

Mediator measurements after daily instillation of allergen: Increased IL-5 and decreased IFN-gamma

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ABSTRACT

This study was designed to measure symptoms, IL-4, IL-5, IFN-gamma, and eosinophilic cationic protein (ECP) in nasal secretions from subjects experiencing an artificial allergy season and to look for evidence of priming. Clinically relevant allergen was administered intranasally out of season to 12 asymptomatic individuals with seasonal allergic rhinitis. These individuals were then randomized to receive allergen or saline daily for the next 7 days. Nasal secretions and scrapings of nasal epithelium were obtained at baseline (day 1), 24 hours after the initial allergen administration (day 2), and 24 hours after the last instillation of allergen or saline (day 9). Nasal symptom scores ($p < 0.0002$), IL-5 mRNA ($p = 0.03$), and ECP ($p < 0.02$) increased after receiving the first challenge (day 2 compared with day 1). In the six subjects randomized to receive seven sequential daily challenges with allergen, symptom scores remained elevated ($p < 0.02$), IL-5 protein increased ($p = 0.02$), and IFN-gamma ($p = 0.02$) levels decreased (day 9 compared with day 1). In the six subjects randomized to receive seven sequential daily challenges with placebo, symptom scores, IL-5, and IFN-gamma levels were not significantly different (day 9 compared with day 1). Compared with the findings at day 2 ($n = 12$), the treated subjects ($n = 6$) had no further increase in symptoms but did show a further increase in IL-5 ($p = 0.01$) and a decrease in IFN-gamma ($p = 0.02$) at day 9. Daily instillation of moderate doses of allergen intranasally is characterized by persistent symptoms, elevation of IL-5, and reduced levels of IFN-gamma.

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Key words: Allergen challenge, cytokines, eosinophilic cationic protein, inflammation, mediators, nasal challenge, priming effect, rhinitis

Allergic rhinitis is estimated to be present in 15–20% of the United States population and continues to increase.^{1,2} This chronic condition has a negative impact on quality of life and often patients are embarrassed or frustrated by their ongoing symptoms.³ Although treatment for allergic rhinitis has improved over the last 10 years, many questions remain unanswered regarding the pathophysiological mechanisms of this disease.

Much of our current understanding of the inflammatory mediators important in allergic rhinitis comes from studies using nasal provocation challenges. Subjects receiving suprathreshold doses of allergen in a nasal challenge model have measurable increases in proinflammatory cytokines in their nasal secretions and increased levels of mRNA (messenger RNA) of proinflammatory cytokines in nasal epithelium and/or nasal mucosa.^{4–6} Nasal secretions can be collected on

filter paper after subjects have undergone nasal provocation and increased levels of IL-1 α , IL-5, IL-6, IL-8, CM-CFS, RANTES, and MIP-1 α have been shown with this method 3–8 hours after challenge.⁵

A model for the production of an “artificial season” has been described in which subjects are challenged with modest amounts of inhalant allergens on a daily basis and experience substantial symptoms of allergic rhinitis.⁷ This model is more reflective of a true allergy season than is the single challenge with a large dose of allergen.

The priming effect is a well-known phenomenon first described by Connell in 1968 and is characterized by an increase in nasal congestion after repeated exposure to allergen.^{8,9} Increased levels of histamine, kinins, and TAME-esterase after repeated exposure to allergen have been reported.¹⁰

In this report, we describe the clinical and immunologic response of 12 subjects in whom the dose of relevant allergen necessary to produce moderate nasal symptoms was determined. They then were randomized to receive daily instillation of either the predetermined dose of allergen or saline. After the initial challenge, there were changes in several parameters of inflammation. Those receiving daily allergen had persistence of symptom scores, further increase in IL-5, and decrease in IL-4, whereas most subjects receiving saline had a return to baseline.

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METHODS

Subjects

Twelve subjects (six men and six women; 34 ± 12 years old) with a history of seasonal allergic rhinitis and known sensitivity to ragweed or grass allergen as documented by previous skin testing were studied out of season. At baseline, all subjects were, at the time studied, free from symptoms suggestive of allergic rhinitis. Subjects were excluded if they had used any nasal corticosteroid preparations or oral antihistamines within 1 month of the study. Institutional Review Board approval was obtained from the National Jewish Medical and Research Center and the University of Colorado Health Sciences Center. All subjects provided informed consent.

