

Prevention of Allergic Eye Disease by Treatment with IL-1 Receptor Antagonist

Andrea M. Keane-Myers, Dai Miyazaki, Grace Liu, Iva Dekaris, Santa Ono, and M. Reza Dana

PURPOSE. To determine the impact of interleukin-1 (IL-1) inhibition using IL-1 receptor antagonist (IL-1Ra) in a mouse model of allergic eye disease.

METHODS. A/J mice sensitized and challenged with cat dander in the eye were treated with topical IL-1Ra or vehicle alone. Control mice were treated with IL-1Ra or vehicle but sensitized and challenged with phosphate-buffered saline alone. Immediately after the final allergen challenge, the mice were observed for behavioral changes and assessed for lid injection and chemosis. The animals were then killed, eyes and attached lids were removed for either RNA extraction or histology, and draining lymph nodes were removed for either RNA extraction or in vitro stimulation assays. Differences in chemokine message between experimental and control groups were determined by RNase protection assays.

RESULTS. Treatment with IL-1Ra in allergen-challenged animals significantly reduced allergen-induced changes in photosensitivity (60%, $P = 0.0002$), chemosis (50%, $P = 0.0151$), and injection (86.7%, $P = 0.0068$) compared with vehicle-treated controls. Interleukin-1Ra reduced the number of degranulated mast cells and caused a significant reduction in the number of eosinophils infiltrating the conjunctival matrix ($P < 0.001$) after allergen challenge. Examination of chemokine mRNA taken from the conjunctiva and draining lymph nodes by RNase protection assay showed a profound decrease in the production of a number of C-C chemokines.

CONCLUSIONS. These findings suggest that IL-1Ra is suppressing allergic eye disease by a down-modulation of the recruitment of eosinophils and other inflammatory cells essential for the immunopathogenesis of ocular atopy. (*Invest Ophthalmol Vis Sci.* 1999;40:3041-3046)

From the Department of Ophthalmology, Laboratory of Molecular Immunology, The Schepens Eye Research Institute, Harvard Medical School, Boston, Massachusetts.

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Corresponding author: Andrea M. Keane-Myers, Laboratory of Molecular Immunology, The Schepens Eye Research Institute, Harvard Medical School, 20 Staniford Street, Boston, MA 02114.
E-mail: akm@vision.eri.harvard.edu

Allergic conjunctivitis (AC), atopic inflammation of the mucous membrane that lines the eyelid and outer eyeball, affects over 40 million patients per year in the United States.¹ Patients with AC experience itching and burning sensations in the eye in response to allergens that are innocuous for normal individuals and show clinical signs of chemosis, tearing, conjunctival hyperemia, and lid edema.¹ The diagnosis of AC is a clinical one mainly based on the history and ophthalmologic findings. Conjunctival scrapings, although not commonly used, are of help because eosinophils are not ordinarily found in the conjunctival scrapings from nonallergic individuals, making the presence of even one eosinophil considerable evidence in favor of a diagnosis of AC.¹

We have developed an experimental murine model for cat-dander-induced AC, which provides the clinical, cellular, and humoral parameters of allergic disease. Sensitized mice are challenged via eyedrops with cat dander extract containing defined amounts of the major cat allergen, a 35-kDa protein known as *Felis domesticus* allergen 1 (*Fel d1*).² As the predominant human IgE binding component in cat dander, *Fel d1* is known to elicit the symptoms of perennial AC.² Sensitization and challenge with cat dander results in significant increases in photosensitivity, itching, chemosis, and conjunctival injection compared with phosphate-buffered saline (PBS)-challenged control animals. These early clinical symptoms correlate with mast cell degranulation observed within 1 hour after challenge, and significant infiltration of inflammatory cells, including eosinophils, 24 hours later. This model enables us to carefully study disease pathogenesis and to evaluate new therapies, for the treatment of AC.

Interleukin 1 (IL-1), a 17.5-kDa cytokine synthesized primarily by activated macrophages, plays a central role in the initiation and coordination of host defenses in response to a range of physiological insults, including trauma, infection, and inflammation. The IL-1 family consists of two agonists, IL-1 α and IL-1 β , and a structurally related specific receptor antagonist, IL-1Ra. Interleukin-1Ra binds with high avidity to IL-1 receptors but fails to trigger intracellular responses, thus acting as a competitive inhibitor.³ Recombinant human IL-1Ra has been used both in vitro and in vivo to block a range of IL-1-induced biological activities, thereby validating its use in determining the precise role of IL-1 in short-term animal models of disease.

