

Particulate *Aspergillus fumigatus* antigens elicit a T_{H2} response in BALB/c mice

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Background: In order to understand the immunoregulation in allergic aspergillosis, a murine model was developed by exposing animals to particulate and soluble antigens of *Aspergillus fumigatus*.

Methods: BALB/c mice were exposed to soluble *Aspergillus* antigen intranasally. Animals showing moderate levels of IgE were subsequently exposed to soluble antigen or antigen conjugated to polystyrene beads intranasally. The IgE and IgG₁ in the sera and the eosinophils in the blood and lungs were studied. The spleen and lung mononuclear cells were stimulated with both concanavalin A and antigen and evaluated for production of interleukin (IL)-4, IL-5, IL-10, and interferon- γ .

Results: Animals exposed to particulate antigens showed more elevated serum IgE levels and increased numbers of eosinophils in the blood and lungs than those exposed to soluble antigen. Lung cell cultures from animals exposed to particulate antigens when stimulated with *Aspergillus* produced IL-4 and IL-5, indicating a T_{H2} type of response. Animals exposed to soluble antigens showed a weaker T_{H2} response, as evidenced by low IgE levels in sera, fewer eosinophils in the blood, and low levels of cytokine production from lung and spleen cells.

Conclusion: The results indicate that the physical nature of the antigen may have a major role in determining the type of immune response of the host. (*J ALLERGY CLIN IMMUNOL* 1994;93:1013-20.)

Key words: *Aspergillus*, Murine model, cytokine, IgE, eosinophil, T_{H2} cell, interleukins, interferon- γ

In a previous study we have shown that BALB/c mice exposed intraperitoneally or intranasally to *Aspergillus fumigatus* (Af) antigens demonstrate high levels of IgE antibody and eosinophilia in the blood.¹ Animals exposed intraperitoneally with alum-precipitated Af antigen showed more IgE than those exposed through other routes. In addition, animals that were previously primed and had circulating IgE and Af-specific IgG₁ on subsequent intranasal exposure to particulate Af an-

Abbreviations used

Af: *Aspergillus fumigatus*
IFN- γ : Interferon- γ
IL: Interleukin
OD: Optical density

tigen demonstrated further enhancement in serum IgE and blood eosinophilia. These animals also showed a marked accumulation of eosinophils in the lungs. When BALB/c mice were challenged intranasally with Af antigen, an increase in eosinophil numbers in peripheral blood, lungs, and bone marrow was noted.¹ Af antigen also induced the differentiation of eosinophils from precursor cells in the bone marrow.²

It is known that CD4⁺ T_{H2} cells are responsible for the IgE and eosinophilia in parasitic infections, and this is well documented in other murine models as well.^{3, 4} However, the roles of the T-cell subsets and specific cytokines in antibody and eosinophil production induced by Af antigen exposure had not been determined. Hence, we have

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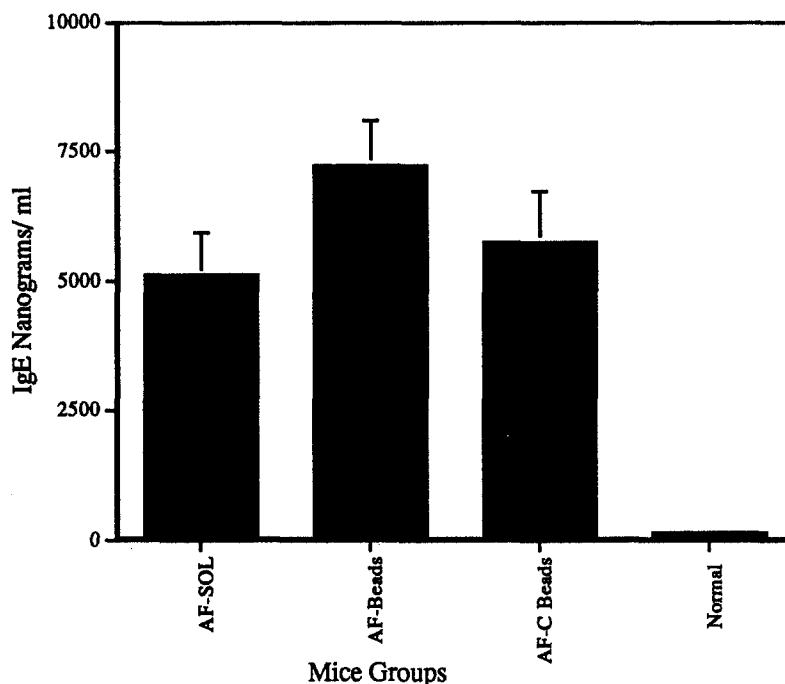