Study Design

On day 1, baseline samples were obtained before challenging subjects with allergen as described in the next section. On day 2, samples were again collected and the subjects were randomized into two separate groups. One group received seven additional daily instillations of allergen at the dose that produced the initial symptomatic response for a total of 8 days. The other group also received seven additional daily instillations but with saline/phenol alone. Excipients were the same in both the allergen group and the saline group (phenol saline). The same lot of allergen was used throughout the study to ensure equal potency. Samples were again collected on day 9, 24 hours after the last instillation of allergen or placebo.

Titration of Intranasal Allergen

One hundred microliters of velvet grass or ragweed extract were administered intranasally at increasing concentrations (ranging from 0.2 to 2000 PNU/mL) at 10-minute intervals until nasal congestion or rhinorrhea was reported as moderate or severe as measured by a score of ≥ 2 on a scale of 0–3 or 5 or more sneezes occurred. The dose at which this threshold was reached was used as the challenge dose throughout the study. This scoring system was used to determine the dose used throughout the study and replicates that described by Anderson *et al.*⁷

Symptom Scores

Daily symptom scores were recorded by subjects on diary cards in the morning and in the evening reflecting the previous 12 hours and were reviewed on a daily basis with the study coordinator. Sneezing was scored using a scale of 0–2, where 0 = no sneezes, 1 = 3–4 sneezes, and 2 = 5 or more sneezes. Nasal congestion was scored on a scale of 0–3, where 0 = no blockage, 1 = breathe with some difficulty, 2 = one

nostril blocked, and 3 = both nostrils blocked. Rhinorrhea was scored on a scale of 0–3, where 0 = no rhinorrhea, 1 = anterior drainage, 2 = posterior drainage, and 3 = both anterior and posterior drainage. Pruritus was scored as present or absent (0 or 1). The maximal total symptom score for each 24-hour period was derived by the sum of the morning score (9) plus the evening score (9) for a possible maximal score of 18. This scoring system was used at baseline (day 1) and daily thereafter and was used in all statistical analysis and data presented.

Collection of Samples

Collection of samples was performed on all subjects on day 1 (baseline), day 2, and day 9. Three filter papers (Whatman No. 42; 7×30 mm) were placed in the anterior nares and were allowed to become saturated.⁴ The saturated filter papers were allowed to dry at room temperature and were stored in microfuge tubes at -70°C for later analysis. After collection of nasal secretions, nasal scrapings were obtained by scraping the inferior aspect of each inferior turbinate twice with a Rhino-probe (Arlington Scientific, Springville, UT).⁶ The samples were immediately immersed in RNAlater (Ambion, Austin, TX) and stored at 4°C . Nasal challenge with allergen or saline was performed within 5 minutes after collection of samples.

Recovery and Assay of Cytokines

Cytokines were eluted from the filter papers using a modification of the technique described by Sim *et al.* except that to improve recovery of cytokines, bovine hemoglobin was substituted for bovine albumin (data not shown). With this buffer, the recovery of cytokines was $58 \pm 9\%$ for IL-4, $72 \pm 8\%$ for IL-5, and $31 \pm 8\%$ for IFN- γ . At each time point, nasal secretions absorbed onto the six filter papers were eluted into a final volume of 1 mL. Immediately after samples were eluted, assays for IL-4, IL-5, and IFN- γ protein were performed using ELISA kits obtained from BD PharMingen (San Diego, CA). The eosinophilic cationic protein (ECP) ELISA kit was purchased from Medical and Biologic Laboratories (Nagoya, Japan). The assays were performed as recommended by the companies except that the standard curves were extended by prolonging the time of color development from the recommended 30 minutes to 60 minutes. This gave increased sensitivity with possible loss of specificity.

Recovery and Assay of mRNA

Nasal scrapings were analyzed for relative amounts of mRNA for IL-4, IL-5, and IFN- γ using real-time fluorescent quantitative polymerase chain reaction with predesigned Taqman primers and probes as previously described.⁶

