 h, the supernatants were collected and analyzed for secreted cytokines by ELISA. The cultures were incubated for up to 9 days to determine total IgE and *Aspergillus*-specific IgG production.

Preparation of Bone Marrow Cells

Bone marrow cells were isolated from the femur of the mice as previously reported [16]. After aseptically removing and repeatedly washing with Hanks' balanced salt solution containing antibiotics, both ends of the bones were cut off and the bone marrow flushed out. The single-cell suspension was washed several times, and the concentration of cells was adjusted by counting the cells in a hemacytometer and suspending them in complete RPMI 1640 medium as described above.

Total Serum IgE and Af-Specific IgG

Total serum IgE and Af-specific IgG levels were measured by ELISA as previously described [6, 17]. Af-specific serum IgG antibody was detected by coating the Af antigen at a concentration of 5 µg/ml onto ELISA plates. Serum dilutions were incubated for 3 h, followed by biotin-conjugated rabbit antimouse IgG and horseradish peroxidase-streptavidin conjugate. Substrate and chromogen were added and the color read in an ELISA reader using a 490-nm filter. The optical density values are presented after subtracting the blanks.

Eosinophil Peroxidase in Bone Marrow Cells

Eosinophil peroxidase in bone marrow cells was estimated using the assay as previously described [12, 18]. Briefly, 100 µl of bone marrow cells (5×10^5 /ml) from mice was incubated in round-bottom microtiter plates. Cultures were set up in duplicate wells. After 48 h of incubation, the plates were centrifuged at 1,000 rpm for 10 min and the medium aspirated, and this was followed by the addition of 100 µl of 1 mM *o*-phenylenediamine in 0.05 mM Tris-HCl, pH 8.0, containing 0.1% Triton X-100 and 1 mM hydrogen peroxide, to each well. After 30 min incubation at room temperature, the color reaction was stopped by addition of 50 µl of 4 M sulfuric acid, and the absorbency at 490 nm was determined using an MR 700 microplate reader (Dynatech Laboratories, Chantilly, Va., USA). The data are represented as optical density after subtracting the blanks.

Cytokines in the Culture Supernatants of Spleen Lymphocytes

Supernatants from spleen cell cultures stimulated with the Af antigens were assayed for IL-4, IL-5, and IFN-γ by means of a two-site sandwich ELISA as previously described [10, 19]. In brief, microtiter plates were coated with monoclonal antibodies against mouse IL-4 (11B11), IL-5 (TRFK-5), and IFN-γ (XMG1.2) in phosphate-buffered saline overnight at 4°C. After washing and blocking the wells, samples were added and incubated for 3 h at room temperature. This was followed by the addition of a biotinylated second antibody and incubation for 1 h. The wells were washed, and streptavidin peroxidase was added and the samples again incubated. After washing the wells, the substrate-chromogen was added, and the developed color was read at 490 nm as described above.

Standard curves of IL-4, IL-5, and IFN-γ were run on each plate and the optical densities of the samples were compared and quantitated.

Cytokine mRNA

Spleen cells from mice sacrificed 7 days after the last exposure of the antigen or saline were prepared in RPMI 1640 medium with antibiotic and washed three times. Cells (10×10^6 /ml/tube) were cultured

for 18 h in polypropylene tubes with concanavalin A (5 µg/ml), *Aspergillus* antigen (10 µg/ml) or medium alone. These cells were collected, washed in phosphate-buffered saline, suspended in lysis buffer, and stored at -70°C until used [20, 21]. For RNA isolation, 0.5 ml of the cell suspension in lysis buffer was thawed and extracted with 500 µl of water-saturated phenol and 100 µl of chloroform-isoamyl alcohol (49:1). The final suspension was shaken vigorously for 10 s, cooled on ice for 15 min, and then centrifuged at 10,000 g for 20 min at 4°C. The aqueous phase was transferred to a new tube, mixed with 500 µl of isopropanol, and held at -70°C for 30 min and then at -20°C for 2 h. This preparation was then centrifuged at 10,000 g for 20 min at 4°C. The RNA pellet was resuspended in 1 ml of 75% ethanol and stored overnight at -20°C. After centrifugation, the tubes were inverted and the alcohol drained. The pellet was air-dried in a waterbath at 37°C and resuspended in 18 µl of diethyl pyrocarbonate treated sterile water.

