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Aspergillus Antigen-Induced Eosinophil Differentiation in a Murine Model

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Eosinophilia is a prominent feature of the cellular response in allergic and parasitic diseases. Allergic bronchopulmonary aspergillosis due to colonization of the lungs of some asthmatics with *Aspergillus fumigatus* is characterized by high levels of serum immunoglobulin E and peripheral blood (PB) and lung eosinophilia. This study investigates the role of eosinophils in the pathogenesis of allergic bronchopulmonary aspergillosis by using a mouse model. BALB/c mice were immunized intranasally and intraperitoneally with *A. fumigatus* antigens (Ag), and the eosinophils in PB and bone marrow (BM) were enumerated. Eosinophilopoiesis in BM cultures was studied in the presence of murine recombinant interleukin-5 (mrIL-5) and supernatants from pokeweed mitogen-stimulated spleen cells as the source of eosinophil differentiation factors. Eosinophils were quantitated by direct counting and by estimating eosinophil peroxidase activity. The results indicate that the percentage of eosinophils in the PB (5.77 ± 1.17) and the BM (11.19 ± 4.31) of mice exposed to *A. fumigatus* Ag was higher than in controls (PB, 2.42 ± 0.76; BM, 5.12 ± 2.79; $P < 0.01$ for both). Similarly, a significant increase in eosinophils was observed in the BM population from mice exposed to *A. fumigatus* Ag compared with that in controls when cultured with murine recombinant interleukin-5 (23.13 ± 7.14 versus 13.77 ± 5.79, $P < 0.01$), indicating that the mice exposed to *A. fumigatus* Ag had significantly greater numbers of eosinophil precursors in their BM. This study demonstrates that *A. fumigatus* Ag may be involved in the in vivo commitment of stem cells in the eosinophil differentiation pathway.

Allergic bronchopulmonary aspergillosis (ABPA) is characterized by episodic wheezing, pulmonary infiltrates on a chest radiograph, central bronchiectasis, elevated specific serum immunoglobulin E (IgE) and IgG antibodies to *Aspergillus* antigens (Ag), and immediate wheal and flare skin reactions to *Aspergillus fumigatus* and increased levels of total serum IgE (4). Peripheral blood (PB) and lung eosinophilia are prominent features of the cellular response in these patients (4). Although the presence of eosinophilia in a variety of diseases has been known for years, the role that eosinophils play in the pathogenesis of diseases has not been completely clarified. This is particularly true for ABPA and related fungal diseases, although considerable information on eosinophils is available from human parasitic diseases and animal models of parasitic infections. Eosinophilia has been infrequently reported in most animal models of ABPA. The present evidence suggests that eosinophils, by virtue of their cytotoxic capabilities, may induce many of the adverse effects associated with allergic inflammatory reactions (19).

The development of animal models of ABPA utilizing monkeys, rabbits, rats, and mice has been attempted via exposure to *A. fumigatus* Ag and other related organisms (6, 10, 12, 14, 15). These studies demonstrated some features of human ABPA, such as elevated levels of total serum IgG, IgE, and *Aspergillus*-specific IgG1, PB lymphocytosis with an increase in lymphocytes in lung lavage fluid, and progressive inflammatory reactions in lung tissue (6). However, the mechanism of eosinophilia was not studied with these models.

In our recent studies on the animal model of ABPA with C3H/HeN and C57BL/6 mice, we have demonstrated *Aspergillus*-specific antibodies (6). However, we did not study eosinophilia in these strains. Studies of cutaneous leishmaniasis (11) revealed that C57BL/6 mice with a predominantly T helper cell 1-type response were resistant, while BALB/c mice with a T helper cell 2-type response developed fatal illness. Hence, to induce eosinophilia, a T helper cell 2-type response, we have used BALB/c mice to develop a model of allergic aspergillosis.

In our present study on a model of ABPA developed by exposing BALB/c mice to *A. fumigatus* Ag, a marked eosinophilia in PB and bone marrow (BM) and elevated serum levels of *Aspergillus*-specific IgE and IgG1 antibodies were demonstrable.