In the study reported in this article, we used recombinant IL-1 receptor antagonist to study the effects of IL-1 inhibition in a mouse model of AC. Interleukin-1 has been found to regulate chemokines and adhesion factors, the upregulation of which has been closely linked to the development of allergic disease.³ Therefore, we hypothesized that treatment with IL-1Ra could suppress ocular allergy. Our results suggest that IL-1Ra can effectively treat allergic eye disease by decreasing the expression of chemokines essential for the recruitment of allergy-inducing inflammatory cells, including eosinophils.

MATERIALS AND METHODS

Animals

Six-week-old A/J mice were obtained from Jackson Laboratory (Bar Harbor, ME). To prevent any potential aerosolization of allergen from affecting PBS-challenged control animals, all mice were kept in cages with filter-topped lids. The studies reported

here conformed to the principles for laboratory animal research outlined by the Animal Welfare Act and the National Institute of Health guidelines for the experimental use of animals and complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We used 10 mice for each experimental group at each time point studied, unless specified otherwise.

Antigen Challenge Protocols

We sensitized the mice to cat dander by local administration via eye drops of prepared cat dander extract containing quantitated amounts of *Fel d1* (ALK; 250 AU/eye) drop-wise in the eye (2.5 μ l/eye) with the use of a p10 micropipette (Eppendorf). Mice were then challenged with the same amount of allergen via eyedrops once per week for the next 4 weeks. Control mice were challenged with a similar volume of PBS (sham challenge).

Administration of IL-1Ra

Two percent IL-1Ra (20 mg/ml) in 0.2% sodium hyaluronate was administered to both cat dander-challenged and sham-challenged (PBS) control mice three times daily for the duration of the experiment (28 days), starting 30 minutes before the initial sensitization. The dosage and administration of IL-1Ra treatment used in these experiments conformed to previous protocols using topical IL-1Ra in models of inflammatory eye disease.⁴ To control for any potential response to sodium hyaluronate alone, two additional groups (either cat dander-challenged or sham-challenged) received a similar volume of vehicle (0.2% sodium hyaluronate).

Clinical Evaluation

In all the challenge protocols, the identity of treatment in each of the cages was masked, and mice were observed by two independent investigators every 5 minutes for the first half hour after the final antigen challenge for changes in behavior (i.e., increased photosensitivity as evidenced by squinting or excessive face washing, suggesting a response to itching eyes). The animals were also assessed by two ophthalmologists (DM and ID), using a slit lamp for assessment of injection and chemosis. Representative photographs of mouse eyes were used to assess disease severity, and all symptoms were recorded as $\pm$ during each 1-minute timed assessment. The percentage of animals per cage experiencing a particular symptom was then recorded. The animals were then killed at 1, 24, 48, or 72 hours after the final antigen challenge. In all cases, the mice were anesthetized with ketamine/xylazine (200 mg/kg ketamine + 10 mg/kg xylazine) and exsanguinated via cardiac puncture for antibody analysis of their sera.

Histology

To assess the cellular infiltrate in the conjunctiva and surrounding tissue, the eyes were removed with the attached lids and intact conjunctiva, immediately fixed in 4% paraformaldehyde, and embedded in Histo-resin (Leica Instruments GmbH, Heidelberg, Germany). Serial sections were cut from each eye and stained with Giemsa for mast cells and hematoxylin and eosin (H&E) or Congo red (both from Sigma) for the identification of eosinophils. All slides were masked, and cell counts were made by two independent investigators. For cellular quantitation,

five sections were examined from each mouse per treatment group ($n = 10$ mice per group), and cells were counted in five 400 $\times$ nonoverlapping fields per eyelid section, for a total of 250 400 $\times$ fields per treatment group. Eosinophils were identified by red eosin staining of their cytoplasmic granules and their distinctive bilobed nuclei. Mast cells were identified by their oval contour, mean diameter of approximately 12 to 13 μ m, and cytoplasm filled with distinctive pink-to-purple granules.

