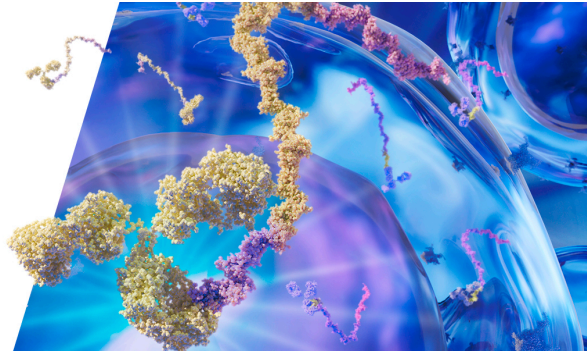


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Resolution of Bronchial Hyperresponsiveness and Pulmonary Inflammation Is Associated with IL-3 and Tissue Leukocyte Apoptosis

Clare M. Lloyd,^{1*} Jose-Angel Gonzalo,* Trang Nguyen,* Tracy Delaney,* Jane Tian,* Hans Oettgen,[†] Anthony J. Coyle,* and Jose-Carlos Gutierrez-Ramos^{2*}

We have used two models of murine pulmonary inflammation to investigate the signals responsible for the resolution of bronchial hyperresponsiveness (BHR). Both protocols involved two sensitizations with OVA followed by serial aerosolized challenge with OVA. We determined that administration of the second sensitization by aerosol (model A) was associated with a transient response, whereas administration by the i.p. route (model B) induced a sustained response, in the form of BHR and eosinophilia. This difference in kinetics was due solely to the route of the second Ag administration and was not associated with Ag dose or adjuvant. Differences in kinetics of lung eosinophilia/BHR were shown to be independent of IgE levels and IL-4 or IL-5. However, IL-3 levels in model A closely correlated with the rate of leukocyte clearance by apoptosis and were observed concomitant with a decline in BHR. Blockage of IL-3 in model B increased leukocyte apoptosis but reduced tissue eosinophilia and BHR. The use of mouse models in which a single different administration of allergen is associated with a failure/success to resolve inflammation and BHR by 72 h postchallenge indicates a link between IL-3 production, leukocyte apoptosis, and BHR responses. *The Journal of Immunology*, 2001, 166: 2033–2040.

Asthma is a disease characterized by reversible airflow and is associated with episodic increases in bronchial hyperreactivity (BHR)³ to nonspecific stimuli. Various studies have shown that this BHR occurs as a consequence of leukocytic infiltration of the airways (1–3). The cellular composition of the inflammatory infiltrate in the lung is characterized by increased numbers of eosinophils, T lymphocytes, neutrophils, monocytes, and mast cells (4, 5). The recruitment of these cells to the site of inflammation and their subsequent activation is thought to be strictly controlled by a complex series of cellular interactions mediated by cytokines and chemokines, secreted by resident lung cells as well as by the infiltrating inflammatory cells (6–9). Following migration to the site of inflammation, leukocytes release proinflammatory mediators (histamines, PGs, leukotrienes, platelet-activating factor, reactive oxygen intermediates, cytokines, and chemokines) that initiate tissue injury, augment the immune response, and ultimately result in BHR (10–12).

Increased BHR in asthmatic individuals has been reported as early as 2 h after provocation, persisting for up to 7 days (13–15). Clinical and experimental studies have outlined mediators that are

thought to be responsible for the initiation of BHR, but there is little information regarding the factors responsible for the resolution of this lung dysfunction. Animal models of the allergic response to inhaled Ags show many similarities to those in human, including the induction of BHR and inflammation (7, 16, 17). In general there is correlation between the degree of BHR and eosinophil infiltration in the majority of models (18, 19). The specific importance of selected mediators, such as particular IgE, IL-4 and IL-5, in the development of the allergic response has been highlighted by various investigators (17, 19–21). However, despite these findings the signals responsible for determining whether BHR resolves or is maintained remain unclear. In the present study, we have addressed some of the issues surrounding the development of airway hyperresponsiveness by investigating the chronological response to inhaled Ag. We have identified two models of murine allergic airway disease (AAD) that, although using similar immunization protocols for disease induction, result in profound differences in BHR, as well as inflammation. This study highlights the importance of the route of Ag exposure in determining the kinetics of BHR and assesses the relative importance of mediators produced by leukocytes in the resolution of this pathophysiology.

Materials and Methods

Induction of AAD

Eight- to 10-wk-old BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and kept in Millennium Pharmaceuticals specific-pathogen free mouse facility (Cambridge, MA). Mice treated with model A were sensitized by OVA, 0.1 mg/mouse i.p. on day 0 (Sigma, St. Louis, MO) and challenged on day 8 (2% OVA aerosolized for 5 min) and daily between day 15 and 21 (1% OVA, aerosolized for 20 min). PBS (i.p. and/or aerosolized) was administered to mice as a negative control. Mice treated with model B were sensitized by OVA, 0.01 mg/mouse in 0.2 ml alum (Au-Gel-S; Boehringer Ingelheim, Ridgefield, CT) i.p. on day 0. Mice were then challenged on day 10 with 0.01 mg/mouse in 0.2 ml alum and daily between day 19 and 24 (5% OVA aerosolized for 20 min). The scheme for both treatment protocols is depicted in Fig. 1. Variations of this

*Millennium Pharmaceuticals Inc., Cambridge MA 02139; and [†]Division of Immunology, Children's Hospital, Boston, MA 02115

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¹ Address correspondence and reprint requests to Dr. Clare M. Lloyd at her current address: Leukocyte Biology Section, Biomedical Sciences Division, Sir Alexander Fleming Building, Imperial College School of Medicine, London SW7 2AZ, U.K. E-mail address: c.lloyd@ic.ac.uk

² Address correspondence and reprint request to Dr. Jose-Carlos Gutierrez-Ramos, Millennium Pharmaceuticals Inc., 75 Sidney Street, Cambridge, MA 02139. E-mail address: gutierrez@mpi.com

³ Abbreviations used in this paper: BHR, bronchial hyperreactivity; AAD, allergic airway disease; BAL, bronchoalveolar lavage; MCh, methacholine; Penh, enhanced pause.

protocol were used, which included injection of 0.01 mg OVA/mouse or 0.1 mg OVA/mouse in the absence of alum on days 0 and 10. Mice were sacrificed by CO₂ asphyxiation at 6, 24, 48, or 72 h after OVA administration on day 21 (model A) or day 24 (model B) and analyzed using the following parameters: bronchoalveolar lavage (BAL), BHR, and histology.