MATERIALS AND METHODS

Animals. Specific-pathogen-free, 6-week-old female BALB/c mice were obtained from Sasco Inc. (Omaha, Nebr.) and used in this study.

Antigens. A 1:1 mixture of Ag from culture filtrate (2 mg of protein per ml) and mycelial extracts (0.36 mg of protein per ml) of *A. fumigatus* was used to sensitize the mice. Culture filtrate Ag were prepared by growing the organism in a synthetic broth (AOAC; Difco Laboratories, Detroit, Mich.) for 3 to 4 weeks at 37°C as previously described (8). The broth was separated from the mycelium after the incubation period and dialyzed extensively against deionized water, and the retentate was lyophilized. Mycelial extract Ag was prepared from 3-day-old growths of aerated cultures of *A. fumigatus* grown in synthetic medium (9). After incubation, the mycelium was separated by centrifugation and washed several times in chilled phosphate-buffered saline (PBS). The mycelial mat was homogenized by using a French press at 10,000 lb/in^2. The extract was then centrifuged at 10,000 rpm

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(17,700 × *g*) for 30 min, and the supernatant was collected and dialyzed. The suitability of the Ag was determined by comparing its reactivity with patient sera by agar gel double diffusion and by enzyme-linked immunosorbent assay (ELISA) (7, 9). The Ag preparations were characterized by their reactivity against patient sera by crossed immunoelectrophoresis and enzyme and protein profiles as previously described (7–9).

Immunization of mice. Mice were lightly anesthetized with CO_2, and 50 μl of the Ag mixture was slowly applied to the nostrils by using a micropipette with a sterile disposable tip (6). After being inoculated, the animals were held upright until all Ag applied to the nostril was completely inhaled. All animals also received 100 μl of the same Ag mixture intraperitoneally. Intranasal injections of *Aspergillus* Ag produce eosinophilia, and the intraperitoneal injections are required to produce the antibody response. Intranasal instillations and intraperitoneal injections were given twice a week to each mouse for 4 weeks. Control animals were immunized identically but with PBS. Both control mice and mice exposed to Ag were sacrificed 3 days after the last administration of Ag or PBS.

Antibody response. Serum IgE and IgG1 antibodies to *A. fumigatus* Ag were measured by a biotin-avidin-linked immunosorbent assay (5) before and after exposure to *A. fumigatus* Ag. Previous studies determined that 4 to 6 injections of *A. fumigatus* Ag yielded a fourfold or greater increase in IgE and IgG1 antibodies and demonstrable inflammatory reactions in the lungs (6).

Eosinophils in PB and BM. Blood was obtained from the tail vein and spread on clean glass slides for eosinophil counting. Eosinophils from the smear stained with Wright's Stain (Baxter Co., Gibbstown, N.J.) were enumerated under a microscope and classified either as immature, which included all stages from promyelocytes to cells with ring-shaped nuclei, or as mature, which corresponded to cells with a segmented nucleus. The percentages of mature and immature eosinophils were determined by counting 200 leukocytes. A cytocentrifuge (Cytospin 2; Shandon, Inc., Pittsburgh, Pa.) was used to make a smear of the nonadherent BM cells (see the description of preparation of BM cells below) on glass slides, fixed with methanol, and stained with Wright's Stain. The percentages of mature and immature eosinophils in BM were determined as described above.