FIG. 1. Total serum IgE in nanograms per milliliter estimated by ELISA in the different groups of mice. All three groups of animals were exposed to Af soluble antigen intranasally, followed by one exposure to soluble Af (AF-SOL), antigen-coated beads (AF-Beads), or uncoated beads (Cont. Beads). IgE in normal control mice (Normal) is also given. Bar indicates standard error of the mean.

measured the cytokines expressed by spleen and lung lymphocytes in animals exposed to Af antigen. The animals exposed to particulate Af antigens demonstrated marked eosinophilia in the blood and lungs and enhanced IgE production in serum but no major difference in the interferon- γ (IFN- γ) production either by lung or spleen lymphocytes. The lung lymphocytes of these animals showed increased expression of interleukin (IL)-4, IL-5, and IL-10 in vitro in response to Af antigen. However, mice challenged with soluble Af antigen intranasally showed consistently low levels of IgE and eosinophil numbers in the blood and failed to express IL-4, IL-5, and IL-10 from lung or spleen cells stimulated with antigen.

METHODS

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.^{5, 6} In brief, Af was grown as stationary culture in a synthetic broth for 2 to 3 weeks at 37°C, and the culture filtrate was dialyzed against deionized water and freeze-dried. The mycelial extract was obtained by homogenizing the washed 72-hour growth from aerated cultures with a French press at 10,000 psi. Both culture filtrate and

mycelial antigens were evaluated for their protein content, sodium dodecylsulfate-polyacrylamide gel electrophoresis profile, and reactivity with patient and normal sera.⁶ The particulate antigen for final intranasal exposure was prepared by coupling Af to polystyrene microbeads (3 mm diameter polybeads; Polysciences, Inc., Warrington, Pa.) with gluteraldehyde according to the instructions of the manufacturer. The amount of antigen bound to the surface of the beads could not be quantitated precisely because of the low amount of proteins and the interference of the beads in the assays. When 1.68×10^9 beads were mixed with a 4 mg/ml (wt/vol) antigen solution, a reduction in the optical density (OD) value at 280 nm from 1.362 to 0.837 was noted after coupling. These coated beads agglutinated a 1:100 dilution of serum containing 90 ng/ml of Af-specific IgG₁ in the serum.

Mice

Female BALB/c/ByJ mice, 10 to 12 weeks old, were obtained from Simonsen Laboratory (Gilroy, Calif.) and were housed in the animal research facility at the DNAX Research Institute. Animal studies were carried out according to National Institutes of Health guidelines for the care and use of laboratory animals.

Immunization of mice with Af antigen

Ten- to twelve-week-old female BALB/c mice were exposed to soluble Af antigen intranasally twice weekly

for 8 weeks. Mice were lightly anesthetized with Metofane (methoxyflurane; Pitman-Moore, Inc., Mundelein, Ill.), and 200 μ g of Af antigen (about 50 μ g of protein) in 50 μ l of phosphate-buffered saline was instilled into the nostrils with a pipetter tip. Animals were held in an upright position for 2 minutes to regain normal breathing. Animals were bled a week after the last antigen exposure, and the antibody levels were determined by ELISA. If the total serum IgE was not increased 20 times or more, animals were further exposed to Af antigen. These animals were subsequently exposed to 10×10^6 antigen-conjugated or unconjugated control beads or soluble antigen intranasally. Animals were killed 10 days after the last antigen exposure.

Eosinophils in the peripheral blood

Mice exposed to Af antigen were bled from the tail vein at intervals, and the eosinophils were estimated by counting on a hemocytometer after staining with Discombe's fluid.⁷ The mean values from five animals were presented.