RNase Protection Assay

To determine relative chemokine mRNA levels, we used the Riboquant Multiprobe RNase Protection Assay system (PharMingen, San Diego, CA). Briefly, draining (cervical) lymph nodes and eyeballs containing conjunctival samples were removed from the mice immediately after they were killed. The conjunctiva was dissected from its attachments to the eyelids. The tissues were placed in 2 ml of RNAsStat 60, homogenized, and immediately frozen on dry ice. The samples were stored at -80°C until used. For use, separate RNA samples from five mice per experimental group were defrosted on ice, and the RNA was extracted with a phenol chloroform procedure using DEPC-treated materials. Aliquots of the samples were run on a 1% agarose gel to determine concentration and ascertain any potential degradation. The samples were then used as per manufacturer's instructions with the mCK-5 probe positive for lymphotaxin (Ltn), Regulated on activation normal T cell expressed (RANTES), eotaxin, macrophage inflammatory peptide-1 α (MIP-1 α), MIP-1 β , MIP-2, interleukin-gamma-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), T cell activation gene-3 (TCA-3), and the housekeeping genes L32 and GAPDH. To semiquantify differences in amounts of RNA, the film was scanned after development, and the bands were subjected to densitometry using Digital Science ID software (Kodak). The numerical values were normalized according to the densitometry of the housekeeping genes GAPDH and L32.

Data Analysis

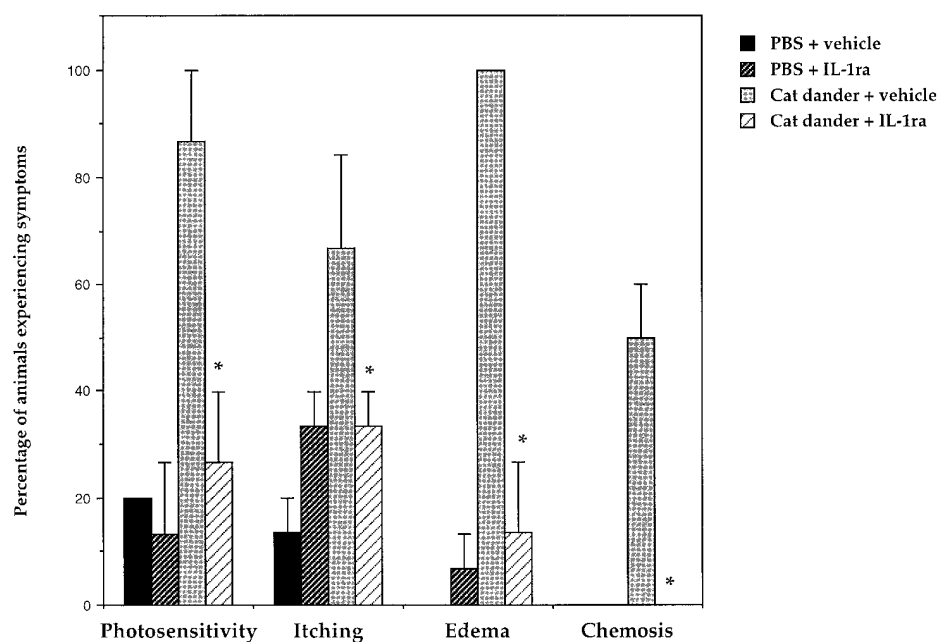
Data are summarized as mean $\pm$ SE. The statistical analysis of the results was performed by ANOVA using Fisher's least significant differences test for multiple comparisons. $P < 0.05$ was considered significant.