Preparation of BM cells and spleen cells. Both femurs from each mouse were flushed out with Hanks balanced salt solution (HBSS) (Sigma, St. Louis, Mo.), and a single-cell suspension was obtained. The cells were washed three times in HBSS and resuspended in RPMI 1640 (Sigma) containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 2 mM glutamine, 5% fetal bovine serum (Biocell Laboratory), penicillin (100 U/ml), and streptomycin (100 μg/ml, Sigma). The cell concentration was adjusted to 10^6 cells per ml. Aseptically removed spleen from both mice exposed to *A. fumigatus* Ag and control mice were teased separately through a no. 100 stainless-steel mesh into HBSS. The cell suspension was allowed to stand for 5 min to allow the large clumps to settle, and the supernatant was then collected and centrifuged at 400 × *g* for 10 min. Erythrocytes were lysed twice with lysing buffer (Sigma). After three more washes in HBSS, the cells were resuspended in complete RPMI 1640 (RPMI 1640, penicillin-streptomycin, 2 mM glutamine, 10% fetal bovine serum) and adjusted to a concentration of 10^6 cells per ml. Cell viability (Trypan blue exclusion method) was always 95% or more in both spleen and BM cells.

Preparation of mitogen-stimulated spleen cell culture supernatants. Mitogen-stimulated spleen cell culture supernatants (MSSS) were used as a source of eosinophil differentiation factors (17). Spleen cells (10^6 cells/ml) were incubated with and without pokeweed mitogen (PWM) (10% vol/vol; GIBCO, Grand Island, N.Y.) for 72 h at 37°C in a humid atmosphere with 5% CO_2. Cell-free supernatants were obtained and stored at −20°C until assayed for eosinophil differentiation factor activity (17). Culture supernatants with PWM were termed MSSS, and those without PWM were termed Control Sup.

Eosinophil precursors in the BM. The numbers of eosinophil precursors in the BM from control mice and the BM from mice exposed to *A. fumigatus* Ag were estimated by stimulating the BM cells with MSSS (20 μl per well) and murine recombinant interleukin-5 (mrIL-5) (25 IU/ml; Genzyme, Boston, Mass.) in microwell cultures as described below. A previous study demonstrated that there was a linear relationship between eosinophil numbers and eosinophil peroxidase (EPO) activity with no interference with similar enzymes present in monocytes or neutrophils (18). However, no reports are available to differentiate the EPO of immature eosinophils from that of mature eosinophils. We have used EPO levels strictly as a measure of eosinophil differentiation factors as described previously by Strath et al. (18). The level of EPO also gave an indirect estimate of the number of eosinophils after 48 h of culture with mrIL-5 or MSSS.

Assay of eosinophil differentiation activity in MSSS. Eosinophil differentiation activity in MSSS was assayed as described earlier (18). Briefly, 100 μl of BM cells (10^6/ml) from mice exposed to *A. fumigatus* Ag and from control mice were incubated in round-bottom microtiter plates (Nunc, Roskilde, Denmark) in the absence or presence of Control Sup, MSSS, PWM (all 10% vol/vol), or mrIL-5 (25 IU/ml). Cultures were set up in duplicate wells. After 48 h of incubation, the plates were centrifuged at 1,000 rpm (400 × *g*) for 10 min and the medium was aspirated; 100 μl of 1 mM *o*-phenylenediamine in 0.05 Tris-HCl (pH 8.0) containing 0.1% Triton X-100 and 1 mM hydrogen peroxide was added to each well. After 30 min at room temperature, the color reaction was stopped by the addition of 50 μl of 4 M sulfuric acid, and the A_{490} was determined by using an MR 700 Microplate Reader (Dynatech Lab. Inc., Chantilly, Va.). The data are represented as net optical density (OD), where the net OD equals the OD in BM cultures with test supernatants or mrIL-5 minus the OD in BM cultures with medium alone.

In order to detect whether the level of EPO in the BM culture was proportional to the level of eosinophil differentiation factor in MSSS, different sources of MSSS preparations were tested with BM cells from both control mice and mice exposed to *A. fumigatus* Ag. Since eosinophil differentiation activity is mainly due to eosinophil differentiation factor, the EPO expressed was a direct measure of eosinophil differentiation activity (18).

Statistical methods. The methods of statistical analysis include Student's *t* test for population means and paired comparison and simple linear regression and correlation using the Spearman rank correlation test (2).