Lung and spleen cell cultures

Spleens and lungs from animals killed at the termination of the experiment were collected aseptically. The organs were cut into small pieces and gently minced against a sterile no. 100 steel mesh screen with the piston of a sterile plastic syringe. The suspended cells were washed in RPMI 1640 medium (Gibco, Grand Island, N.Y.). Erythrocytes were lysed with Tris-buffered ammonium chloride, and the cells were washed in RPMI 1640 medium. The cells were counted on a hemocytometer, and 2×10^6 cells/ml was cultured in complete RPMI 1640 medium with 10% fetal bovine serum, glutamine, 2-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 μ g/ml) as described previously.⁸ The purity and viability of lymphocytes were always over 90%. The cultures were inoculated with 5 μ g of concanavalin A or 10 μ g of antigen per milliliter in a 24-well plate. After 60 hours, the supernatants were collected and analyzed for cytokine secretion by ELISA.

Isotype specific ELISA

Serum IgE and IgG₁ levels were measured by isotype-specific ELISA as described previously.⁹ The Af-specific IgG₁ in the sera was detected by ELISA as described previously.¹ The Af-specific IgG₁ standards were prepared by absorbing the specific antibody on Affi-gel beads (Bio-Rad Laboratories, Hercules, Calif.) and eluting the bound Af-specific IgG₁. The OD values were converted to nanograms from a standard curve obtained with Af-specific IgG₁.

Cytokine determination in the culture supernatants of spleen and lung lymphocytes

Samples were assayed for IL-4, IL-5, and IFN- γ by means of two-site sandwich ELISA.^{10,11} In brief, micro-

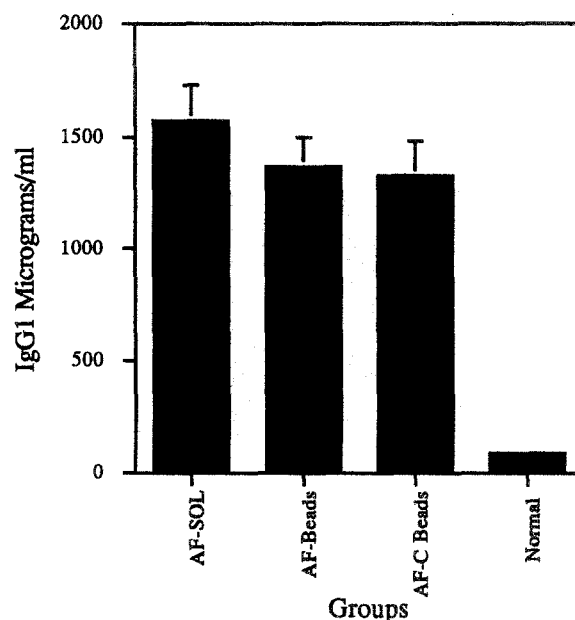


FIG. 2. Total serum IgG₁ in micrograms per milliliter, estimated by ELISA, is given. All groups of animals are the same as in Fig. 1. Bar indicates standard error of the mean.

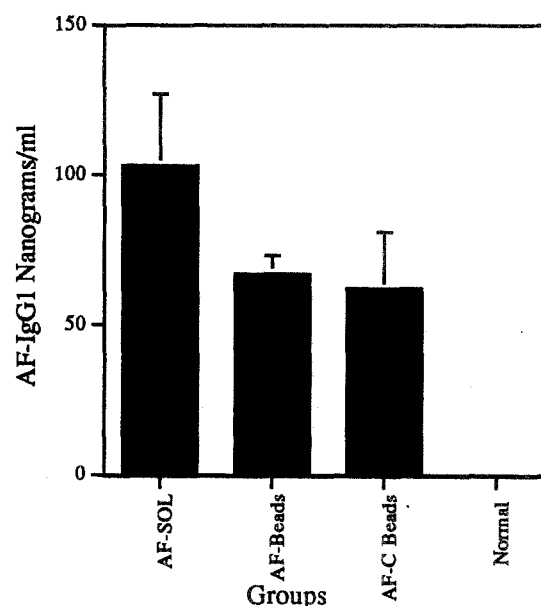


FIG. 3. Estimation of Af-specific IgG₁ in nanograms per milliliter of serum. All groups of animals are the same as in Fig. 1. Bar indicates standard error of the mean.

titer plates were coated with monoclonal antibodies against mouse IL-4 (11B11), IL-5 (TRFK-5), IFN- γ (XMG1.2), or IL-10 (SXC₂) in phosphate-buffered saline overnight at 4° C. After washing and blocking the wells, samples were added and incubated for 3 hours at room temperature. This was followed by the addition of