Mice deficient in IgE were obtained from Dr. H. Oettgen (Children's Hospital, Boston, MA) and were subjected to protocol B as described above (22). Control age- and sex-matched mice of the same strain (129/SVEV) were obtained from Taconic (Germantown, NY).

For IL-3 blockage experiments, mice were treated with protocol B as described above, until 24 h after the final OVA challenge on day 24 when one group was given 50 µg/mouse of goat anti-mouse IL-3 Ab (R&D Systems, Minneapolis, MN) intranasally. Another OVA-treated group was given 50 µg of goat Ig (Jackson ImmunoResearch, West Grove, PA) as a control. BHR was measured at 6, 24, 48, and 72 h postchallenge when mice were sacrificed and their lungs analyzed.

Bronchoalveolar lavage

BAL was performed as described (6). Briefly, the airways of the mice were lavaged via a trachea cannula with 1 ml PBS. The resulting BAL fluid was immediately centrifuged (700 × g, 5 min at 4°C) and cells were then washed and resuspended in 1 ml PBS. Total BAL cell counts were performed, and aliquots (5 × 10⁵ cells/slide) were pelleted onto glass slides by cytocentrifugation. Differential counts were performed on Geimsa-stained cytopins, and percentages of eosinophils, lymphocytes, neutrophils, and macrophages was determined by counting their number in eight high-power fields (×40 magnification; total area 0.5 mm²) per area randomly selected and dividing this number by the total number of cells per high-power field (7). To obtain the absolute number of each leukocyte subtype in the lavage, these percentages were multiplied by the total number of cells recovered from the BAL fluid.

Bronchial hyperreactivity

The degree of bronchoconstriction (BHR) was measured at 6, 24, 48, and 72 h in the same mice after the last aerosolized Ag challenge by recording respiratory pressure curves by whole-body plethysmography (Buxco Technologies, Sharon, CT) in response to inhaled methacholine (MCh; Aldrich Chemical, Milwaukee, WI) at a concentration of 3 × 10⁻² M for 1 min, as described previously (18). BHR was expressed as enhanced pause (Penh), a calculated value, which correlates with measurement of airway resistance, impedance, and intrapleural pressure in the same mouse: $Penh = (T_e/T_r - 1) \times (P_{ef}/P_{if})$, where T_e is the expiration time, T_r is the relaxation time, P_{ef} is the peak expiratory flow, and P_{if} is the peak inspiratory flow × 0.67 coefficient (23). The relaxation time is the time it takes for the box pressure to change from a maximum to a user-defined percentage of the maximum. Here, T_r measurement begins at the maximum box pressure and ends at 40%. Values were expressed as the percentage shift from baseline, which was measured by comparing the Penh of mice before (i.e., baseline) and after stimulation with MCh.

Histology

Lung sections from the different experimental groups of mice were prepared as described (7). Briefly, lungs were fixed in 10% neutral-buffered formalin (J.T. Baker, Phillipsburg, NJ) and paraffin embedded, and sections (4 µm) were stained with hematoxylin/eosin according to standard protocols. A semiquantitative scoring system was used to grade the size of lung infiltrates, where +5 signified a large (>3 cells deep) widespread infiltrate around the majority of vessels and bronchioles and +1 signifies a small number of inflammatory foci.

Cytokines

Levels of IL-4, IL-5, GM-CSF, IL-3, and IFN-γ were assessed in bronchiolar lavage samples by ELISA according to the manufacturers instructions (Endogen, Woburn, MA).

IgE

Serum levels of total IgE were measured by ELISA using paired Abs according to the manufacturer's instructions (BD Pharmingen, San Jose, CA). Serum levels of anti-OVA IgE were measured by ELISA, and Ab titers were related to pooled standards generated in the laboratory, then were assigned the arbitrary values U/ml.

Apoptosis staining

An estimation of the degree of apoptosis was made in paraffin-embedded lung sections from mice after protocol A or B at 6, 24, 48, and 72 h

following the final challenge, using an Apotag in situ detection kit (Oncor, Gaithersburg, MD) according to the manufacturer's instructions. Briefly, sections were rehydrated, digested with proteinase K (20 µg/ml, 15 min), and treated to block endogenous peroxidase (3% hydrogen peroxidase in PBS, 5 min). Sections were then incubated with TdT enzyme (37°C, 1 h), washed and incubated with antidigoxigenin conjugate (30 min), and color was developed with diaminobenzidine substrate kit (6 min) and counterstained with hematoxylin. Apoptotic cells were visualized by the brown reaction product on a blue background. Apoptosis was quantified by counting the number of positive and negatively stained leukocytes within peribronchiolar infiltrates in five high-power fields per section (each field = 0.5 mm² at ×400). Apoptosis was then expressed as a percentage in 2.5 mm² for each mouse.

Statistics

All results are expressed as mean + SEM. Student's *t* test or ANOVA were used to determine statistical significance between groups of mice, using between 12 and 20 mice in a group. Values of *p* < 0.05 were considered significant.

