Inflammatory mediator release on conjunctival provocation of allergic subjects with allergen

David Proud, PhD,* James Sweet, MD, Paul Stein, PhD,*****
Russell A. Settipane, MD, Anne Kagey-Sobotka, PhD,***
Mitchell H. Friedlaender, MD,* and Lawrence M. Lichtenstein, MD, PhD***
Baltimore, Md., and La Jolla, Calif.

To evaluate the role of inflammatory mediators in the pathogenesis of the ocular allergic response, 23 subjects with positive histories of allergies to either cat dander or ragweed pollen and positive skin tests to the appropriate allergen extract were recruited and were subjected to conjunctival provocation. The tear duct of the left eye of each subject was blocked with a collagen plug while the right eye was left unplugged. In all cases, the eye was initially provoked with saline and subsequently with the appropriate allergen extract. Nonallergic subjects, or allergic subjects provoked with nonrelevant allergen, were used as control subjects. After each provocation, symptoms were recorded, and tears were collected with preweighed strips of filter paper (Schirmer strip). Each strip was placed into a tared tube containing fluid appropriate for the optimal preservation of the mediator to be measured. It was therefore possible to calculate the weight of tears collected and to express mediator levels per milliliter of tears. All allergic subjects demonstrated a positive symptomatic response to allergen challenge, whereas the control subjects remained asymptomatic. Blockage of the tear duct did not significantly alter the response. For allergic subjects, the levels of histamine, kinins, prostaglandin D_2, albumin, and TAME-esterase activity were all significantly ($p < 0.005$ in each case) greater after allergen challenge than after saline challenge. Furthermore, levels of each of these mediators after allergen challenge (expressed as increases above levels after saline provocation) were significantly greater for allergic subjects than for control subjects ($p < 0.005$ in each case). Thus, the clinical response to conjunctival provocation with allergen is associated with increases in the levels of inflammatory mediators in tears. (J ALLERGY CLIN IMMUNOL 1990;85: 896-905.)

The eye is a common target organ of the allergic response and is readily accessible to study. Despite this accessibility, however, there have been relatively few studies of the pathogenesis of the ocular allergic response, and the allergic manifestations of ocular hay fever have been monitored largely by subjective measures. Although Bisgaard et al.[1] have observed increased levels of immunoreactive leukotrienes in tear fluids after conjunctival provocation, and it has been reported that histamine levels in tear fluids are not elevated in ocular hay fever,[2] there has been no systematic attempt to relate the clinical response to allergen to the generation of inflammatory mediators. In other tissues, including the lung,[3-6] nose,[7-10] and skin,[11-14] the ability to determine the levels of mediators generated during the allergic response has not only provided a more objective means of monitoring

Abbreviations used

PGD_2, PGE_1, PGF:	Prostaglandin D_2, E_1, F_1
HPLC:	High-performance liquid chromatography
RIA:	Radioimmunoassay
LTC_4, LTD_4, LTE_4:	Leukotrienes C_4, D_4, E_4

From the *Division of Clinical Immunology, The Johns Hopkins University School of Medicine, Baltimore, Md., and **Division of Allergy and Immunology, ***Department of Molecular and Experimental Medicine, and Division of Ophthalmology, Scripps Clinic and Research Foundation, La Jolla, Calif.
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Reprint requests: David Proud, PhD, The Johns Hopkins Asthma and Allergy Center, Clinical Immunology Unit office 2, 301 Bayview Boulevard, Baltimore, MD 21224.
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the allergic reaction but has also increased our understanding of the pathogenesis of the allergic response in each tissue. Furthermore, the measurement of inflammatory mediators during pharmacologic intervention studies in these tissues has provided useful information regarding the mechanisms of actions of various antiallergic drugs.[15, 16] One particularly important observation to arise from studies of mediator generation during allergic events is that tissue-specific differences are observed. For example, while PGD_2 is generated concomitantly with histamine during the immediate allergic response in the upper airways,[7] this eicosanoid is not detectable until considerably later after antigen provocation in the skin and does not display the same time course of generation and persistence as histamine.[14] Such tissue-specific responses may result, in part, from the demonstrable heterogeneity of mast cells from different tissues[17-20] and clearly emphasize the importance of examining each target organ of interest, rather than trying to extrapolate from one tissue to another.