RESULTS

Antibody responses. None of the animals demonstrated significant *Aspergillus*-specific antibodies in the preimmunization sera. After immunization with *A. fumigatus* Ag,

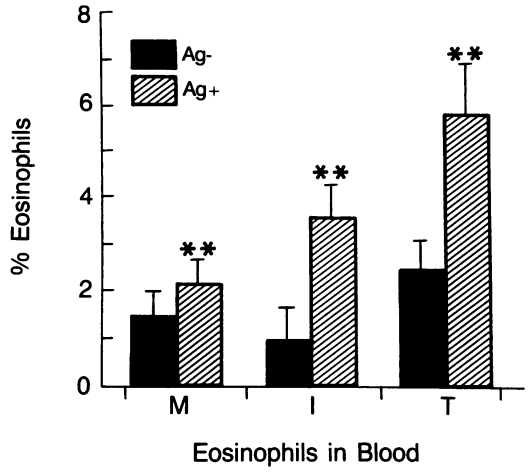

FIG. 1. Effect of *A. fumigatus* Ag exposure on the percentage of eosinophils in the PB of BALB/c mice. See Materials and Methods for details of exposure. Each bar represents the mean and SD for the control group (Ag−; $n = 13$) or the group exposed to Ag (Ag+; $n = 13$). A statistically significant difference ($P < 0.01$) in the percentages of mature (M), immature (I), and total (T) eosinophils was observed for the Ag+ and Ag− groups, as indicated by asterisks.

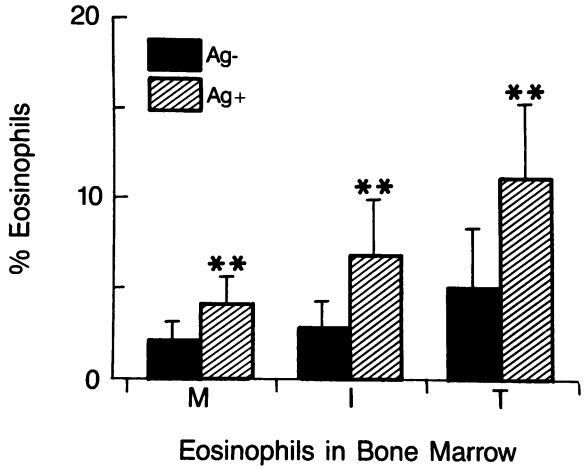

FIG. 2. Effect of *A. fumigatus* Ag exposure on the percentage of eosinophils in the BM of BALB/c mice. See Materials and Methods for details of exposure. Each bar represents the mean and SD for the control group (Ag−; $n = 13$) or the group exposed to Ag (Ag+; $n = 13$). A statistically significant difference ($P < 0.01$) in the percentages of mature (M), immature (I), and total (T) eosinophils was observed for the Ag+ and Ag− groups, as indicated by asterisks.

specific IgE and IgG1 levels increased (OD [mean ± standard deviation (SD)], IgE, 0.009 ± 0.003 to 0.051 ± 0.025; IgG1, 0.07 ± 0.032 to 0.42 ± 0.32) over preimmunization levels as determined by ELISA. No difference in antibody levels in control mice before and after treatment was observed.

Eosinophils in PB and BM. The percentage of eosinophils in PB from control mice and mice exposed to *A. fumigatus* Ag is shown in Fig. 1. The percentages of both mature and immature eosinophils were significantly higher in mice exposed to *A. fumigatus* Ag (mean ± SD, 2.19% ± 0.66% and 3.58% ± 0.95%; $P < 0.01$) than in the control mice (1.46% ± 0.63% and 0.96% ± 0.72%). The percentage of eosinophils in the BM cell preparation before culture is shown in Fig. 2. The percentages of both mature and immature eosinophils were higher in mice exposed to *A. fumigatus* Ag (4.19% ± 1.58% and 7.00% ± 3.10%; $P < 0.01$) than in control mice (2.23% ± 1.42% and 2.88% ± 1.65%). The results indicated that the percentages of immature eosinophils were higher than those of the mature cells in both the PB (3.58 versus 2.19%, $P < 0.001$) and the BM (7.00 versus 4.19%, $P < 0.01$) of mice exposed to *A. fumigatus* Ag. In the control mice, the numbers of PB immature eosinophils were lower than those of the mature eosinophils (0.96 versus 1.46%, $P < 0.05$); however, no significant differences between mature and immature eosinophils could be detected in the BM (2.23 versus 2.88%, $P > 0.05$). These results indicated that exposure to *A. fumigatus* Ag resulted in eosinophil accumulation in both PB and BM and that immature eosinophils were favored over mature eosinophils. The percentages of total eosinophils in both the PB and the BM of mice exposed to *A. fumigatus* Ag were significantly higher than those in the PB and the BM of control mice (Fig. 1 and 2).