Results

Model A induces transient AAD whereas model B results in a sustained AAD

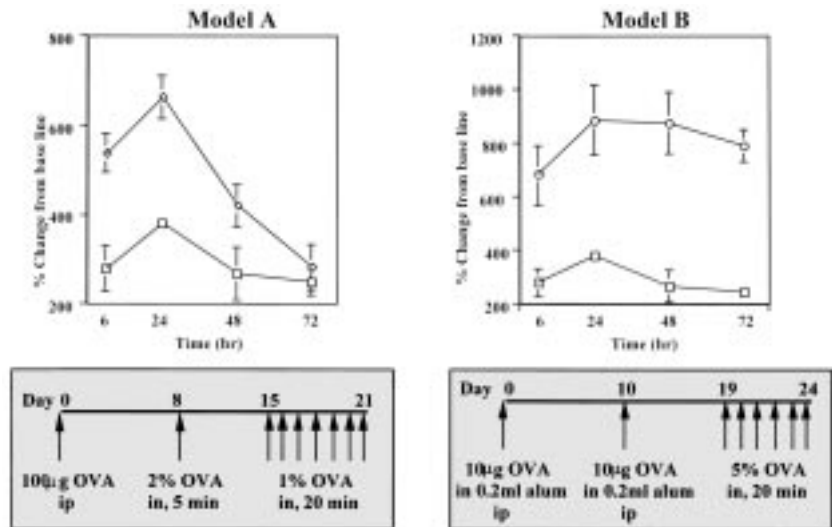
We examined different models of AAD from the literature with respect to the pathophysiological parameters BHR and eosinophil inflammation and identified two models that differed profoundly in the kinetics of BHR. Subsequently, these models were termed model A (6) and model B (24). Both models were induced by two sensitizations with OVA followed by multiple aerosolized challenges and resulted in BHR with associated pulmonary inflammation (Fig. 1 and data not shown). However, when BHR was measured at timed intervals after the final aerosolized challenge, we determined that model A resulted in an increase in BHR that peaked at 24 h but that had returned to baseline by 72 h (Fig. 1). In contrast, model B resulted in a sustained in BHR that was maintained for at least 72 h (Fig. 1). The magnitude of the responses at peak BHR was similar in both models with a 700% shift from baseline achieved in model A (baseline Penh = 0.43 ± 0.01, increasing to 2.7 ± 0.2 at 24 h) and a 900% shift in baseline seen in model B (baseline Penh = 0.39 ± 0.01, moving to 2.96 ± 0.5 at 24 h). Although the difference in magnitude was not statistically different at 24 h (*p* > 0.1), it was highly significant at 72 h (*p* < 0.001).

The presence of alum did not account for the difference in kinetics of BHR development between model A and B. This was determined in mice sensitized with either high (100 µg) dose OVA (termed model B/100) or low (10 µg) dose OVA (termed model B/10) in the absence of alum. Both groups developed sustained hyperreactivity to MCh over 72 h (data not shown), similar to those mice given protocol B proper. The degree of hyperreactivity was most pronounced in those mice receiving the higher dose OVA, with the percentage change from baseline reaching comparable levels to those achieved in model B proper (baseline Penh = 0.41 ± 0.01, moving to 2.81 ± 0.41 at 72 h). Thus, although alum seemed to compensate for a lower dose of OVA (model B/100 compared with model B), it did not account for differences in kinetics.

Kinetics of eosinophilic inflammation reflects kinetics of BHR

To determine whether the difference in kinetics of BHR observed between model A and B correlated with the pattern of inflammation, we determined the extent of leukocytic inflammation in both lavage and tissue in mice at the same time intervals used to examine BHR. Model A resulted in an increase in total leukocytes from as early as 1 h post challenge as previously described in B6 mice (7). These numbers peaked at 6 h but declined to prechallenge levels by 72 h (Fig. 2A). This increase in cells was primarily

FIGURE 1. BHR and protocols for induction of AAD. Female BALB/c mice were treated with either protocol A or B, and hyperreactivity to MCh was measured at time points following the final allergen challenge (6, 24, 48, or 72 h). Results shown are from four different experiments with either protocol with four mice in each group during each experiment. Model A (*left*) results in an acute transient BHR, whereas model B (*right*) induces chronic maintained BHR. The percentage change in Penh was calculated from values taken before and after provocation with 20 mg/ml MCh for mice immunized with OVA and challenged with PBS (□) or immunized and challenged with OVA (○). Bars represent SEM.



due to a pronounced eosinophilia and neutrophilia at 6 h, with eosinophils predominating at 24 h (Fig. 2*B* and data not shown). In contrast, model B resulted in a prolonged accumulation of cells in the lavage, where there was no significant decrease in cells over the 72-h period (Fig. 2*A*). Again the primary cell type responsible for this increase in cellularity in response to aerosolized Ag was eosinophils (Fig. 2*B*). The difference in cell numbers between model A and B at 72 h was highly significant with $p < 0.0001$.

The profile of BAL cell infiltration was found to be independent of either the dose of OVA used for immunization or the use of alum. Low-dose OVA in the absence of alum still resulted in an eosinophilia that was maintained at 72 h, with $6.9 \pm 1.2 \times 10^5$ eosinophils remaining at this time point.

The extent and anatomical location of eosinophilia in the airway interstitium was examined in sections from mice sacrificed at time points following the last aerosolized Ag challenge. Leukocytes were observed around vessels and airways from as early as 6 h postchallenge after protocol A as well as after protocol B (Fig. 2*C*). However, the peak eosinophilia was reached at 24 h for protocol A and 72 h for model B. The primary cell type making up the infiltrates were eosinophils, although some neutrophils were observed with both protocols at 6 h. Although the anatomical location of infiltrates did not differ discernibly between protocols, the peak infiltrate was considerably larger in model B than in A (average score of +5 vs +3, respectively). Most importantly, the infiltrate remained at 72 h in model B, but was cleared by 72 h in model A.