RESULTS

IL-1Ra Significantly Reduced Clinical Signs of Allergic Eye Disease

To control for any potential effects of either IL-1Ra treatment or vehicle, a second set of mice were sham-challenged with PBS. Previous studies in our laboratory did not find any discernible difference between PBS-challenged and naive control animals. Antigen treatment of sensitized mice caused significant increases in clinical signs of AC, including an increase in photosensitivity (86.6%, $P = 0.0001$) indicated by marked squinting, itching as evidenced by vigorous face-washing and scratching about the eyes⁵ (66.6%, $P = 0.001$), and chemosis (50%, $P = 0.001$) and injection (100%, $P = 0.0001$) of the conjunctiva compared with sham-challenged control animals. The allergic symptoms began within 5 minutes after antigen

FIGURE 1. Interleukin-1Ra treatment significantly decreases the clinical symptoms of AC. Allergen challenge significantly increased photosensitivity, itching, injection, and chemosis in A/J mice. Treatment with IL-1Ra significantly decreased these clinical signs to levels observed in PBS control mice. * $P < 0.05$ compared with sham-challenged, cat dander-treated animals.



challenge and peaked 20 minutes after the final challenge (Fig. 1). Control mice sham-challenged with PBS displayed none of these symptoms and appeared physiologically normal. Treatment with IL-1Ra in allergen-challenged animals significantly reduced allergen-induced changes in photosensitivity (60%, $P = 0.0002$), chemosis (50%, $P = 0.0151$), and injection (86.7%, $P = 0.0068$), reducing symptoms to levels indistinguishable from the sham-challenged control groups.

Reduction in Allergen-Induced Eosinophil Infiltration and Mast Cell Degranulation after IL-1Ra Treatment

To assess the cellular infiltration in the conjunctiva and surrounding tissue, the intact eyes and lids were sectioned and stained with either hematoxylin and eosin or Congo red for the assessment of eosinophils, or with Giemsa for the assessment of intact mast cells. The groups were masked, and slides were counted by two independent investigators. There was a significant increase in the numbers of degranulated mast cells in the matrix surrounding the conjunctiva after allergen challenge compared with sham-challenged controls (301%, $P = 0.03$; Fig. 2A). Treatment with IL-1Ra caused a modest, statistically insignificant decrease in mast cell degranulation in cat dander-challenged animals ($P = 0.1846$; Fig. 2A).

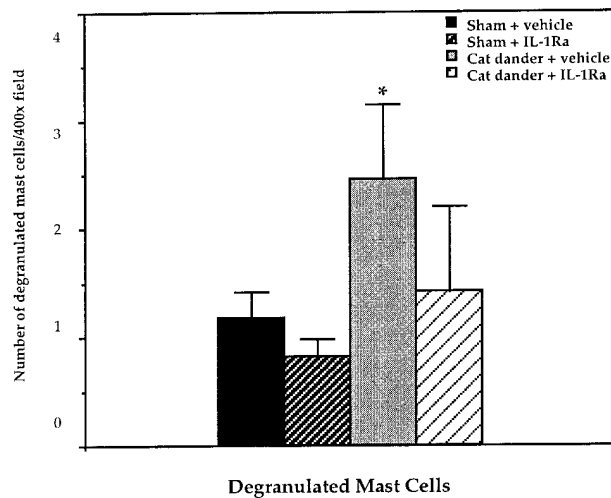
Sham-challenged control mice had few eosinophils in their eyelids and conjunctiva. At 24 hours after the final allergen challenge, there were significant increases in the total number of eosinophils infiltrating into the matrix surrounding the conjunctiva, and especially in the fornical region, compared with sham-challenged controls (Fig. 2B) (598%, $P = 0.0001$). Cell counts on conjunctiva from animals treated with IL-1Ra showed a reduction in the numbers of eosinophils to levels similar to that observed in the sham-challenged control animals ($P = 0.998$), suggesting an ablation of antigen-induced eosinophil influx.

IL-1Ra Treatment Reduces the Transcription of Allergen-Induced Chemokines

The comparatively small amount of tissue available from the mouse conjunctiva resulted in relatively low total concentrations of RNA and therefore weaker overall signals than what could be observed from other tissues, including the draining lymph nodes (Fig. 3). However, we were able to observe a reduction in eotaxin and RANTES mRNA in the conjunctiva after IL-1Ra treatment in both the allergen-challenged and sham-challenged control groups (Fig. 4A). The levels of gene expression for eotaxin and RANTES in cat dander- and IL-1Ra-treated conjunctival samples were comparable to levels seen in sham-challenged control animals.