The correlation and regression analysis between the percentages of PB and BM eosinophils showed a significant linear correlation between these two sources of eosinophils with regard to mature ($r = 0.412$, $P < 0.05$), immature ($r = 0.562$, $P < 0.01$), and total ($r = 0.645$, $P < 0.01$) eosinophils. This suggests that the increase of total eosinophils in PB was probably a result of an increase in eosinophils in BM.

Eosinophil precursors in BM. Figure 3 shows the percentage of eosinophil granulocytes in BM cells stained with Wright's Stain after 48 h of culture with mrIL-5. The results demonstrate that the levels of both mature and immature cells in mice exposed to *A. fumigatus* Ag (6.04% ± 2.70% and 17.08% ± 5.75%) were significantly higher than those in control mice (4.08% ± 2.86% and 9.69% ± 3.69%). The

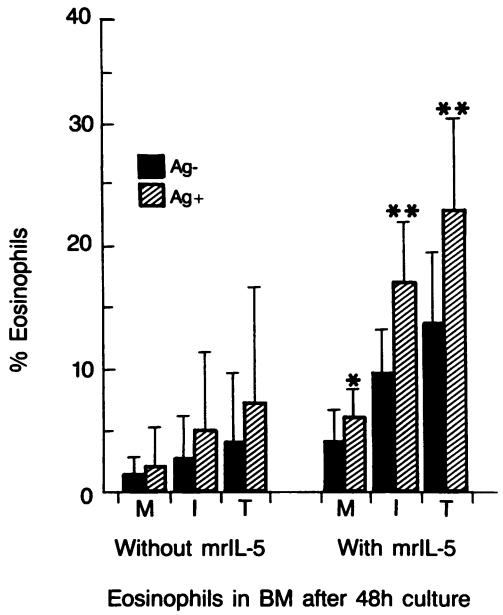

FIG. 3. Eosinophils in BM from mice exposed to *A. fumigatus* after 48 h of culture with mrIL-5. Culture conditions are described in Materials and Methods. Each bar represents the mean and SD for the control group ($n = 13$) or the group exposed to Ag ($n = 13$). M, I, and T are described in the legend for Fig. 1. The values for Ag+ mice which are significantly different from those for Ag− mice are marked as follows: *, $P < 0.05$; **, $P < 0.01$.

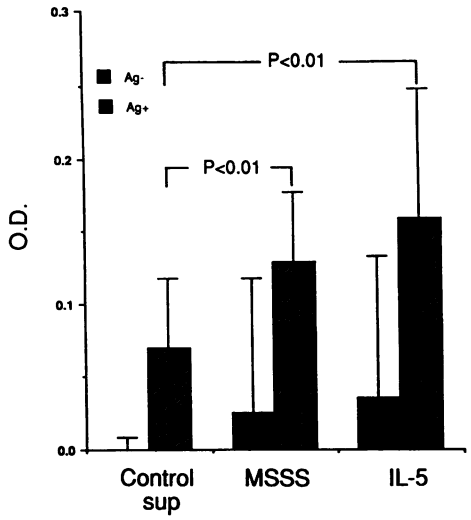

FIG. 4. Eosinophil peroxidase levels in BM from mice exposed to *A. fumigatus* Ag after 48 h of culture with Control Sup, MSSS, and mrIL-5. Culture conditions are described in Materials and Methods. Each bar represents the mean and SD for the control group (n = 13) or the group exposed to Ag (n = 13).