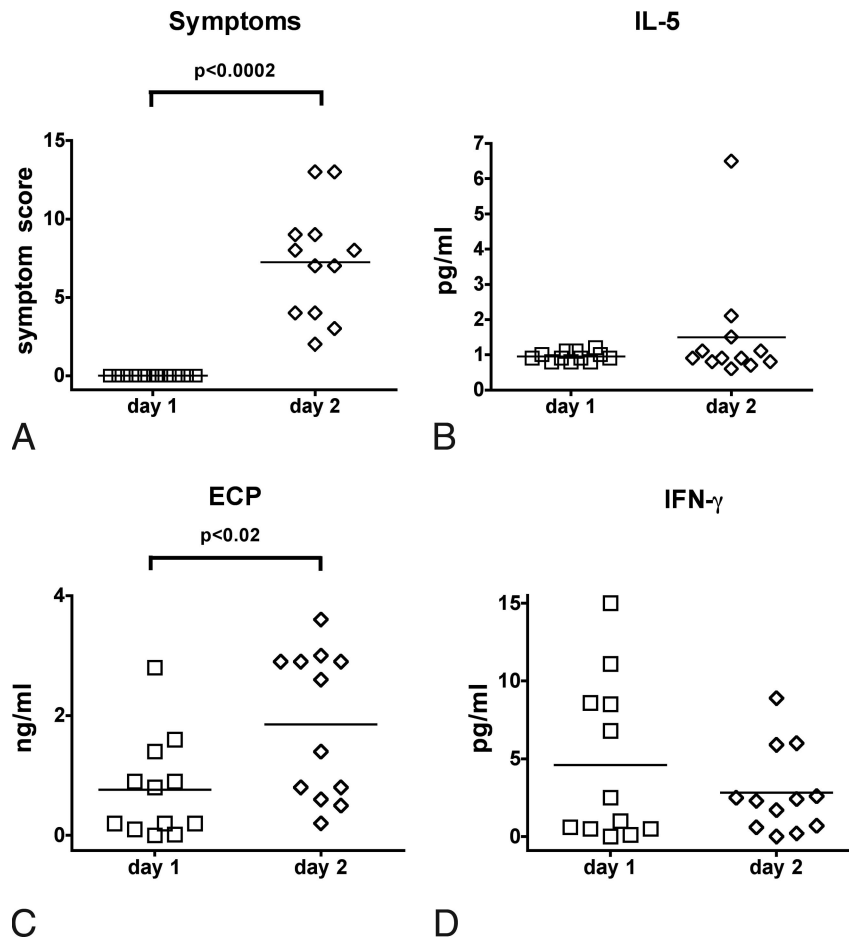


Figure 1. Symptom scores and mediators on day 2 compared with day 1. On day 1, samples were obtained before instillation of allergen (open squares). Day 2 is 24 hours after allergen challenge (open diamonds). The bars represent mean values. Significant differences are indicated. (A) Symptom scores; (B) IL-5; (C) ECP; (D) IFN- γ . Absolute values (mean $\pm$ SD; $n = 12$) were symptom scores, 0.0 on day 1 and 7.3 ± 3.6 on day 2; IL-5, 0.95 ± 0.13 on day 1 and 1.5 ± 1.6 on day 2; ECP, 0.76 ± 0.84 on day 1 and 1.8 ± 1.2 on day 2; IFN- γ , 4.6 ± 5.2 on day 1 and 2.8 ± 2.7 on day 2.

Statistics

Data obtained in placebo and active groups were compared at day 1 (baseline), day 2, and day 9. Data from the active group at day 9 also were compared with the data from the entire group at day 2. Data for mediators on day 2 compared with day 1 are presented as concentrations of cytokines in the final volume of eluate. For further comparisons (days 2 and 9), the data are presented as "fold increase" compared with baseline (day 1). This compensated for variability among subjects at baseline. Statistical comparisons with baseline values were made using a two-tailed Wilcoxon signed rank test if baseline values were all the same value. Other comparisons were analyzed with a two-tailed, unpaired t -test. Values of $p < 0.05$ were considered statistically significant. All analyses were generated with GraphPad Prism version 4.0b software (GraphPad Software, San Diego, CA).

RESULTS

Effect of Initial Challenge

At baseline (day 1), all 12 subjects were without symptoms. Twenty-four hours after titration to an effective dose of allergen (day 2), there was a significant increase in symptom scores ($p < 0.001$) and ECP levels ($p < 0.02$) compared with baseline (Fig. 1, A and C). There were no significant changes in levels of protein for IL-5 or IFN- γ (Fig. 1, B and D) or for IL-4 (data not shown). The relative amount of mRNA for IL-5 was increased (median, 2.45-fold increase; $p = 0.002$; data not shown). Levels of mRNA for IL-4 and IFN- γ were not significantly changed (data not shown).