Because there are a large number of articles showing that chemokine expression in the draining lymph node of an inflamed organ affects the expression of immunity and inflammation in the organ itself⁶, we also examined the level of chemokine expression in the draining (cervical) lymph nodes. Cat dander challenge caused significant increases in the gene expression of the chemokines RANTES, eotaxin, MIP-1 α , and IP-10 in the draining lymph nodes (Figs. 3 and 4B) compared with sham-challenged PBS controls. Interleukin-1Ra treatment substantially reduced the expression of these chemokines in both the cat dander-challenged mice and in the sham-challenged (PBS) mice. We used densitometric analysis of the autoradiogram with Digital Science ID software to approximate differences in chemokine mRNA. RANTES production was decreased 15% (Fig. 4) in samples taken from draining lymph nodes of mice that had been treated with IL-1Ra compared with control animals, although the actual differences may have been greater because the bands in the cat-dander + vehicle group appeared to have reached saturation. Differences in eotaxin and MIP-1 α expression between allergen-challenged animals treated with IL-1Ra and controls were even more striking, with a 41% and a 47% decrease, respectively, in allergen-challenged IL-1Ra-treated animals compared with controls. Levels of IP-10 after IL-1Ra treatment showed the biggest

A.



B.

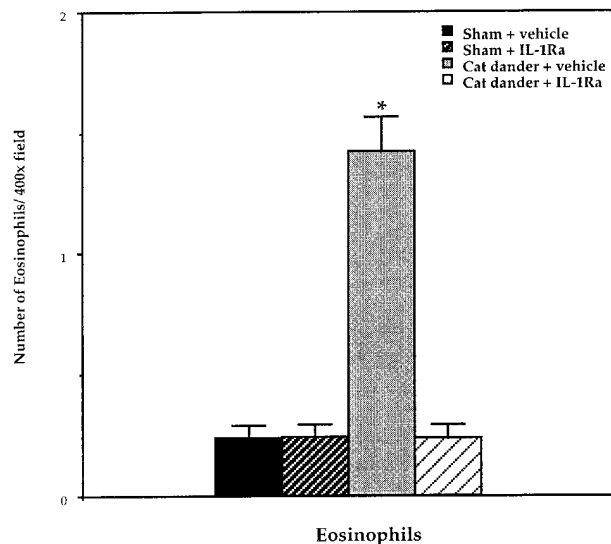


FIGURE 2. (A) Interleukin-1Ra does not significantly alter mast cell degranulation in conjunctival samples taken 24 hours after final allergen challenge. Sensitization and challenge with cat dander caused a significant increase in the number of degranulated mast cells observed in the conjunctival matrix. * $P < 0.05$ compared with sham-challenged control animals. (B) Interleukin-1Ra treatment ablates allergen-induced eosinophilia in the eye. Sensitization and challenge with cat dander resulted in a significant infiltration of eosinophils into the conjunctival matrix within 24 hours after the final allergen challenge. Treatment with IL-1Ra significantly reduced the number of eosinophils to what was observed in sham-challenged control groups. * $P < 0.05$ compared with sham-challenged control animals.

differences, with a 50.7% decrease compared with vehicle-treated animals.

DISCUSSION

Increased levels of IL-1 have been found in late phase reactions in the skin after allergen skin challenge in sensitive humans,³ suggesting an important role for IL-1 in allergic disease. More-

over, a critical element of all immunoinflammatory responses, including allergic disease, is the recruitment of leukocytes by chemokines and upregulation of adhesion factors. Interleukin-1 increases chemokine production, increases adhesion factors, increases macrophage infiltration and activity, and increases lymphocyte proliferation,³ all of which play a role in the immunopathogenesis of allergic disease.

The administration of IL-1Ra used in the present study proved effective in significantly reducing the clinical stigmata