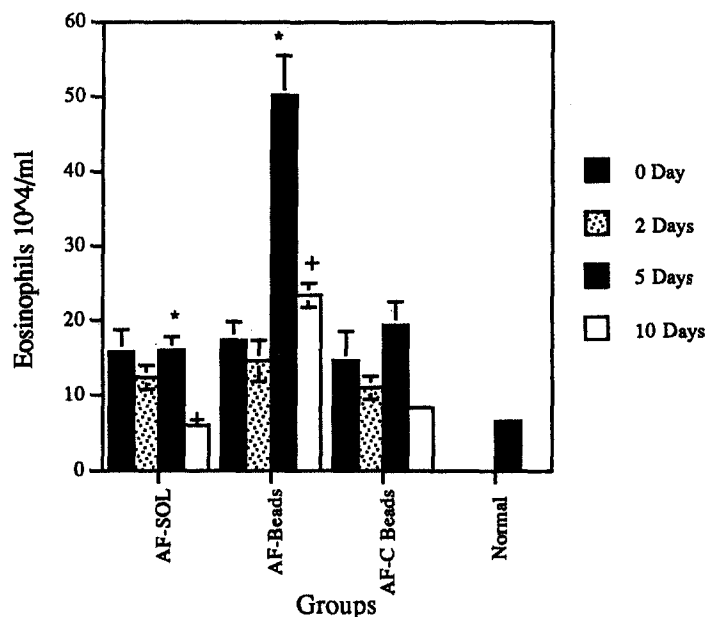


FIG. 4. Peripheral blood eosinophils estimated on days 0, 2, 5, and 10 days after the final exposure of antigen in the different groups of mice. Details about the groups are given in the legend to Fig. 1. Bar indicates standard error of the mean. Fifth day (*) and tenth day (+) eosinophil counts in the peripheral blood were significantly increased in the Af antigen-coated bead versus soluble antigen-exposed mice ($p < 0.01$).

TABLE I. Cytokines produced by spleen and lung cells stimulated with concanavalin A and *Aspergillus fumigatus* antigens

Animal group (mice)	Cytokine	Lung cells stimulated with		Spleen cells stimulated with	
		Con A	Af Ag	Con A	Af Ag
Normal mice		<0.41	<0.41	<0.41	<0.41
Af Ag sol		1.05 ± 0.15	<0.41	<0.41	<0.41
Af Ag beads	IL-4 (ng/ml)	1.9 ± 0.5	0.8 ± 0.12	1.65 ± 0.4	<0.41
Control beads		<0.41	<0.41	2.0 ± 0.3	<0.41
Normal mice		<0.246	<0.246	<0.246	<0.246
Af Ag sol		0.8 ± 0.08	<0.246	<0.246	<0.246
Af Ag beads	IL-5 (ng/ml)	2.74 ± 0.83	0.31 ± 0.13	2.28 ± 0.25	1.4 ± 0.25
Control beads		2.36 ± 0.71	<0.246	1.03 ± 0.3	<0.246
Normal mice		<4	<4	6.74 ± 4.4	<4
Af Ag sol		11 ± 8	<4	30.7 ± 7	<4
Af Ag beads	IL-10 (U/ml)	28 ± 2	17 ± 2.45	44 ± 9	11 ± 4
Control beads		23 ± 6	9 ± 4.48	29 ± 6	21 ± 5
Normal mice		2.04 ± 0.17	1.60 ± 0.19	24 ± 2.74	3.43 ± 0.75
Af Ag sol		3.7 ± 1.03	1.37 ± 0.41	31 ± 3.24	3.5 ± 0.72
Af Ag beads	IFN-γ (ng/ml)	1.95 ± 0.47	0.98 ± 0.09	27.7 ± 2.44	2.7 ± 0.42
Control beads		2.8 ± 1	1.7 ± 0.2	27 ± 1.74	2.5 ± 0.47

Con A, Concanavalin A; Ag, antigen; sol, soluble.

a biotinylated second antibody and incubation for 1 hour. The wells were washed, and streptavidin peroxidase was added and incubated as before. After washing the wells, the substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), was added, and the

color developed was read at 450 nm with an automated ELISA reader (Molecular Devices, Menlo Park, Calif.). Standard curves of IL-4, IL-5, IL-10, and IFN-γ were run on each plate and the OD values of the samples were compared and quantitated.