percentage of immature eosinophils was higher than that of mature eosinophils (17.08 versus 6.04%, $P < 0.001$) in the BM cells from mice exposed to *A. fumigatus* Ag after incubation with mrIL-5 and was comparable to the results in Fig. 1 and Fig. 2 described above. This indicates that mrIL-5 could induce both proliferation and maturation of eosinophils in the BM cells of mice exposed to *A. fumigatus* Ag and that the pattern of distribution of immature and mature eosinophils was similar to that observed in freshly isolated BM cells; i.e., there was a predominance of immature over mature cells. However, upon culturing BM from control mice and mice exposed to *A. fumigatus* Ag with medium alone, there was no significant difference between the percentages of mature and immature eosinophils in the two groups (Fig. 3).

EPO in BM cells. The levels of EPO in BM after 48 h of culture with MSSS (from mice exposed to *Aspergillus* Ag) and with mrIL-5 are shown in Fig. 4. As described above, the level of EPO paralleled the relative number of total eosinophil precursors (both mature and immature) in 48-h cultures. EPO levels were significantly higher in the BM from mice exposed to *A. fumigatus* Ag, whether cultured with MSSS (0.129 ± 0.048, $P < 0.01$) or with mrIL-5 (0.158 ± 0.089, $P < 0.01$), than in the BM of control mice cultured with either MSSS (0.025 ± 0.093) or mrIL-5 (0.035 ± 0.097). Furthermore, MSSS and mrIL-5 induced significant levels of EPO in BM from Ag-exposed mice and not from control mice (Fig. 4). The ratios of EPO levels in mice exposed to *A. fumigatus* Ag and control mice were 5.16 in cultures with MSSS and 4.5 in cultures with mrIL-5. From Fig. 4, it is evident that the EPO values of BM cells cultured with MSSS are almost identical to those of BM cells cultured with mrIL-5. This observation, as well as the above ratios, suggests that part of the eosinophil differentiation activity in MSSS may be due to IL-5. However, PWM alone did not change the EPO activity relative to Control Sup (data not shown).

Eosinophil differentiation activity from different sources of MSSS. Eosinophil differentiation activity levels in MSSS from mice exposed to *A. fumigatus* Ag and MSSS from control mice were determined by measuring EPO levels. A total of 10^5 BM cells per well were cultured in duplicate in 96-well round-bottom plates with MSSS (10% vol/vol) from control mice and mice exposed to *A. fumigatus* Ag. The data were statistically analyzed by using the paired comparison t test. No significant differences ($P > 0.05$) in eosinophil differentiation activity levels between MSSS from mice exposed to *A. fumigatus* Ag assayed on BM cells from control mice (0.025 ± 0.093) or from mice exposed to *A. fumigatus* Ag (0.129 ± 0.048) and MSSS from control mice assayed on BM cells from control mice (0.033 ± 0.091) or mice exposed to *A. fumigatus* Ag (0.112 ± 0.049) were observed. This suggests that MSSS from control mice and from mice exposed to *A. fumigatus* Ag have similar eosinophil differentiation factor activities. The data also indicate that when MSSS from the same source was tested, significantly higher levels of EPO production by BM from mice exposed to *A. fumigatus* Ag than by BM from control mice were observed. This suggests that Ag exposure may be an essential factor for eosinophil differentiation in BM.