cDNA Preparation

One microgram of oligo-dT (Promega, Madison, Wisc., USA) was added to the suspension which was then incubated at 65°C for 10 min. After the suspension was cooled on ice, 400 U of Moloney murine leukemia virus reverse transcriptase (Life Technologies GIBCO/BRL, Grand Island), 24 U of RNasin (Promega), 2 µl of 10 mM dNTPs (Perkin Elmer, Hayward, Calif., USA), and 6 µl of 5× reverse transcription buffer (Life Technologies) were added. The mixture was incubated at 42°C for 60 min and heated at 65°C for 10 min. The cDNA was stored at -20°C until used.

Polymerase Chain Reaction (PCR)

cDNA from each sample was amplified using 5' sense and 3' antisense primers for murine β-actin, IFNγ, IL-4, IL-5, and IL-10 (all primers made by National Bioscience, Plymouth, Minn., USA) [21, 22]. In brief, 3 µl of cDNA, prepared as described above, was added to 2 µl of 10 mM four dNTPs mix, 2 µl of 25 mM MgCl₂ (Perkin Elmer Cetus, Emeryville, Calif.), 1 µM of each primer, 2.5 U of Ampli Taq polymerase (Perkin Elmer), 2 µl of 10× PCR buffer (Perkin Elmer), and 6.5 µl of sterile distilled water. The mixture was amplified in a GeneAmp PCR system 9600 (Perkin Elmer) for 31 cycles (20 s at 94°C, 20 s at 63°C, 20 s at 72°C with 1 s increment on subsequent cycles). The amplified PCR fragment was analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The size of the PCR product was confirmed by comparing with a DNA ladder (GIBCO/BRL).

Histological Examination of Lungs

The animals were sacrificed at the termination of the experiment, and their lungs were fixed in 10% buffered formalin. Sections were stained with hematoxylin and eosin and Gomori methenamine silver for histological study. The eosinophils were counted and graded as follows: 0 = no, 1–3 = occasional, 3–10 = few, 11–30 = moderate, and > 31 = numerous eosinophils.

Statistics

The mean and average were calculated for each group, and the groups were compared by paired t test analysis.

Results

Total Serum IgE and Af-Specific IgG

All animals exposed to different Af antigens showed 10- to 35-fold increases in total serum IgE when compared with their preimmunization levels (fig. 1). Animals exposed to soluble antigen exhibited significantly higher IgE levels as compared with groups exposed to spores or beads (fig. 1). The IgE levels were greater on day 7 as compared with day 2 in all antigen-exposed groups. Af-specific IgG was detected only in minute quantities in the groups challenged with spores or antigen-coupled beads as compared with those challenged with Af-soluble antigen (fig. 2).

Eosinophils

On day 2, following challenge with antigen-coated beads or spores, the eosinophil numbers in the blood of animals increased markedly as compared with animals given soluble antigen (fig. 3). However, by 7 days, the eosinophil numbers in soluble antigen challenged animals surpassed those of the other two groups of mice when a decline was evident.

Eosinophil Peroxidase in Bone Marrow

All animals challenged with Af antigens, irrespective of their physical nature, demonstrated an increase in eosinophil peroxidase in their bone marrow cells. This increase was maximum after 2 days of the last exposure (fig. 4).

Cytokine Production by Spleen Cells Stimulated with Antigen

The cytokines expressed by the antigen-stimulated spleen cells on day 7 are shown in figure 5. The patterns of IL-4 and IL-5 production on day 7 in the different groups of animals matched with the IgE levels (fig. 1) and eosinophil numbers (fig. 3), respectively. Mice exposed to soluble antigen produced higher levels of IL-4 as compared with those treated with beads. No major differences were detected in the levels of IL-4 and IL-5 produced by animals challenged with particulate antigens or with spores. The IFN- γ production was negligible in all antigen-challenged animals when compared to controls (fig. 5).