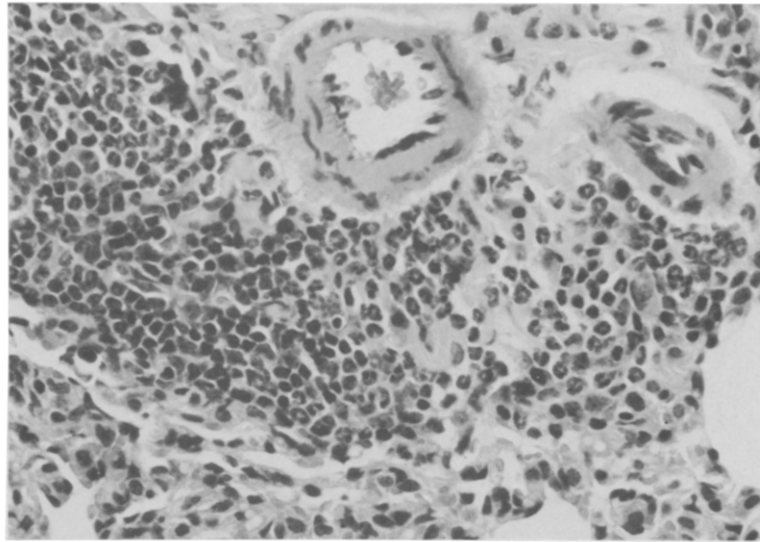


FIG. 5. Section of the lung from a mouse exposed to polystyrene beads conjugated with Af antigen showing peribronchiolar and perivascular infiltrates containing numerous eosinophils. (Hematoxylin and eosin stain; $\times 400$.)

Histologic examination of lungs

Animals were killed on termination of the experiment, and lungs were fixed in 10% buffered formalin. Sections were stained with hematoxylin-eosin and examined for histologic changes.

Statistical analysis

The antibodies in the sera and eosinophils in the peripheral blood in different groups of animals were compared by means of paired *t* test analysis.

RESULTS

Antibody response to Af antigen

All mice exposed to soluble Af antigen intranasally for 8 weeks demonstrated elevated levels of total serum IgE (Fig. 1). The serum IgE levels showed a 32-fold increase, from 160 to 5113 ng/ml. On the other hand, total serum IgG₁ showed only a 16-fold increase (Fig. 2). Af antigen-specific IgG₁ antibody was also detected in the sera of all animals exposed to Af antigen intranasally (Fig. 3). Among animals with moderately elevated IgE and IgG₁ levels on subsequent challenge with Af antigen demonstrated a mean IgE level of 5113 ng/ml in those exposed to soluble antigen and 7226 ng/ml in those exposed to Af-coupled beads, a 41% increase over mean levels in the mice exposed to soluble antigen (Fig. 1). Although not statistically significant, this increase was consistent in all mice challenged with Af-coupled beads. Mice injected intranasally with soluble antigen, on further exposure to con-

trol beads or soluble antigen, showed no change in serum IgE levels. The Af-specific IgG₁ showed a slight increase in animals exposed to soluble antigens intranasally, whereas those animals exposed to antigen-coupled and uncoupled beads showed no change (Fig. 3).

Eosinophil response to soluble and particulate antigens in animals exposed intranasally

An increase in the number of blood eosinophils occurred in all animals exposed to Af antigen. This increase in eosinophil numbers was detected after intranasal exposure of the antigen and increased progressively for 5 to 7 days and thereafter returned to baseline in 2 weeks' time.¹ In animals challenged with antigen-coupled beads, the increase in eosinophil numbers was significantly greater on day 5 ($p < 0.001$) than in animals exposed to soluble antigen (Fig. 4). Animals exposed to control beads without Af antigen invariably showed eosinophil numbers in the blood similar to those of animals exposed to soluble antigen. The eosinophilia persisted for a longer time in animals challenged with antigen-coupled beads than in the other groups of animals.