Effect of Sequential Daily Challenges Compared with Baseline

Subjects who received allergen on day 1 and then placebo for the next 7 days did not have a significant

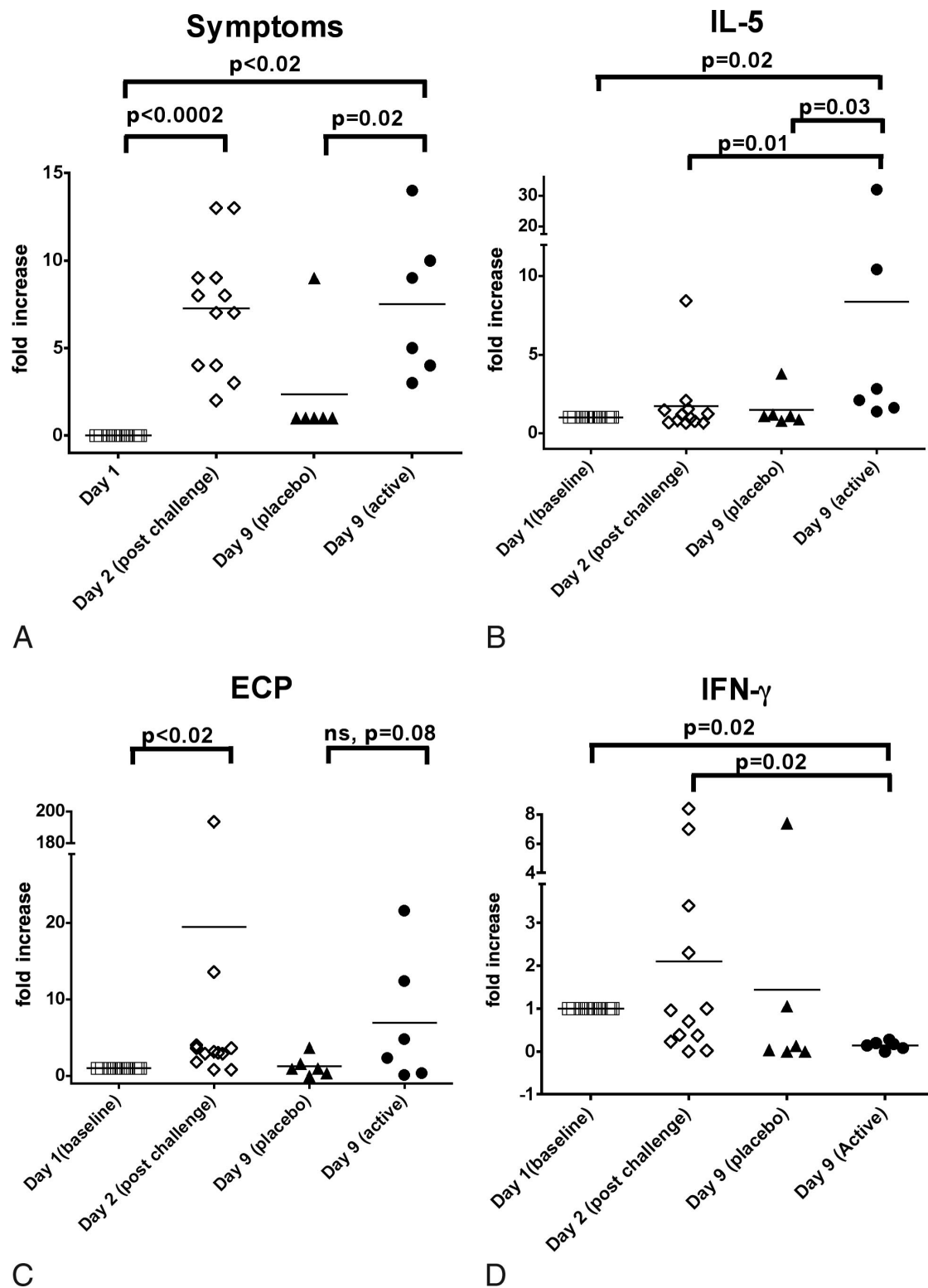


Figure 2. Changes in symptom scores and mediators on days 2 and 9 shown as “fold-change” compared with day 1. On day 1, samples were obtained before instillation of allergen (open squares). On day 2, samples were obtained 24 hours after initial allergen challenge (open diamonds). Day 9 is 8 days after the single instillation of allergen and 24 hours after the last of seven additional daily instillations of placebo (closed triangles) or allergen (closed circles). Bars represent mean values. Significant differences are indicated. Absolute values (mean $\pm$ SD; $n = 6$) in the placebo group: Symptom scores, 1.50 ± 3.67 on day 9; IL-5, 1.5 ± 1.1 on day 9; ECP, 1.24 ± 1.31 on day 9; IFN- γ , 1.43 ± 2.95 on day 9. Absolute values (mean $\pm$ SD; $n = 6$) in the active group: symptom scores, 7.50 ± 4.23 on day 9; IL-5, 8.4 ± 12 on day 9; ECP, 6.9 ± 8.5 on day 9; IFN- γ , 0.15 ± 0.09 on day 9. Absolute values for days 1 and 2 are given in the Fig. 1 legend.