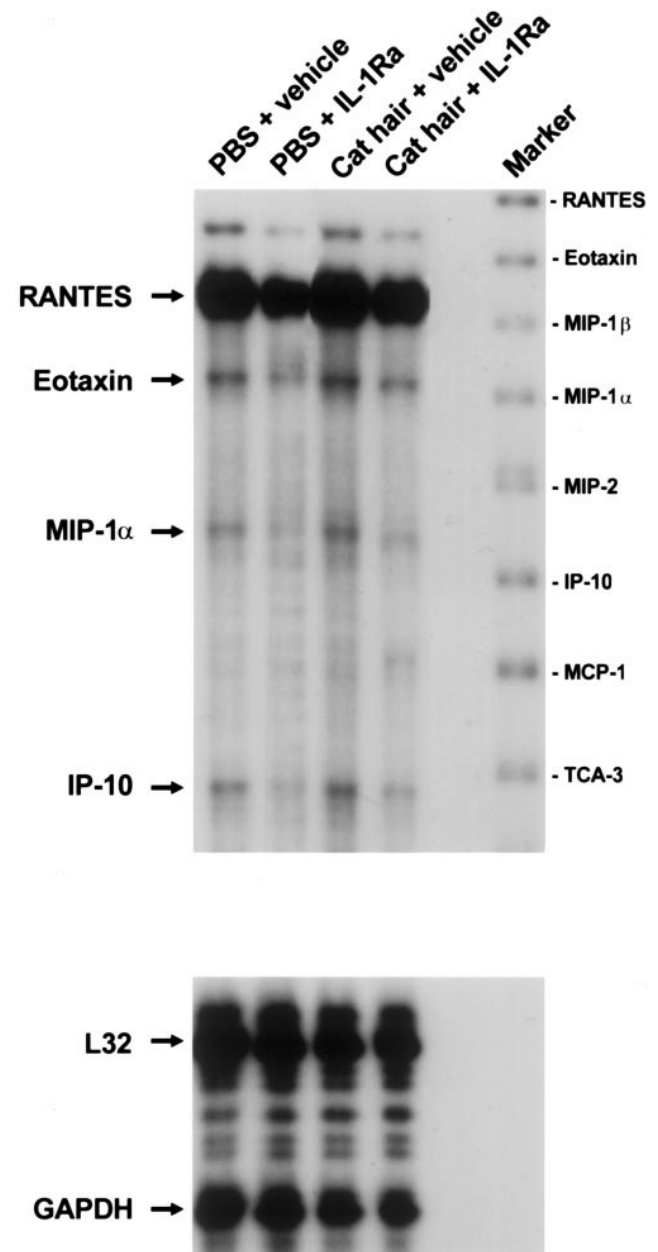


FIGURE 3. Interleukin-1Ra treatment reduces the allergen-induced increases in the chemokines RANTES, eotaxin, MIP-1 α , and IP-10. To determine relative chemokine mRNA levels, we used the Riboquant Multiprobe RNase Protection Assay system from Pharmingen and the values for the housekeeping genes L32 and GAPDH. Allergen challenge resulted in an increase in RANTES, eotaxin, MIP-1 α , and IP-10. Treatment with IL-1Ra significantly reduced such allergen-induced increases in chemokine message.