Cytokine production by lung and spleen cells

The lung lymphocyte cultures from mice challenged with Af antigen-coupled beads produced IL-4 and IL-5 on stimulation with Af antigen in

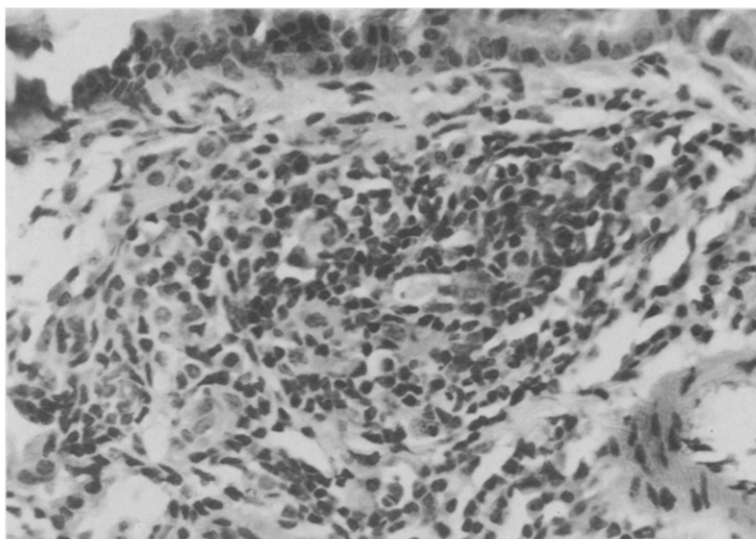


FIG. 6. Section of the lung from animals exposed to soluble antigen showing very few eosinophils. (Hematoxylin and eosin stain; $\times 400$.)

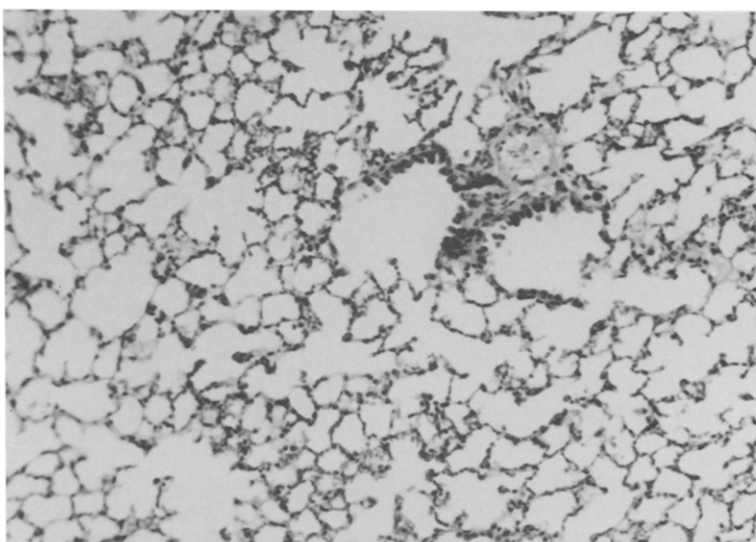


FIG. 7. Section of a lung from a normal mouse. (Hematoxylin and eosin stain; $\times 160$.)

vitro (Table I). None of the other groups of animals expressed detectable levels of IL-4 and IL-5 in cultures of lung cells stimulated with Af. Detectable levels of IL-10 were produced by lung cells of animals exposed to both antigen-coupled and control beads, but not from lung cell cultures of soluble antigen-challenged mice. Measurable amounts of IFN- γ from lung lymphocyte cultures stimulated with concanavalin and Af were detected in all groups of mice studied, including normal mice (Table I). Spleen cells from mice treated with Af-coupled beads also showed IL-5

and IL-10 production in vitro when stimulated with Af. Like lung cells, all spleen cell cultures also produced IFN- γ when stimulated with mitogen or antigen. The in vitro expression of cytokines by the lung lymphocytes in response to antigen stimulation demonstrates a predominantly T_{H2} response.

Histologic examination of the lungs

Animals exposed to Af antigen showed extensive chronic inflammatory infiltrates mainly representing lymphocytes, plasma cells, and varying

numbers of eosinophils. These inflammatory cells were frequently located around perivascular or peribronchiolar areas. The eosinophils were more numerous in animals exposed to Af-coated beads (Fig. 5) than in mice challenged with soluble Af at the last exposure (Fig. 6). In addition, there was interstitial nonnecrotizing granulomatous inflammation, consisting of lymphocytes, histiocytes, and occasional multinucleated giant cells. The results of histologic examination of the normal control animals were normal (Fig. 7).