To evaluate the role of inflammatory mediators in the pathogenesis of the ocular response to allergen, we have therefore monitored the levels of histamine, kinins, PGD_2, sulfidopeptide leukotrienes, TAME-esterase activity, and albumin in recovered tear fluids from allergic and nonallergic subjects after conjunctival provocation with saline and, subsequently, with an appropriate allergen. We now report that a positive symptomatic response to allergen provocation is associated with significant increases in the concentrations of all of these parameters (with the exception of leukotrienes) in tears.

METHODS

Subjects

Healthy male and female volunteers between the ages of 18 and 55 years were recruited. No subjects with vernal or contact lens conjunctivitis were used. Allergic subjects were defined as individuals having a positive clinical history of allergic symptoms (not necessarily conjunctival) to an antigen to which they had a positive intradermal skin test (>6 mm wheal to 0.5 allergy units per milliliter or less of allergen extract). In the present study, 14 subjects (four male and 10 female subjects) were allergic to cat dander, and nine subjects (three male and six female subjects) were allergic to ragweed. The control population consisted of 19 nonallergic subjects (five male and 14 female subjects with no clinical history of allergic symptoms and negative skin tests to the allergens used) and three of the cat-allergic subjects who were exposed to a nonrelevant antigen (ragweed). Several subjects were challenged on more than one occasion. In such instances, challenges were separated by and the study protocol was approved by the Human Subjects Committee of the Scripps Clinic and Research Foundation.

Challenge procedure

Ragweed-pollen extract was obtained from Hollister-Stier Laboratories (Spokane, Wash.), and cat-hair and dander-allergenic extract were obtained from ALK America, Inc. (Milford, Conn.). On entering the laboratory, all subjects were examined to establish that no visible ocular symptoms were present at the time of challenge. The protocol then involved applying a collagen plug to block the tear duct in the left eye. A 40 μl drop of the normal saline diluent used for the allergen extracts was then administered to each eye. After a brief pause (approximately 5 minutes), symptoms were evaluated by the investigator, and tears were collected by placing a preweighed strip of filter paper (Schirmer strip) into the inferior fornix. After again determining that subjects were asymptomatic, a 40 μl drop of allergen extract was then administered into the eye. The concentration of allergen extract used was usually 30,000 to 50,000 times that required to elicit a threshold skin response. Thus, the actual dose of allergen administered to the eye was in the range of 500 to 1000 allergy units. In allergic subjects, symptoms usually developed within minutes after allergen challenge. Symptoms were graded by the investigator, and tears were collected as described above. Symptoms assessed were hyperemia, chemosis, and itching. Examinations included slit lamp evaluations. Each symptom was graded on a scale of 0 to 4 (i.e., the maximum possible score was 12) with 0 indicating no symptoms and 1, 2, 3, and 4 representing mild, moderate, moderately severe, and severe symptoms, respectively. Since each filter paper strip was used for the measurement of no more than two mediators (see below), several individuals were challenged on more than 1 day to allow the collection of sufficient data for each mediator. In a limited number of instances, an allergic subject was challenged with the protocol described earlier, but three separate filter paper strips were superimposed and then placed in the inferior fornix. The strips were then separated and placed into different tubes containing the appropriate solutions for each mediator assay. In this manner it was possible to demonstrate that the entire spectrum of mediators being assayed were all generated in the same sample of tear fluid.

Sample processing

Each preweighed filter paper strip was placed immediately into a preweighed tube containing 300 μl of the appropriate collection fluid for the mediators to be measured. Samples for histamine and TAME-esterase activity were collected into isotonic saline. Samples for kinins and albumin were collected into saline containing 40 mmol/L of ethylenediaminetetraacetic acid, whereas samples for PGD_2 and immunoreactive leukotrienes were collected into 95% ethanol. In each case, the tubes were weighed again immediately after placement of the filter strip into the tube

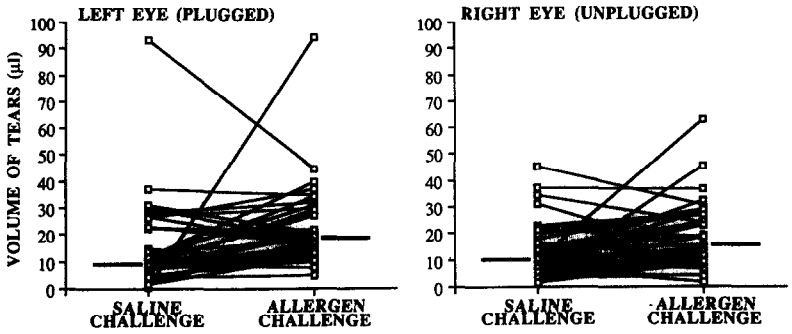

FIG. 1. Volume of tear fluids collected after challenge of collagen-plugged and unplugged eyes from the same allergic subjects. Tear volumes were determined by weight subtraction. *Horizontal bars* represent medians. Tear volumes were not different after saline challenge of the two eyes, and allergen challenge resulted in significant increases in tear weights in both eyes ($p < 0.02$ in unplugged eyes and $p < 0.005$ in plugged eyes). These allergen-induced increases above saline challenge were not different between the two eyes.