Cytokine mRNA in Spleen Cells

A representative result of IL-4, IL-5, IL-10, and IFN- γ mRNA is shown in figure 6. IL-4, IL-5, and IL-10 were detected in all concanavalin A stimulated cells (fig. 6, lane b). The spleen cells stimulated with Af antigen also showed mRNA transcript of IL-5 (fig. 6, lane c). No IL-5 mRNA was detected in antigen-stimulated spleen cells from control

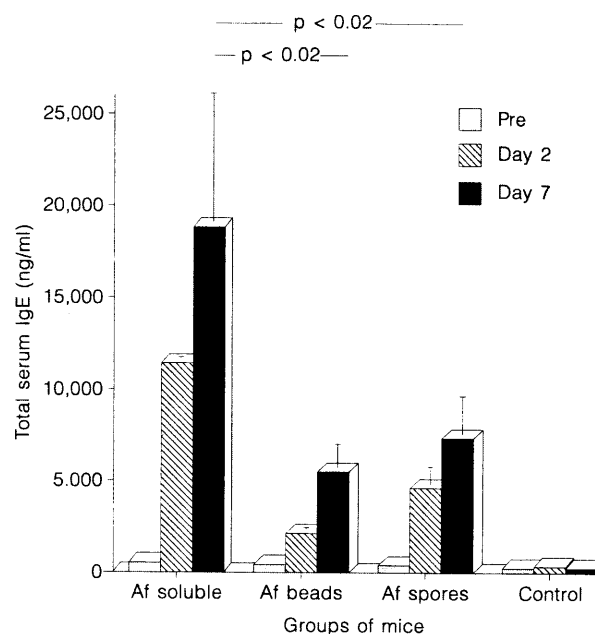


Fig. 1. Total serum IgE response in different groups of animals. The columns represent IgE before and after immunization. The bars represent standard error of the means.

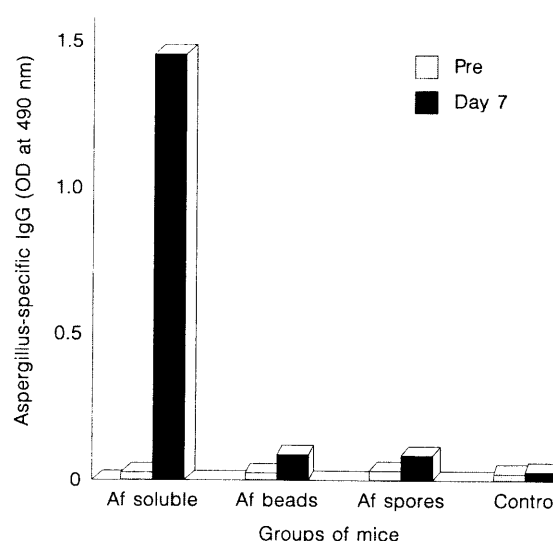


Fig. 2. *Aspergillus*-specific IgG in different groups of animals; all details are the same as in figure 1. OD = Optical density.

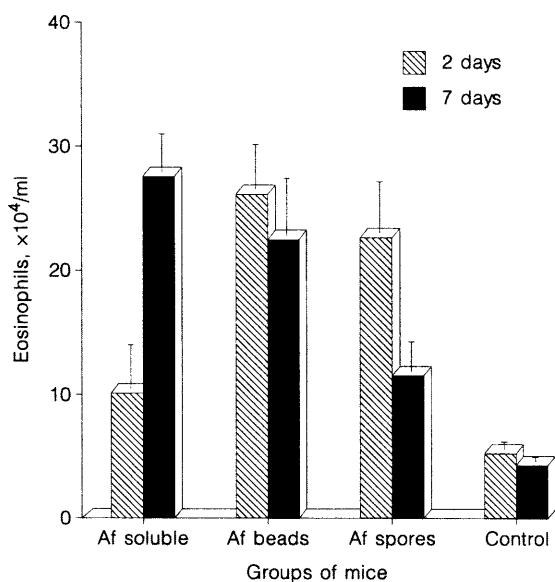


Fig. 3. Peripheral blood eosinophils enumerated by staining with Discombe's stain.