In considering the extent of cellular inflammation between models, model B resulted in a greater degree of eosinophilia compared with model A, but interestingly this was not reflected in a significantly greater peak in BHR. However, persistence of inflammatory infiltrate did correlate with the persistence of hyperreactivity.

Persistence of BHR and inflammation is not due to enhanced IgE production in model B

Elevated levels of serum IgE have been reported to be important in the development of an allergic pulmonary response. Therefore, we evaluated whether the difference in kinetics between models could be explained by IgE production. Both model A and B resulted in the secretion of serum IgE, which did not change over the 72-h period following the final aerosolized Ag challenge (Fig. 3*A*). However, the administration of the second OVA challenge via the peritoneal route (model B) induced 10-fold higher levels of IgE than when administered via aerosol (model A). This difference was not due to the presence of alum because comparable levels of IgE

were induced even in the absence of alum (data not shown). OVA-specific IgE varied significantly between the models, with model B eliciting several thousandfold more U/ml of OVA-IgE than model A (Fig. 3*B*). We hypothesized that the difference in specific IgE levels might be responsible for the different kinetics of the models. Thus, we took advantage of gene-targeted mice that lack the facility to produce IgE. We induced model B in these knockout mice to determine whether their response to OVA followed the kinetics of model B or A due to their lack of IgE. In fact, absence of IgE did not change the kinetics of the response, with maximal BHR and inflammation still occurring at 72 h postchallenge (Fig. 3, *C* and *D*, respectively). Thus it seems that the delayed resolution in model B is not due to the heightened IgE response observed with this protocol.

The kinetics of inflammation is not dependent on T cell cytokine responses

For the generation of an efficient immune response, the induction of a strong T cell response is critical. We compared T cell responses during both protocols by measuring levels of T cell cytokines during the challenge phase of both models. Levels of IL-4, IL-5, and IFN- γ were measured in BAL fluid collected at different time points following the final aerosolized challenge during model A or B. Both models induced the secretion of IL-4 and IL-5 into the BAL, with the peak of cytokine production occurring in both cases at 6 h and decreasing to control levels by 72 h (Fig. 4). However, model A induced 10-fold less of both IL-4 and IL-5 compared with model B, and both models showed 10-fold more IL-5 than IL-4. Levels of IFN- γ were comparable between models. Importantly, the kinetics of production followed a similar pattern in both models throughout the 72-h period following challenge. Similarly, *in vitro*-stimulated splenocytes from mice treated with protocol B showed a 300-fold increase in IL-4, a 5-fold increase in IL-5, and a 1.5-fold increase in IFN- γ compared with those prepared from model A (data not shown). Our data suggest that sensitization solely via the peritoneal route in model B leads to a response that is more Th2-like compared with that elicited after sensitization via the peritoneum plus aerosol as in model A.

Role of leukocyte survival and clearance in determining kinetics of inflammation

The early peak in eosinophils observed at 24 h in model A compared with a sustained eosinophilia in model B at 72 h may represent a more efficient resolution of the inflammatory response by