then stored at −80° C until assay. When samples were thawed, fluids for the determination of histamine, TAME-esterase activity, kinins, and albumin were aliquoted and assayed at appropriate dilutions. The samples for arachidonic acid metabolites were thawed, and the strips were removed. The samples were then centrifuged at 10,000 *g* for 5 minutes, and the supernatants were removed and concentrated to dryness with a vacuum concentrator (Savant Instruments, Inc., Hicksville, N.Y.). Each sample was then reconstituted with 0.5 ml of phosphate-buffered saline and assayed immediately for immunoreactive PGD_2 and LTC_4.

Mediator assays

Histamine was measured in 50 μl aliquots of fluid with a radioenzymatic assay sensitive to 50 pg/ml.[21, 22] This assay uses partially purified histamine *N*-methyl transferase from rat kidney and S-adenosyl-C-[^{3}H]-methyl-methionine (New England Nuclear/DuPont, Boston, Mass.) to convert histamine to [^{3}H]-methylhistamine, which is then isolated from other radiolabeled materials and quantified by liquid scintillation counting. A standard curve ranging from 50 pg/ml to 10,000 pg/ml was used in each experiment, and unknown values were determined by reading from the linear portion of the standard curve. The identity of measured histamine was confirmed by also assaying selected samples in a fluorometric assay[23] and by demonstrating that treatment with diamine oxidase destroyed the measured material.

Kinins were assayed with a competitive RIA sensitive to 20 pg/ml of bradykinin.[8] The antibody used does not discriminate, on a molar basis, between bradykinin, lysylbradykinin, and methionyllysylbradykinin. The only other materials known to cross-react in the assay are the human kininogens, which display <2% cross-reaction on a molar basis. The assay has inter- and intra-assay coefficients of variation that are both <5%. The identity of the kinins present in tear fluids after antigen provocation was deter-

0.025% triethylamine, pH 2.5 (solution A), kinins were eluted from the cartridge with 50% solution A per 50% solution B (solution B: 80% acetonitrile in solution A) and concentrated to 50 to 100 μl with a vacuum concentrator. Recovery of [^{3}H]-bradykinin with this method was 90%. Samples were centrifuged at 10,000 *g* for 2 minutes to remove any particulate material before application to the HPLC column. HPLC separation was achieved with a 3.9 by 300 mm μ Bondapak C_{18} column (Waters Associates) that was eluted at a flow rate of 1 ml/min with a mobile phase consisting of 76% solution A and 24% solution B. Absorbance of standard bradykinin, lysylbradykinin, and methionyllysylbradykinin was monitored at 210 nm. Fractions were collected and assayed at appropriate dilutions to confirm the immunoreactivity of eluted materials. To ensure that there was no carryover of standard kinins into tear-fluid samples, the sample loop was thoroughly washed with acetonitrile after each run, and a negative control run was performed before administration of tear samples. For all tear samples the identity of kinins present was determined by immunoassay of recovered fractions and comparison to the retention times of authentic kinins.

Enzymes that have arginine esterase activity were assayed according to the method of Imanari, et al.,[24] which is based on the liberation of tritiated methanol from the synthetic substrate ^{3}H-TAME. In brief, duplicate 40 μl aliquots of appropriately diluted samples are incubated with 10 μl of 0.2 mol/L of Tris buffer, pH 8.0, and 10 μl of ^{3}H-TAME for 60 minutes at room temperature. The reaction is then terminated, and tritiated methanol is partitioned into an organic scintillation fluid and quantified by liquid scintillation counting. Results are recorded as counts per minute above background (corrected for dilution). Although the enzymes responsible for the TAME-esterase activity in tears have yet to be identified, during the immediate allergic reaction in the upper airways, TAME-esterase activity is largely a reflection of the activities of a plasma kallikrein/α_2-

fidopeptide leukotrienes were measured with a previously described RIA.[28] The antibody used was raised against LTC_4, and cross-reactivity with LTD_4 and LTE_4 was 68% and 25%, respectively. The sensitivity limit of the assay is 200 pg/ml of LTC_4. PGD_2 was measured with an RIA capable of detecting 50 pg/ml of PGD_2.[29] The antibody used demonstrates <1% cross-reaction with PGE_1, PGE_2, thromboxane B_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$.