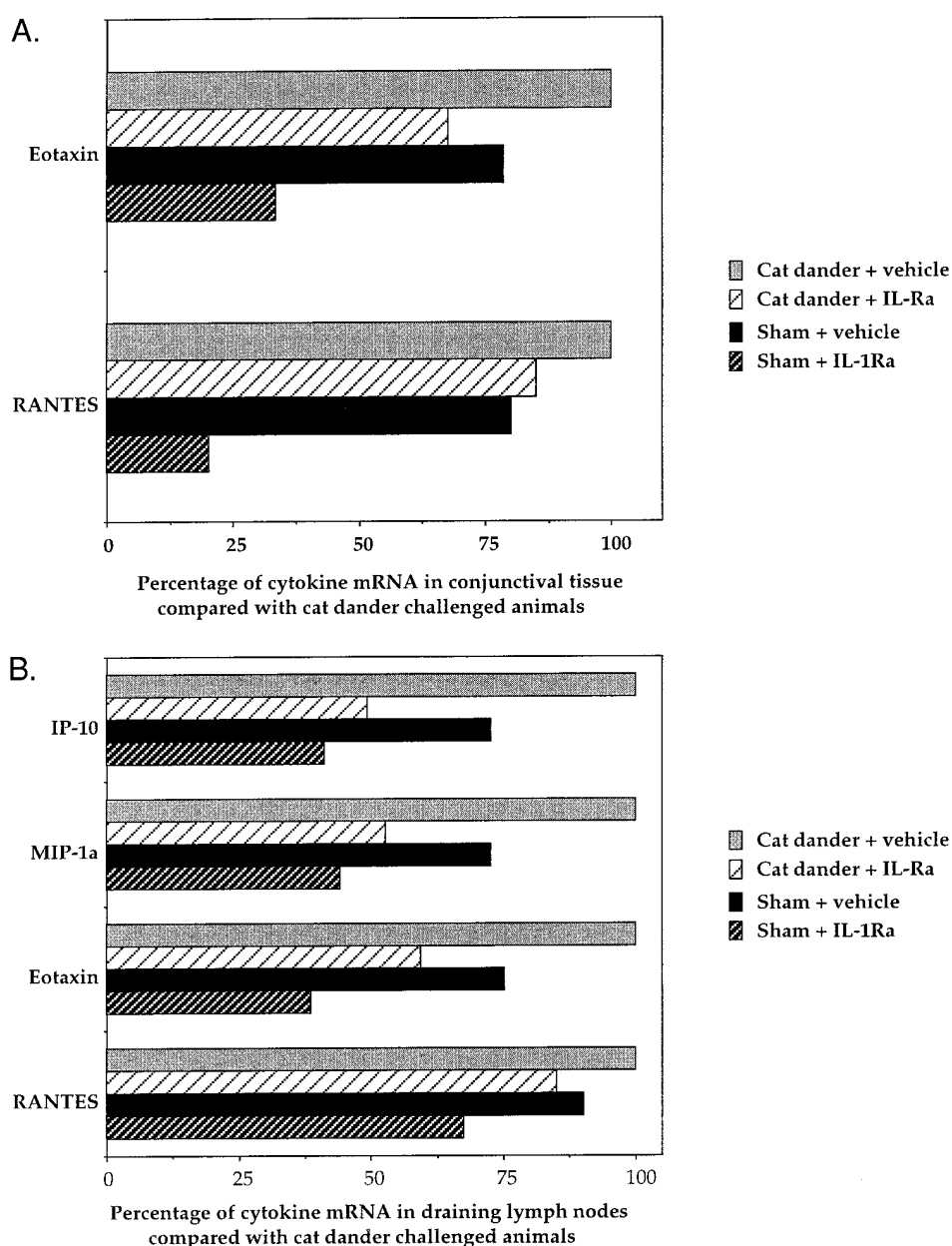


FIGURE 4. Densitometry for IL-1Ra data in conjunctiva (A) and draining lymph nodes (B). To assess semi-quantitative differences in amounts of RNA, the film was scanned after development, and the bands were subjected to densitometry using Digital Science ID software (Kodak). The numerical values were normalized according to the densitometry of the housekeeping genes *GAPDH* and *L32*. The amount of chemokines produced in mice given the maximal stimulus (cat dander + vehicle) was set as 100%. Interleukin-1Ra significantly reduced the amount of RANTES, eotaxin, MIP-1 α (MIP-1a), and IP-10 in both allergen- and sham-challenged control groups. In addition, IL-1Ra treatment reduced the concentrations of eotaxin and RANTES found in conjunctival samples in both cat dander-treated and sham-challenged control groups.

of allergy in response to cat dander. In contrast, treatment with vehicle alone did not significantly alter responses in either sham-challenged or cat dander-challenged mice. The reduction in clinical symptoms after IL-1Ra treatment coincided with the ablation of the allergen-induced eosinophilia, because the number of eosinophils in allergen-challenged IL-1Ra-treated animals was similar to that seen in the sham-challenged control animals. A number of studies in recent years have shown the infiltration of eosinophils and the subsequent release of eosinophil-derived granular proteins and lipid metabolites to be central in the pathogenesis of allergic disease.⁷ Initially, we thought the decrease in eosinophil infiltration into the conjunctiva after IL-1Ra treatment was due to a modification in cytokine levels. A number of articles have stressed the importance of T_H2 cytokines, especially IL-4 and IL-5, in the induction of eosinophilia.⁷ However, although we did see an allergen-induced increase in the concentrations of IL-4 and IL-5 in

lymphocyte cultures from draining lymph nodes after allergen challenge, blockade of IL-1 did not significantly alter IL-4 and IL-5 cytokine production in this model (data not shown).