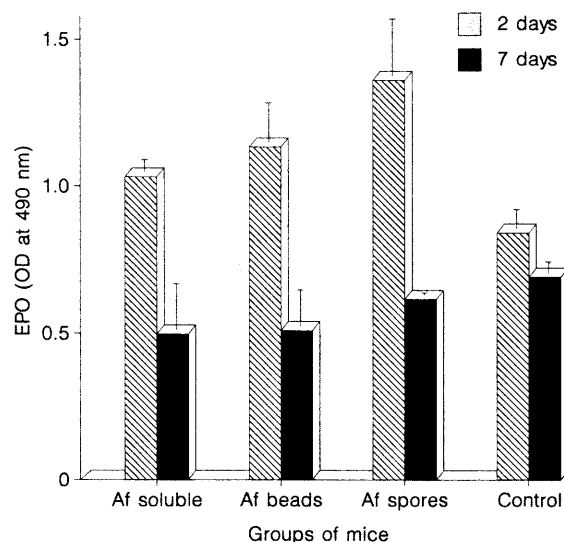


Fig. 4. Eosinophil peroxidase (EPO) detected as optical density (OD) values at 490 nm from the bone marrow of different groups of animals.

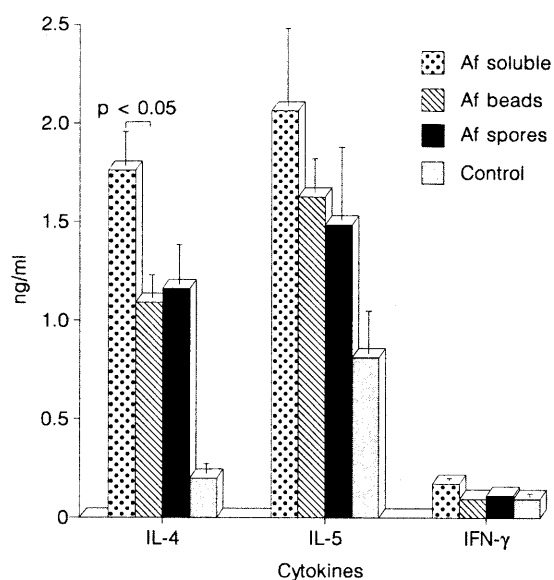


Fig. 5. IL-4, IL-5, and IFN-γ in spleen cell culture supernatants.

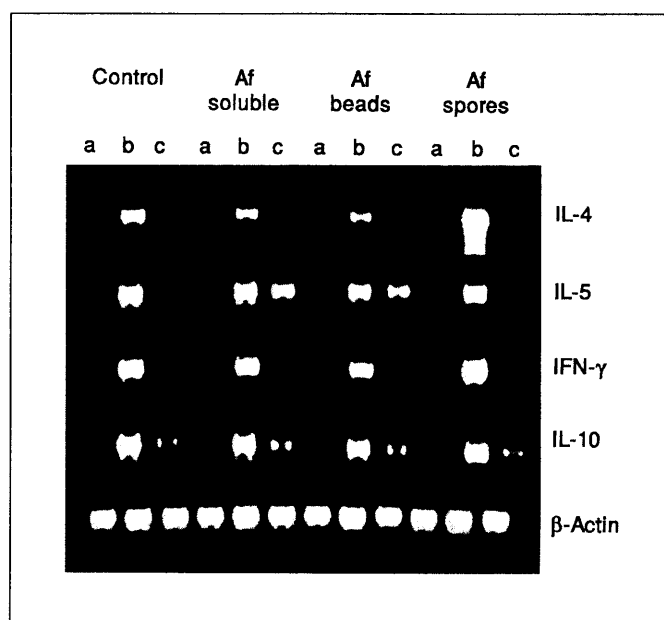


Fig. 6. mRNA transcripts of IL-4, IL-5, IL-10, IFN-γ, and β-actin in spleen cells of different groups of mice are shown. The lanes a, b, and c represent unstimulated spleen cells, cells stimulated with concanavalin A, and cells stimulated with *Aspergillus* antigen, respectively.

Fig. 7. Section of the lung, 2 days after last challenge with beads, reveals a prominent perivascular chronic interstitial inflammatory infiltrate with numerous eosinophils, HE. $\times 150$. The inset shows eosinophils at higher magnification. HE. $\times 300$.