Statistical analysis

All data were corrected to be expressed as concentrations in original tear fluids (i.e., not diluted by the collection fluid) except TAME-esterase activity, which was expressed as counts per minute generated by a 40 μl aliquot of tears. Comparisons of mediator values after saline versus allergen challenges in each group of subjects were performed nonparametrically with the Wilcoxon matched-pairs sign-ranks test. This test was also used for comparing weights of tears, baseline mediator levels, and allergen-induced increases in mediators (above saline challenge) between plugged and unplugged eyes. The degree of association between mediator levels and symptoms was evaluated with Spearman's rank correlations. Across-group analysis (allergic subjects versus control subjects) of allergen-induced increases in mediators (above saline challenge) was also performed nonparametrically with the Mann-Whitney U test. Statistical significance was presumed to be achieved for values of $p < 0.05$. Statistical analysis was performed on a Macintosh computer (Apple Computer, Inc., Cupertino, Calif.) with Statview software (Brainpower, Inc., Calabasas, Calif.).

RESULTS

In our initial analysis of these data obtained in the present study, we evaluated the effects on conjunctival provocation of the use of collagen plugs to block the tear duct. Our purpose was to determine if quantitative recoveries of tear fluids and mediators could be obtained without occluding the tear duct. We analyzed the effects of collagen plugging on the weights of tear fluids recovered in all challenge experiments (n = 37 for allergic subjects and n = 26 for control subjects). In control subjects there was no significant difference in tear weights after saline challenge in plugged versus unplugged eyes (median tear weights, 16.7 mg and 14.4 mg, respectively). Furthermore, there was no significant increase in tear weights in either eye after allergen challenge of control subjects, and the postchallenge tear weights were not different between plugged and unplugged eyes (median, 14.8 mg and 12.3 mg, respectively). The tear weight data for allergic subjects are illustrated in Fig. 1. Again, no significant differences were observed in tear weights after saline provocation of plugged and unplugged

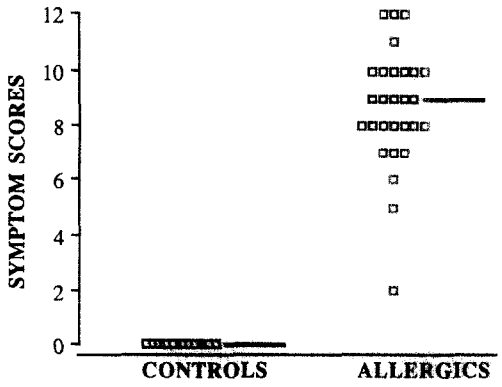

FIG. 2. Distribution of symptom scores after allergen challenge of allergic subjects. Symptom scores are reported on a scale of 0 to 12. The median is represented by a *horizontal bar.*

unplugged eyes ($p < 0.005$ for collagen-plugged eyes and $p < 0.02$ in unplugged eyes). Although postallergen-challenge tear weights were statistically different in the two eyes (median, 18.1 mg in plugged eyes and 14.3 mg in unplugged eyes; $p < 0.005$), the magnitude of the difference was modest, particularly in view of the inherent inaccuracies of the method for determining tear weight and appears unlikely to be of biologic significance. Moreover, when a comparison was made of the allergen-induced increases in tear weights above increases recorded for saline challenge in plugged and unplugged eyes, no significant effects of collagen plugging were observed.

Next, a comparison of mediator levels in plugged versus unplugged eyes of the same subjects was performed. No significant differences were observed in the levels of baseline mediators in plugged versus unplugged eyes. Similarly, allergen-induced mediator levels were analyzed in terms of mediator generation above that induced by saline provocation of the same subjects (n = 12 to 14 for each mediator in allergic subjects and n = 8 for each mediator in control subjects). No significant differences for plugged versus unplugged eyes were observed for any mediator in either allergic or control subjects. Taken in its entirety, we interpreted these data to indicate that collagen plugging of the tear duct had little or no effect on the response to allergen provocation. Accordingly, for all subsequent analyses, symptom score and mediator data from plugged and unplugged eyes were combined.