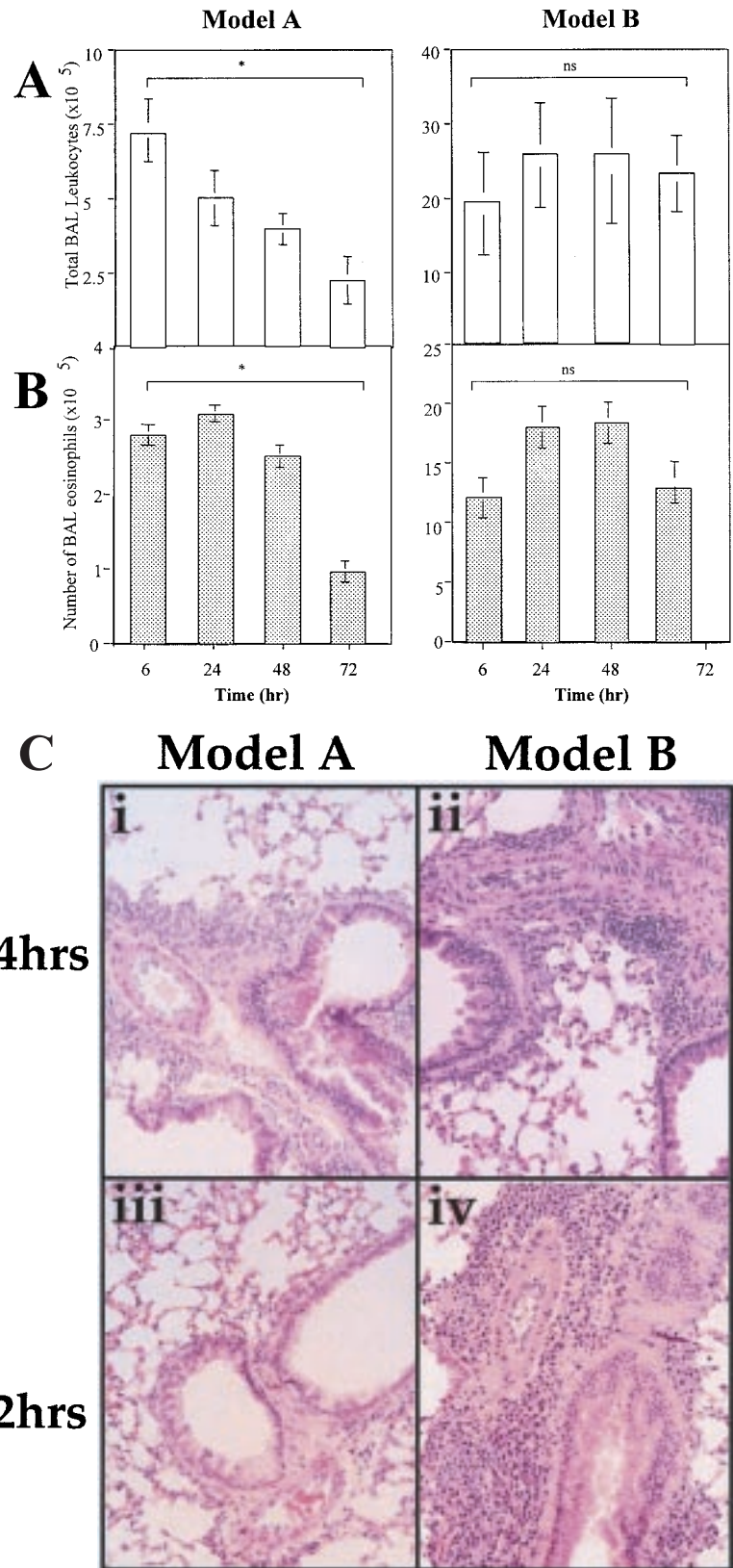


FIGURE 2. BHR differences between models are also reflected in kinetics of pulmonary inflammation. Model A (*left*) results in an acute transient inflammation, whereas model B (*right*) induces chronic persistent inflammation. *A*, Total leukocyte numbers were counted in lavage fluid recovered from mice sacrificed at the indicated time points following the final OVA challenge. *B*, Total numbers of eosinophils were calculated from differential stained BAL cytospins. Bars represent the mean (\pm SEM) number of cells per group of mice ($n = 8$). *, $p < 0.01$. *C*, Histology from mice sacrificed at 24 h after the final aerosolized challenge following protocol A (*i*) or protocol B (*ii*) or 72 h after challenge with protocol A (*iii*) or protocol B (*iv*). Representative hematoxylin and eosin-stained sections are shown (original magnification, $\times 400$).

clearance of leukocytes. To examine this hypothesis, we looked at mechanisms of leukocyte survival and clearance. Clearance may occur as a result of enhanced apoptosis due to decreased survival factors. Thus we examined levels of the prototypical eosinophil survival factors GM-CSF and IL-3 in lung lavage fluid, as well as the numbers of apoptotic cells in lung tissues. Although levels of

GM-CSF were higher in model B compared with model A (Fig. 5a, 10-fold at 24 h), the kinetics of production were similar, with the highest levels found at 6 h after challenge in both models. However, when levels of IL-3 were examined we saw that peak levels were comparable between model A and B but interestingly the peak production in model A occurred at 6 h (Fig. 5b), coinciding with the

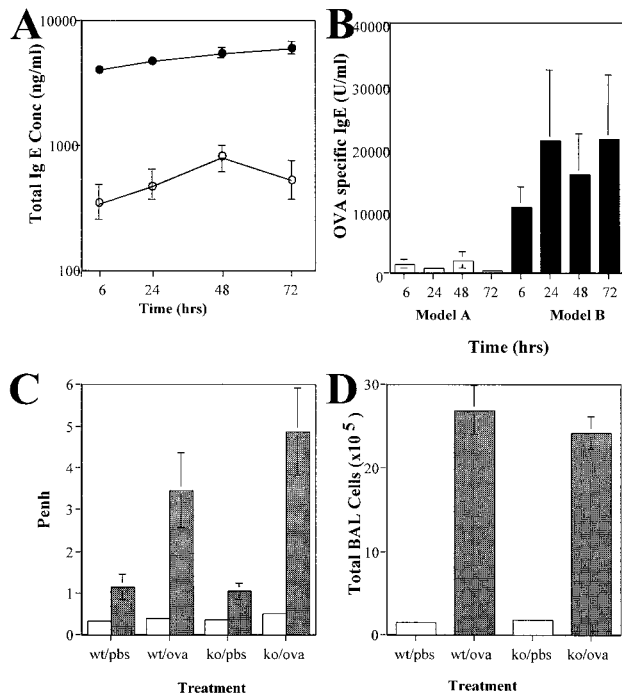


FIGURE 3. Persistence of BHR and inflammation is not due to enhanced IgE production in model B. IgE production was measured in serum samples obtained from mice sacrificed at the indicated time points following the final aerosolized OVA challenge. Levels of total (A) and OVA-specific IgE (B) were measured during protocol A or B. In A, circles represent mean \pm SEM of each group of mice treated with protocol A (○) or protocol B (●). In B, bars represent mean \pm SEM of each group of mice treated with protocol A (□) or protocol B (■). To determine whether the difference in persistence of BHR and inflammation was due to this differential IgE production, the kinetics of BHR (C) and BAL cellularity (D) was determined in IgE knockout mice after induction of protocol B. Both wild-type and IgE-deficient mice were sensitized with OVA, according to protocol B. Thereafter, wild-type ($n = 10$) and IgE knockout mice ($n = 7$) were challenged with aerosolized OVA, while further groups of wild-type ($n = 9$) and IgE knockout mice ($n = 6$) were challenged with PBS as controls. BHR was measured as described in *Materials and Methods* and is shown as mean \pm SEM Penh for each group of mice stimulated with MCH (■) or at baseline (□).

peak eosinophilia and BHR, but decreased to baseline by 72 h. In contrast, maximal IL-3 levels occurred at 72 h postchallenge in model B. Again, this time represents the highest point of inflammation and BHR (Fig. 5*b*).