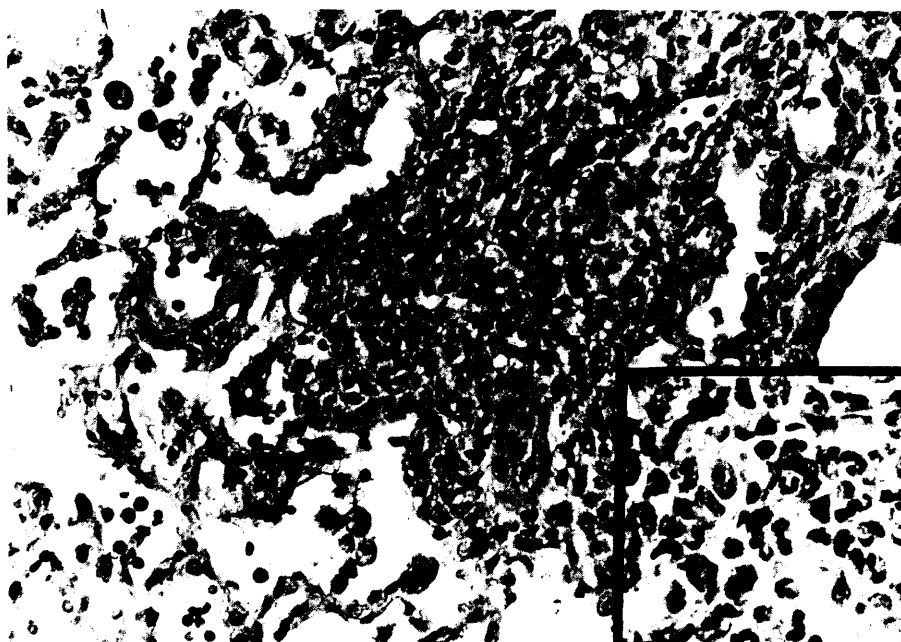
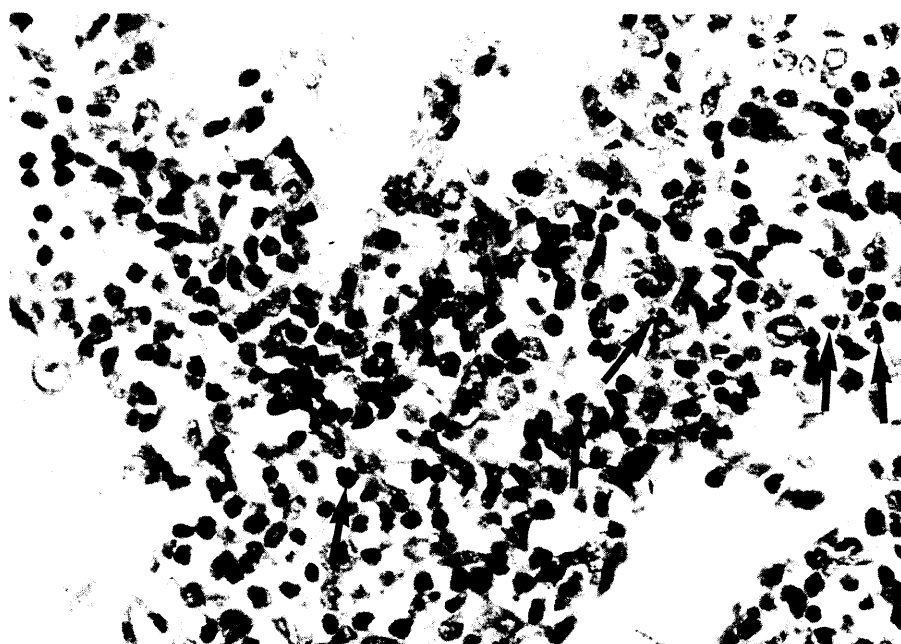


Fig. 8. Section of the lung, 2 days after exposure to soluble antigen, shows similar perivascular chronic inflammatory infiltrates, but only a minimal number of eosinophils (arrows). HE. $\times 300$.



mice (fig. 6, lane c). The cytokine mRNA transcription of IL-4 showed considerable variation in antigen-stimulated spleen cells (fig. 6, lane c). mRNA of IFN- γ was negative in controls as well as in *Aspergillus* antigen stimulated cells, while mRNA of IL-4 was positive in animals inoculated with spores (fig. 6, lane c) and rarely only in culture filtrate antigen and Af beads challenged animals.