Administration of saline or allergen to the conjunctiva of nonallergic individuals failed to induce any detectable symptoms. Similarly, administration of a nonrelevant allergen to the eyes of allergic subjects

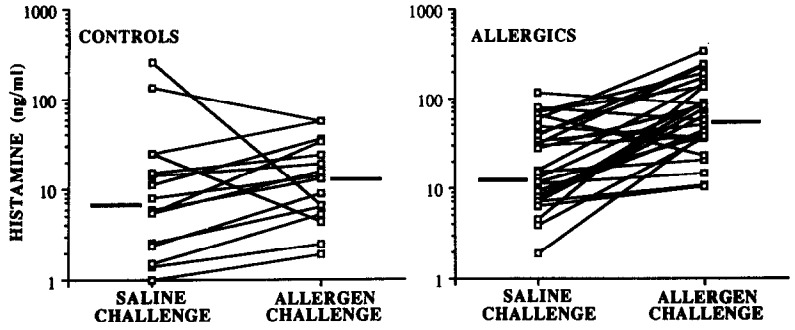

FIG. 3. Histamine levels in tear fluids from allergic and control subjects. Median levels are demonstrated by *horizontal bars.* Histamine levels increased significantly after allergen challenge of allergic subjects ($p < 0.001$) but not control subjects. Histamine levels after saline challenge were also significantly elevated in allergic subjects compared to control subjects ($p < 0.05$).

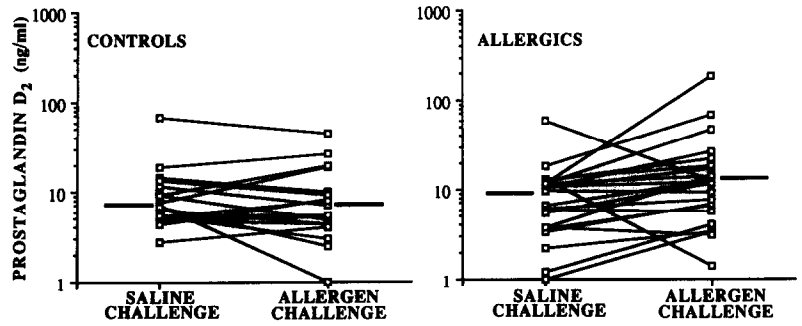

FIG. 4. PGD_2 levels in tear fluids from allergic and control subjects. Median levels are illustrated by *horizontal bars.* Levels after saline challenge are not different for allergic and control subjects. Allergen challenge induced a significant increase in PGD_2 levels in allergic subjects ($p < 0.005$) but not in control subjects.

allergen led to itching, chemosis, and hyperemia in all subjects tested. Although there was some variation in the responses observed (Fig. 2), the median composite symptom score of 9 was consistent with a moderately severe clinical reaction.

While histamine levels (Fig. 3) were not significantly affected after allergen challenge of control subjects when levels were compared to levels measured after saline provocation (median, 13.6 ng/ml versus 6.9 ng/ml, respectively), significant elevations ($p < 0.001$) were observed for allergic subjects (median, 60 ng/ml after allergen versus 15.6 ng/ml after saline). Interestingly, the concentration of histamine detected after saline challenge of allergic subjects was also significantly elevated ($p = 0.048$) compared to that of control subjects. This was the only mediator for which saline challenge induced a significant difference between allergic and nonallergic subjects.

mediator in tear fluids (Fig. 4). No effect of allergen provocation was observed in control subjects (median, 7.0 ng/ml after allergen versus 6.2 ng/ml after saline), but a significant effect was observed for allergic subjects (median, 12.3 ng/ml after allergen versus 9.6 ng/ml after saline; $p < 0.005$). In contrast to PGD_2, immunoreactive leukotrienes were not significantly increased after challenge of either control (median, 5.3 ng/ml after allergen challenge versus 4.6 ng/ml after saline) or allergic (median, 12.7 ng/ml after allergen versus 7.3 ng/ml after saline; $p = 0.073$) subjects (Fig. 5).

Changes in vascular permeability were also observed in response to allergen provocation (Fig. 6). Although a slight increase in the concentration of albumin relative to saline provocation was noted in control subjects (median, 1.4 mg/ml versus 0.6 mg/ml; $p < 0.05$), perhaps signifying some irritant effect of

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