Numbers of apoptotic cells were determined by TUNEL-staining lung sections at intervals following challenge and then counting the number of positively stained cells per field. There were very few apoptotic cells in lungs from PBS-treated mice, but after OVA challenge with model A or B there was a significant increase in the number of cells within peribronchiolar infiltrates that were undergoing apoptosis (Fig. 5, *c* and *d*). The percentage of apoptotic cells was calculated by counting numbers of positively and negatively stained cells in peribronchiolar infiltrates over the same absolute area in each section. It is important to note that the average number of cells counted within the 2.5 mm² in model B was 10-fold greater than in A (e.g., 2500 cells/2.5 mm² in B compared with 220 cells/2.5 mm² in A at 72 h). A significant increase in apoptotic cells occurred at 48 h in model A, which coincides with a decreased BHR and a decrease in lavage and tissue cellularity. This level of apoptosis in model A was significantly higher than that in model B at the same time point ($p < 0.05$). At 72 h, the

percentage of apoptotic cells was still high, although the total number of infiltrating cells had decreased by this time and BHR was back to baseline levels. A different pattern was observed with model B in that the percentage of apoptotic cells increased slightly but never reached the degree observed in A. Although the percentage of apoptotic cells in B did not differ discernibly between 48 and 72 h, the absolute number of apoptotic cells rose because the number of infiltrating cells was still increasing.

Role of IL-3 in mediating resolution of pulmonary inflammation

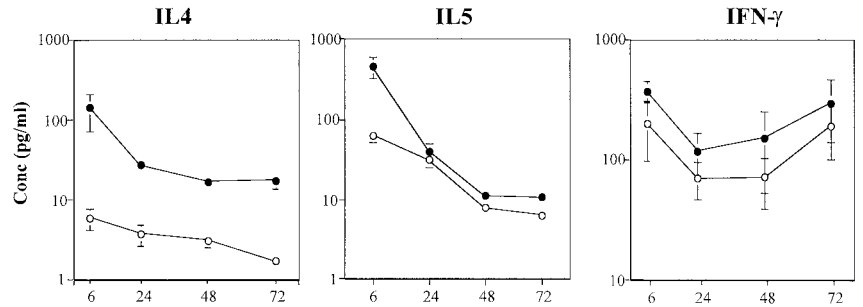
To determine whether IL-3 plays a functional role in the resolution of BHR, we administered a single dose of neutralizing Abs to this cytokine 24 h after the final challenge in mice subjected to model B. This time point was chosen to ascertain whether a single dose of Ab had an effect once inflammation and BHR were established. Control mice received either PBS or goat Ig. Although BHR in this particular experiment was somewhat lower than in previous experiments, a similar degree of BAL eosinophilia was induced in these control mice compared with mice in Fig. 2 (13.5×10^5 eosinophils at 72 h in Fig. 2 and 16.4×10^5 eosinophils at 72 h in Fig. 6). When compared with mice similarly treated with control Ig, the anti-IL-3-treated group showed reduced BHR (Fig. 6A) at both 48 and 72 h postchallenge. This was associated with a significant decrease in the percentage of eosinophils within peribronchiolar infiltrates located within lung sections (Fig. 6B, $p = 0.001$). Interestingly, when the degree of apoptosis in peribronchiolar infiltrates was examined, neutralization of IL-3 was seen to significantly increase the levels of apoptosis within infiltrates (Fig. 6C, $p = 0.001$). The increase in apoptosis observed after IL-3 neutralization was concomitant with the decrease in BHR and tissue eosinophils and was reminiscent of the endpoints observed for model A.

Discussion

In recent years, there has been an intensive investigation of the mechanisms involved in the induction of AAD. These data suggest an important role for various mediators in initiating this complex response. In contrast, there is little information regarding the signals responsible for resolution of Ag-induced BHR. After a comprehensive analysis of several models of AAD, we have identified two models of murine AAD in which airway hyperresponsiveness and lung eosinophilia occur concomitantly after sensitization and challenge with OVA. However, the rate at which these pathophysiological phenomena occurs differed considerably between the two similar models. One model was associated with an acute, transient BHR, which resolved completely by 72 h, while the other protocol induced a sustained BHR, which developed less rapidly but reached a plateau by 72 h. After identifying these differences, which we anticipate will be critically important in the understanding of AAD, we sought to investigate the reasons for these differences with the view of identifying factors involved in mediating resolution of BHR and pulmonary inflammation.

Both protocols resulted in the development of BHR in conjunction with eosinophilia. Although the kinetics of the BHR differed considerably, the peak Penh values were similar in both models, even though the size of the lavage and tissue eosinophilia in model B was greater than that in model A (Fig. 2). Thus, although the presence of eosinophilia correlates with development of BHR, the size of the eosinophilia is not proportional to the decrease in lung function. The main difference in the protocol for inducing disease was the addition of alum in the sensitization period in model B but not in A. However, when alum was subsequently omitted from the protocol there was no difference in the kinetics of either eosinophilia or BHR. Thus the degree of both BHR and size of infiltrate

FIGURE 4. The kinetics of inflammation is not dependent on T cell cytokine responses. Levels of T cell cytokines were determined by ELISA in isolated BAL samples after treatment with protocol A or B. Each point represents the mean (\pm SEM) of groups of mice ($n = 8$) subjected to protocol A (\square) or protocol B (\bullet).



was dependent upon the dose of Ag rather than the presence of alum. Adjuvants are used to increase the antigenicity of a particular Ag, and thus one might expect a stronger anti-OVA response after model B compared with A; this would be reflected in the humoral and cellular responses to OVA. We found that although both protocols increased serum IgE levels, protocol B induced a 10-fold increase when compared with model A (Fig. 3). Moreover, measurement of OVA-specific IgE showed that mice produce greater amounts of OVA-specific IgE after model B compared with A. This probably reflects a more aggressive response to OVA in mice that are primed with OVA in the presence of alum, leading to enhanced priming of B cells to produce IgE. A number of investigators have shown IgE to be a critical component of the allergic response (16, 25). Although passive transfer experiments using allergen-specific IgE Abs indicate a direct role for IgE in both inflammation and BHR (26), blocking experiments have not

been so clear. Neutralization of IgE with nonanaphylatogenic Abs leads to decreased eosinophilia and BHR (17); however, IgE knockout mice still develop allergen-induced BHR and eosinophilic inflammation (22). We used IgE knockout mice to determine whether the difference in model pathophysiologies was due to the differential IgE production. This was not the case because IgE-deficient mice still developed a sustained inflammation and BHR after protocol B (Fig. 3). Thus, although the observed differential IgE expression may account for the difference in magnitude of eosinophilia, it does not provide an explanation for the delay in resolution in model B compared with A.