Lung Histology

The lungs of mice studied 2 days after challenge with particulate Af antigens revealed prominent interstitial chronic inflammatory infiltrates, particularly around the vessels and bronchioles, along with numerous eosinophils (fig. 7). Mice treated with spores, in addition, showed a granulomatous reaction with giant cells and many neutrophils. However, only a mild infiltration of eosinophils was seen in animals treated with soluble culture filtrate antigen, al-

Fig. 9. Lung tissue, 7 days after treatment with spores, shows a moderate interstitial inflammation with a few eosinophils (long arrows) and many neutrophils (short arrows) HE. $\times 185$.

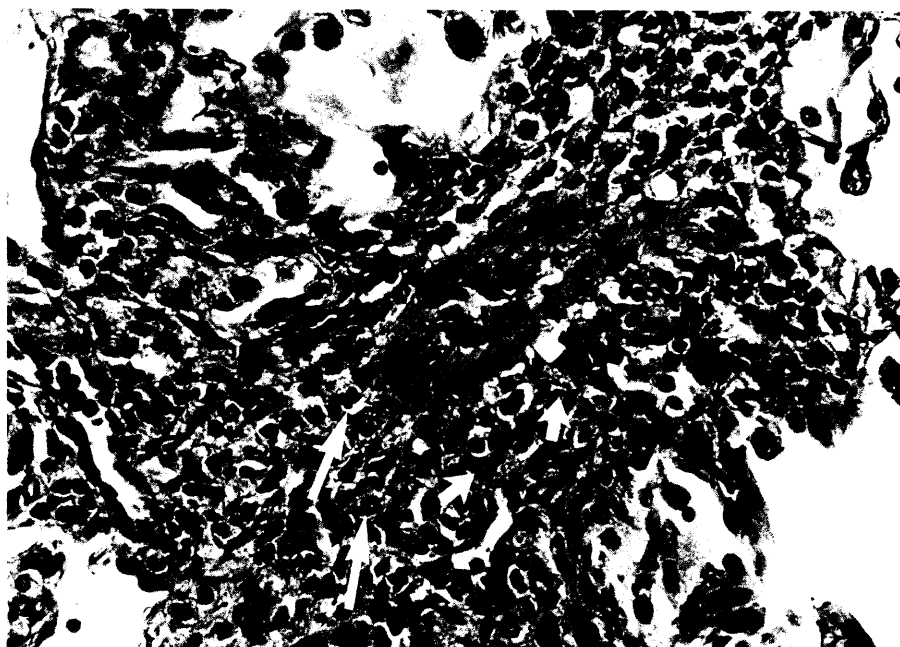
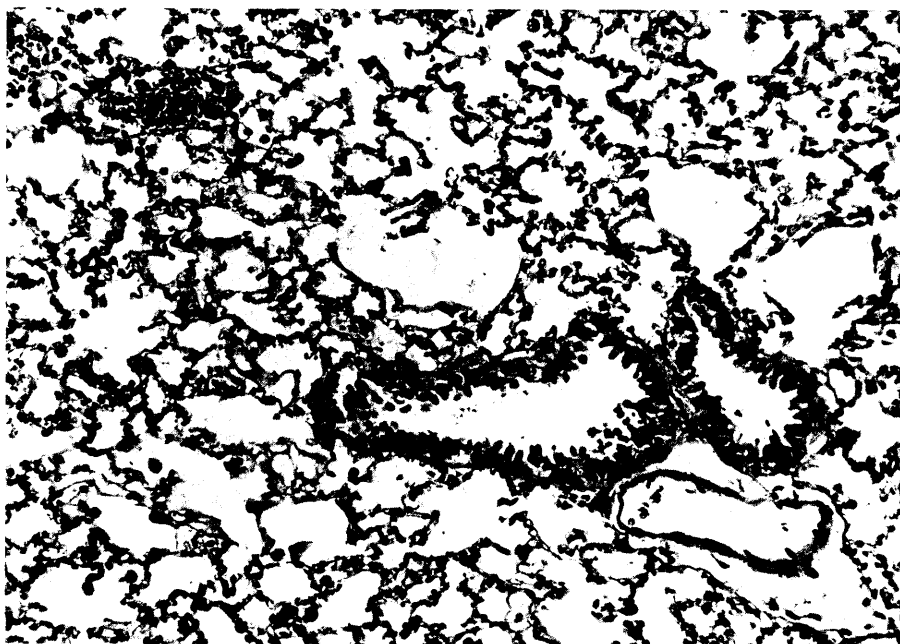