DISCUSSION

The data presented above clearly show that exposure to Af antigen resulted in the production of elevated levels of IgE in the sera of mice and enhanced eosinophil numbers in the lungs and blood of BALB/c mice. Our previous studies have demonstrated that the antibody levels were higher when different methods of priming with Af were used.¹ However, methods such as intraperitoneal injection of the antigen are not comparable to natural exposure in patients with allergic bronchopulmonary aspergillosis, which is usually through the respiratory tract by inhalation of particulate antigen.¹²⁻¹⁴ Only a moderate increase in IgE and IgG₁ levels was noted in the present study in which intranasal exposure of Af antigen was compared with intraperitoneal injections of Af.¹ In contrast, mice injected intraperitoneally with alum-precipitated Af antigen produced increased levels of IgE and IgG₁ (80-fold and 53-fold, respectively) compared with the intranasal exposure used in the present study, in which a lesser increase in IgE and IgG₁ levels (32-fold and 15-fold, respectively) was detected.¹ Exposure to soluble antigen resulted in a slight increase in the levels of IgG₁ and a slight reduction in the levels of IgE compared with exposure to particulate antigens. A similar trend was also noted with Af-specific IgG₁, but none of these differences were found to be statistically significant.

The antigen-coated polystyrene beads, which were instilled intranasally into mice, are almost identical in size to Af spores.¹³ The inert particles were selected over the viable Af spores for the development of an allergic aspergillosis model because viable spores given intranasally may result in invasive aspergillosis instead of allergic bronchopulmonary aspergillosis. Killed spores may have altered antigenicity; hence, we used the antigen extracted from the organism for coupling the beads and for intranasal exposure. This method could be used in the future to study the

role of individual peptides in producing allergic responses and to preselect appropriate peptides for immunodiagnosis or even for immunotherapy. The group of mice exposed to particulate antigens intranasally showed a moderate increase in IgE antibody levels and more prolonged eosinophilia than other groups of animals (Figs. 1 and 4). The T_{H2}-specific cytokines, IL-4, IL-5, and IL-10 were also demonstrable in cell cultures from the lungs stimulated with concanavalin or Af antigen. Although these cytokine values were low, they were produced consistently on exposure to particulate antigen. On the other hand, the cytokine levels were below detectable limits in the Af antigen-stimulated lung cell cultures from animals treated with soluble Af antigen. Thus the overall immunostimulatory effects of particulate antigen are more pronounced than soluble antigen administered intranasally and represent a T_{H2} pattern.

It has been reported that rats inoculated intrabronchiolarly with dextran beads showed increased eosinophils in lung lavage fluid, blood, and lung parenchyma.¹⁵ We have not seen any increase in the eosinophil numbers in normal animals exposed to control beads. Although the size of the beads used in the study by Kubin et al.¹⁵ was not specified, it is assumed that dextran beads are several times larger than the beads we used. The large size of the dextran beads, which can be trapped in the lung capillary bed, may be responsible for the eosinophilia reported in rats.¹⁵

The cytokine expression in response to antigen and mitogen by spleen and lung lymphocytes *in vitro* showed differences among different groups of animals. Both spleen and lung cell cultures produced low levels of IFN- γ when stimulated with concanavalin A, but little if any of this production could be ascribed to the effects of antigen exposure. These levels were consistently lower than those detected in T_{H1} response.⁸ Detectable levels of IL-4 and IL-5 were produced *in vitro* by lung cells after stimulation with Af by all animals exposed to antigen-conjugated beads and not by any other groups. However, the spleen cells failed to produce IL-4 *in vitro* when stimulated with Af antigen. This suggests a somewhat localized T_{H2} response in the organ directly challenged with particulate Af antigen.

The eosinophilia demonstrated in the peripheral blood is also corroborated by the histologic findings in the lungs. However, substantial numbers of eosinophils remained in the lungs even after the peripheral eosinophil numbers reached the baseline value (Figs. 4 and 5). By the time the

animals were killed, the eosinophils in the peripheral blood had returned to almost normal levels; but histologic examination of the lungs showed persistence of a large number of eosinophils in the granulomas (Fig. 4). It can be concluded from the results that the physical form of antigen may contribute to determining the immunoregulation leading to allergic or nonallergic states.

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