Development of an allergic response is associated with a strong Th2 response as determined by T cell cytokine profiles (27, 28). We observed that although 10-fold more IL-4 and IL-5 was found in the BAL fluid of model B compared with A, the kinetics of production in lavage were similar, with the peak production

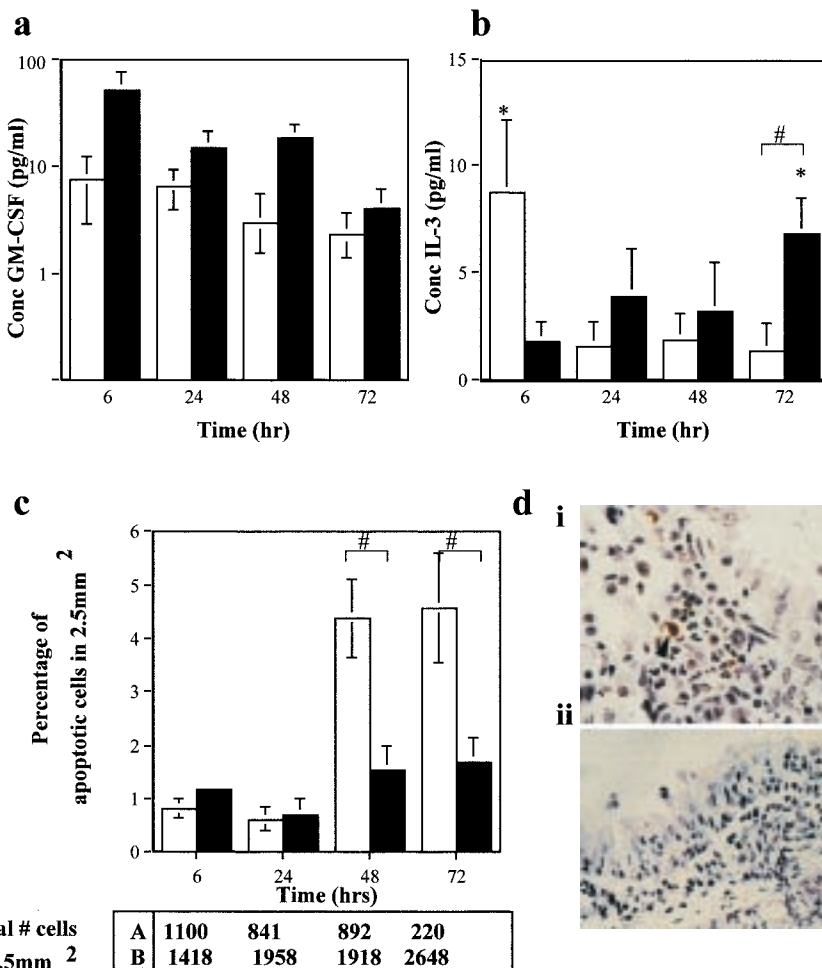


FIGURE 5. Role of leukocyte survival and clearance in determining kinetics of inflammation. Levels of the cytokines GM-CSF (a) and IL-3 (b) were measured in pulmonary lavage samples by ELISA. Bars represent the mean (\pm SEM) of groups of mice subjected to protocol A (\square) or protocol B (\blacksquare). c, The percentage of peribronchiolar leukocytes undergoing apoptosis was determined histologically in lung sections from mice at various time points following challenge with protocol A (\square) or protocol B (\blacksquare). Each bar represents the mean percentage of apoptotic cells (\pm SEM), which was calculated after counting positively stained cells within peribronchiolar infiltrates in the same number of randomly selected high-power ($\times 400$) fields per section (total area of 2.5 mm²). The mean total number of cells counted in peribronchiolar infiltrates within this area is shown for each model below each bar for comparison. *, $p < 0.05$ compared with unchallenged mice; #, $p < 0.05$ model A vs B. d, Apoptosis was detected by TUNEL staining. i, Lung taken at 72 h post-challenge in model B, arrowhead shows positively stained cell; ii, a negative control, TdT was omitted (original magnification, $\times 400$).