Fig. 10. The control mouse showing a normal structure of the lung. HE. $\times 120$.



though this group also showed a similar degree of peribronchiolar and perivascular infiltrates, similar to other groups (fig. 8). The inflammatory changes in the lungs remained the same 7 days after the last antigen exposure, but few eosinophils only were detected among the interstitial infiltrates present in the lung parenchyma of all the three groups of mice (fig. 9). The infiltrates including eosinophils were uniformly present as perialveolar in the both groups studied. All normal controls showed no changes (fig. 10).

Discussion

The results presented indicate an enhanced total serum IgE response in animals challenged intranasally with soluble Af antigen as compared with animals challenged with antigen-coupled beads or spores. This was probably related to the antigen load rather than the physical nature of the antigen. To expose animals to a comparable amount of soluble antigen, a substantial quantity of spores and antigen-cou-

pled beads would be necessary. The Af-specific serum IgG antibody response also showed an identical trend as with the serum IgE levels in all three groups of animals. The results thus indicate that an IgE response was induced in mice in response to Af antigen whether the antigen was coupled to inert particles, given as aqueous solution, or as spores.

All antigen-challenged animals showed eosinophilia during the observation period. In addition, the results indicate that eosinophilia occurred early following particulate antigen or spore exposure as compared with soluble antigen exposure (fig. 3). The presence of elevated eosinophil levels in the bronchoalveolar lavage fluid and peripheral blood has been shown to correlate with allergic inflammatory disease such as asthma [23–25]. Activated eosinophils produce a variety of proinflammatory mediators such as eosinophil cationic protein, major basic protein, eotaxin, eosinophil peroxidase, and eosinophil neurotoxin [23, 26, 27]. Some of these mediators can directly damage airway epithelium and induce contraction of bronchial smooth muscles. Several factors are considered to be important in eosinophil recruitment and homing [28–30]. Eosinophilia in mice exposed to particulate Af antigen in the form of antigen-coated beads or Af spores precedes increases in serum IgE levels. This finding is similar to what is observed in human allergic aspergillosis, where exacerbation, resulting from exposure to antigen, results in enhanced sputum eosinophil and peripheral blood eosinophil levels. The marked enhancement of lung and blood eosinophils subsequent to Af antigen challenge suggests a major role for eosinophils in inducing the asthmatic reactions even when IgE levels are low, as is seen in some cases of ABPA.

On stimulation with Af antigen, IL-4 and IL-5 were secreted in the spleen cell culture supernatants of the immunized animals, while only low levels of IFN- γ were detect-

ed. These cytokine levels correlated with the serum IgE and eosinophilia in the different test groups. Similarly, mRNA transcripts of IL-5 were detected in antigen-stimulated spleen cells from Af-exposed mice. The histology of the lungs revealed peribronchiolar and perivascular infiltration with eosinophils in all experimental animals. Eosinophilia, IgE responses, and the cytokine profile detected in these animals represent a T helper 2 type of response and is in conformity with other previous reports [6, 10, 11].

The natural exposure of patients with ABPA to Af may not be strictly comparable to the present experiments. It is difficult to assess the quantity of spores or antigens inhaled by patients, although exposure in most cases is of a low level but continues for prolonged periods of time [1]. The present results suggest that the early response of exposure to Af is probably eosinophilia followed by IgE production, together leading to pulmonary hyperresponsiveness via inflammation. The enhanced eosinophil recruitment by particulate antigens and the eosinophil factors produced in the lungs [24, 25, 26] may lead to inflammation and more antigen absorption. The increased antigen load in the lungs may result in the persistence of the symptoms in active disease or leads to exacerbations in patients in remission of the symptoms.

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