The traffic of eosinophils to the sites of allergic reactions is presumed to be regulated at several levels, including the expression of chemokines.⁸ Increased levels of RANTES, eotaxin, and MIP-1 α have been detected in the nasal secretions of atopic patients exposed to allergen challenge and have been reported to be increased in nasal washings of ragweed-sensitive subjects during the pollen allergen season.⁹ In this model of AC, several chemokines including RANTES and eotaxin are upregulated in the conjunctiva of sensitized mice after allergen challenge. Treatment with IL-1Ra substantially decreases the concentrations of these chemokines, both of which have been found to be important in eosinophil chemotaxis.⁸

Additionally, examination of chemokine profiles in the draining lymph nodes shows a substantial reduction in the

allergen-induced chemokines RANTES, MIP-1 α , eotaxin, and IP-10. All of these chemokines are thought to play an important role in the allergic inflammatory process, because they are chemotactic for activated T cells, eosinophils, basophils, and monocyte/macrophages.⁸ We do not contend that chemokine patterns in the draining lymph nodes directly influence cell traffic into the conjunctiva. However, the importance of lymph node chemokine data is underscored by the central role lymph nodes play in bringing together mature antigen presenting cells and naive T cells whose priming in the nodes leads to clonal proliferation of effector T cells.⁶ Accordingly, suppression of RANTES and MIP-1 α gene expression in the lymph node as a result of IL-1Ra treatment may affect immune responses by the down-modulation of the activation and eventual recruitment of T cells to the eye, which could, in turn, influence the infiltration of histamine-releasing cells such as eosinophils into the conjunctiva. Interestingly, the reduction in RANTES and eotaxin production was even more striking in the IL-1Ra-treated, sham-challenged groups in both the conjunctiva and the draining lymph nodes. This finding suggests that IL-1Ra was able to down-modulate both the endogenous and the antigen-induced gene expression of chemokines.

Interleukin-1 has been shown to regulate the expression of eotaxin in epithelial and endothelial cells in vitro,¹⁰ again suggesting a strong association between IL-1 production, eosinophil infiltration, and allergic disease. Unlike the more general effects of the other chemokines examined, eotaxin exclusively attracts eosinophils when applied in vivo, and its expression is enhanced in animal models of allergic inflammation and in tissue cells at sites of eosinophil accumulation. Because of this preferential and potent action on eosinophils and its occurrence in different species, eotaxin is considered perhaps the most relevant chemokine in the pathophysiology of allergic conditions and asthma.⁸ The major cellular sources of eotaxin are thought to be the epithelia and activated infiltrating leukocytes like eosinophils. The localization of eotaxin production is important not only to our understanding of the basic mechanisms of tissue eosinophilia and tissue damage but also to the design of drug delivery, which can now target the specific cells that generate the signal responsible for the selective recruitment of eosinophils.⁸ IL-1Ra treatment yielded a substantial decrease in eotaxin levels in conjunctiva and draining lymph nodes compared with allergen-challenged animals that received vehicle alone.

Conjunctiva tends to be a relatively fragile tissue to work with, particularly in small rodent species. It is for this reason that we were not able to extract chemokine proteins themselves from our murine samples. However, because we observed such a striking decrease in clinical symptoms as well as an ablation of eosinophil infiltration into the conjunctival matrix after IL-1Ra treatment, we propose that the differences in

mRNA assayed were biologically relevant and that they explain, at least in part, the observed striking suppression of clinical and histologic parameters of allergic eye disease.

The highly variable efficacy and myriad side effects of topical and systemic nonspecific antiinflammatory pharmaceuticals including corticosteroids are well known to clinicians who use these agents to arrest allergic disease. The observations implicating eosinophils in the pathogenesis of allergy make it likely that understanding the mechanisms of eosinophil recruitment into the conjunctiva will offer new therapeutic approaches for the treatment of this disease. Because chemokine production appears to be essential for the recruitment of eosinophils in the allergic response, strategies that block chemokine production may be effective in treatment of allergic disease. Using an in vivo model of allergic eye disease, we have shown that antagonism of IL-1 activity by the topical administration of IL-1Ra offers an effective means of suppressing eosinophilia and the resultant allergic response in the eye. Our data strongly suggest that specific molecular targeting strategies, such as the use of IL-1Ra to suppress IL-1 activity, may eventually offer a novel approach to the management of AC.

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