DISCUSSION

The present study demonstrates that exposure to *A. fumigatus* Ag by intranasal instillation and intraperitoneal injection can result in an increase in *Aspergillus*-specific IgE and IgG1 antibodies and eosinophilia in the PB and the BM of BALB/c mice (Fig. 1 and 2). The positive correlation between the numbers of total eosinophils in the PB and the BM supports the assumption that an increase in circulating eosinophils could be the result of increased eosinophil differentiation in hemopoietic stem cells (1). Although it is not known whether there is an increase in eosinophils in the BM of ABPA patients, there is a marked increase in the number of eosinophils in PB and lungs (4). Our data concur with those of other studies on parasitic infections and allergic diseases, suggesting that a sustained increase of eosinophils in the circulation would be the result of the increase of eosinophil differentiation in the BM (3, 17). The increases in both mature and immature eosinophils in the PB and the BM indicate that proliferation of eosinophils is a result of exposure to *A. fumigatus* Ag (Fig. 1 and 2). This is consistent with a previously made observation for the acute phase of schistosomiasis (3), although the response in the present model was not as pronounced.

On stained smears, a higher percentage of immature eosinophils than of mature eosinophils in the PB and the BM of mice exposed to Ag was demonstrated (Fig. 1 and 2). We cultured BM cells in the presence of IL-5, a well-characterized eosinophil differentiation factor (13). If the cells were already committed in vivo to an eosinophilic differentiation pathway because of the Ag exposure of the mice, a further increase in eosinophils with IL-5 in vitro is not expected. To our surprise, we observed that in vitro, IL-5 induced significant eosinophil differentiation and proliferation only of BM cells from mice exposed to *A. fumigatus* Ag and not of BM cells from control mice (Fig. 3 and 4). The need for the continuous presence of IL-5 in culture to induce eosinophil differentiation suggests that Ag exposure may be a necessary but not sufficient condition for the eosinophilia in our model.

PWM-stimulated spleen supernatant is recognized as a source of eosinophil differentiation factors (17). We have studied MSSS from both control mice and mice exposed to *A. fumigatus* Ag for their eosinophil differentiation activities on BM cells. We used the eosinophil peroxidase-inducing

ability as an indirect measure of eosinophil differentiation factor levels in culture supernatants (18). Eosinophil peroxidase activity is proportional to the number of eosinophils per well (18). Hence, the increase in the percentage of eosinophils upon Wright's staining which we observed is an actual increase in the number of eosinophils due to the exposure of mice to *Aspergillus* Ag and not due to a decrease in any other cell population. Upon culture with MSSS from mice exposed to Ag, BM cells had EPO levels similar to those for IL-5. Interestingly, BM cells from mice exposed to Ag responded significantly to both MSSS and IL-5, while cells from control mice did not. This and other observations noted above prompt us to hypothesize that exposure to Ag may trigger early events promoting differentiation of eosinophils and requiring a constant presence of factors for terminal differentiation. In our hands, MSSS acts equivalently to IL-5 as an eosinophil differentiation factor. The development and differentiation of eosinophils are promoted by three cytokines: granulocyte macrophage colony-stimulating factor, IL-3, and IL-5 (16). Studies are in progress to determine the role of each of these cytokines in the eosinophil differentiation activity of MSSS.

We finally proceeded to determine whether there were any differences in the eosinophil differentiation activity in MSSS on the basis of its source. MSSS from both control mice and mice exposed to *A. fumigatus* Ag were tested for their abilities to induce EPO. No differences in eosinophil differentiation activity in the different MSSS were observed. However, BM cells from mice exposed to Ag had consistently higher EPO levels than those from control mice. This observation further supports our hypothesis that exposure to *Aspergillus* Ag is crucial in the eosinophil differentiation pathway in this model of ABPA.

In conclusion, we describe here a mouse model of allergic aspergillosis exhibiting significant levels of *Aspergillus*-specific IgE and IgG1 antibodies in serum and eosinophilia in PB and BM. In this model, exposure to *A. fumigatus* Ag may be critical to the early stages of eosinophil differentiation in vivo. Terminal differentiation into immature and mature eosinophils requires the continuous presence of eosinophil differentiation factors. MSSS had activity similar to that of IL-5, although additional factors which may promote eosinophil differentiation may be present. Studies are in progress to identify the factors in MSSS which participate in eosinophilopoiesis in this model.

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