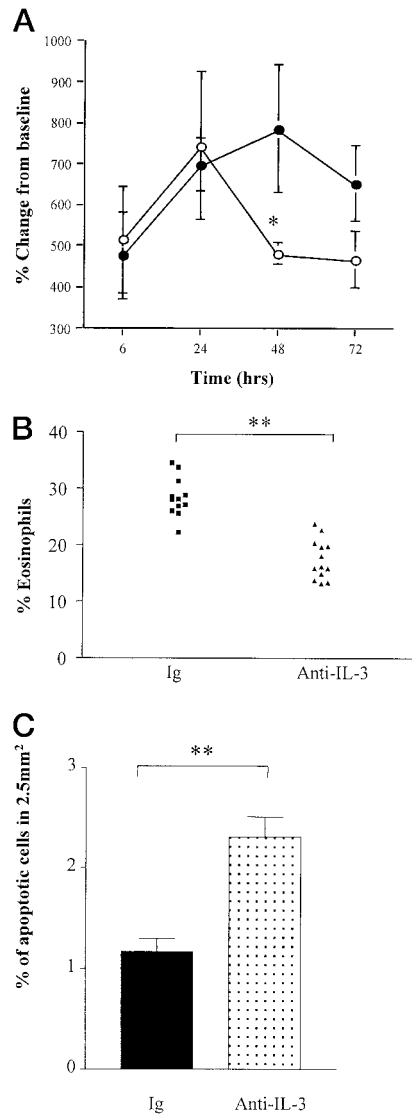


FIGURE 6. Role of IL-3 in mediating resolution of BHR. Neutralizing anti-IL-3 Abs were given to mice 24 h after the final challenge on day 24 of protocol B. A, BHR was monitored at intervals following the final challenge in groups of mice given anti-IL-3 (○, $n = 6$) or goat Ig (●, $n = 6$). B, Tissue eosinophilia was calculated by counting the proportion of eosinophils within five peribronchiolar infiltrates at a magnification of $\times 400$ in sections from mice treated with either anti-IL-3 (▲, $n = 12$) or Ig (■, $n = 13$). C, The percentage of peribronchiolar leukocytes undergoing apoptosis was determined histologically in lung sections from mice treated with either anti-IL-3 (▨) or Ig (■). Each bar represents the mean percentage of apoptotic cells (\pm SEM), which was calculated after counting positively stained cells within peribronchiolar infiltrates in the same number of randomly selected high-power ($\times 400$) fields per section (total area of 2.5 mm²). *, $p < 0.05$; **, $p < 0.001$, treated vs untreated mice, Student's t test. Results shown are pooled from two separate experiments.

occurring early after challenge in both cases. Th2 cells and the cytokines they produce have been described as central players in the generation of a pathophysiological response to allergen (29, 30). Indeed, significant BHR and eosinophilia occur after allergen challenge in mice in which Ag-specific T cells have been transferred, but in the absence of prior priming with Ag (31).

Eosinophils have been implicated in the development of airway hyperreactivity, and there is correlation between their presence in the lung interstitium and the decline in lung function (18, 32, 33). In our study, one of the most profound differences in pathological

outcome between protocol A and B was the kinetics of eosinophil migration to the lung interstitium and into the airway lumen, as determined by BAL. In addition, the kinetics of eosinophil accumulation in the lung directly correlated with the development of BHR in both models and the resolution of the response in model A. This difference in the kinetics of eosinophil accumulation in the interstitium and airway lumen may reflect the rate of clearance of the infiltrate. Cell survival represents a mechanism by which the duration of eosinophil-mediated inflammation can be controlled (34). IL-3, IL-5, and GM-CSF have been found to increase dramatically the life span of eosinophils by inhibiting their death by apoptosis (35–38). We found increased levels of all three of these cytokines in lavage from mice after induction of either protocol, as well as an increase in leukocyte apoptosis in peribronchial areas (Figs. 4 and 5, *a* and *b*). Although production of IL-5 and GM-CSF showed comparable kinetics in both models, there was a clear difference in the pattern of IL-3 secretion. The peak of IL-3 production after model A occurs at 6 h and declines by 24 h. Although the main source of IL-3 is likely to be T cells, eosinophils also produce IL-3 (39). Therefore, eosinophils themselves could maintain inflammation through an autocrine mechanism. In fact, as IL-3 levels decline there is an increase in leukocyte apoptosis in model A (at 48 h) that coincides with a decrease in BHR and ultimately in the resolution of inflammation. However, in model B, IL-3 levels steadily increase to a peak at 72 h. At this point the absolute number of apoptotic leukocytes is also increasing, and in contrast to model A tissue infiltration is at its height. Thus the level of apoptosis may not be sufficient to clear the larger numbers of leukocytes present, presumably because the relevant growth factors are still present. The fact that the percentage of apoptotic cells is higher in model A than B suggests that there are fewer survival signals in the airways of mice given protocol A. It is tempting to speculate that there is a signal for IL-3 to be down-regulated in model A allowing for increased clearance of cells by apoptosis, whereas in model B such a signal is not received by 72 h.

To determine whether reduced IL-3 could influence the resolution of BHR and inflammation, we performed blocking experiments giving a single dose of neutralizing anti-IL-3 Ab to mice during model B. Blockage of IL-3 was seen to reduce both BHR and tissue eosinophilia, but increased the percentage of apoptotic leukocytes within infiltrates. Taken together, these results indicate that IL-3 plays a role in prolonging tissue leukocyte infiltration in model B by limiting clearance by apoptosis. IL-3 has been shown to prevent cell death by apoptosis, either directly or by sensitizing cells to survival factors present in the serum (40). In particular, the effect of IL-3 on eosinophil viability has previously been demonstrated in vitro (41) where addition of IL-3 to eosinophil cultures was found to decrease apoptotic eosinophil numbers. The authors suggested that eosinophil viability could be differentially regulated by cytokines produced during the immune response. Although in our study we were unable to determine the individual cell type undergoing apoptosis, the majority of infiltrating cells at this time point are eosinophils. However, we have shown that blockage of IL-3 in model B changed the course of disease to reduce eosinophilia and BHR at 72 h. This effect may be associated with the role of IL-3 in promoting leukocyte clearance by apoptosis, and the potential link between leukocyte survival signals and resolution of BHR merits further study.

We have determined that although the immune response to OVA results in BHR and pulmonary inflammation, these pathophysiological responses may be transient or persistent depending upon the protocol used to induce AAD. We have shown that these

differences in Ag administration lead to a differential T cell cytokine response. In conclusion, i.p. administration of allergen is associated with a failure to resolve inflammation and BHR by 72 h postchallenge, which is, at least in part, mediated by a low IL-3 production and apoptosis. Investigation of these differences may lead investigators in the field to a greater understanding of how the immune response to inhaled Ags develops and, perhaps more importantly, the mechanisms by which hyperreactivity and inflammation resolves.

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