elevation in symptoms or levels of inflammatory mediators at day 9 compared with baseline (day 1; Fig. 2 A). Those who received allergen on day 1 and continued to receive allergen for the next 7 days had elevated symptom scores ($p < 0.01$; Fig. 2 A), elevated IL-5 protein ($p = 0.02$; Fig. 2 B), and decreased IFN- γ levels ($p = 0.02$; Fig. 2 D), compared with baseline (day 1). When compared with the placebo group, the group receiving sequential allergen challenges had increased symptoms ($p = 0.02$; Fig. 2 A) and elevated levels of IL-5 ($p = 0.03$; Fig. 2 B). mRNA data beyond day 2 did not show statistical significances (data not shown).

Effect of Sequential Daily Challenges Compared with the Effect of One Challenge

To determine if sequential daily challenges with allergen results in a priming effect, we compared symptoms and mediator levels at day 9 with those at day 2. In five of the six subjects who received a total of seven daily instillations of placebo after the initial allergen challenge, symptom scores that were elevated at day 2 returned to baseline by day 9 (Fig. 2 A). In all of the six subjects who continued to receive daily instillations of allergen, symptom scores remained elevated on day 9 but there was no further increase in symptom scores compared with day 2 (Fig. 2 A). In the placebo group, mediator levels (Fig. 2, B–D) returned to near baseline levels on day 9 compared with day 2. For the active group there was no further increase in ECP on day 9 compared with day 2, but there was a significant further increase in IL-5 ($p = 0.01$; Fig 2 B) and a decrease of IFN- γ ($p = 0.02$; Fig. 2 D). There also was an increase in the IL-5/IFN- γ ratio on day 9 compared with day 2 for the active group ($p = 0.006$; data not shown).

Correlative Tests of Symptoms and Immunologic Data

Symptom scores were examined for correlations with each immunologic parameter. At day 2 ($n = 12$) and at day 9 (active group, $n = 6$), no correlations reached significance. The best correlation was between symptom scores and the ratio of IL-5 to IFN- γ on day 2 (Spearman $r = 0.52$; $p = 0.08$; data not shown).

DISCUSSION

Symptom scores and IL-5 protein levels in nasal secretions were increased and IFN- γ levels in nasal secretions were decreased in subjects receiving eight sequential daily intranasal instillations of moderate doses of allergen (Fig. 2). Although increases in proinflammatory cytokines after nasal provocation have been shown in previous investigations,^{4,11–16} those studies relied on single instillations of large quantities of nasal allergen administered over a short period of time. Intranasal immunotherapy also has been pro-

posed as an effective way to desensitize individuals with allergic rhinitis to pollens. The doses of allergen extract in this study were chosen based on inducing symptoms versus small incremental dose increases over an average of 36 days in nasal immunotherapy as a means of desensitization. We chose to study daily instillations of moderate doses of allergen as proposed by Anderson *et al.* as a model of an allergy season.⁷ Moderate quantities of allergen administered over several days may more accurately reflect the physiological response to allergen encountered in the environment than does a single nasal allergen provocation with a large allergen dose.

One day after initial allergen challenge (day 2), subjects in this investigation experienced increased symptoms and had increased levels of ECP and mRNA for IL-5. There was no significant increase in IL-5 protein or significant decrease in IFN- γ protein on day 2 compared with day 1 (Fig. 1). However, in the group receiving eight daily instillations of allergen, significant changes in the protein levels for each of these cytokines were apparent on day 9 compared with day 1 (Fig. 2). To further test the hypothesis that sequential daily administration of allergen leads to augmented symptoms and/or expression of nasal cytokines, we compared findings at day 9 with those on day 2 (Fig. 2). Subjects randomized to receive daily allergen did not have a further increase in symptom scores due to sequential instillation of allergen but did have a demonstrable increase in IL-5 and decrease in IFN- γ when comparing day 2 versus day 9 (Fig. 2). These data do not establish clinically significant priming but are suggestive that priming has occurred at an immunologic level. These statistical comparisons are summarized in Table 1.

There are several possibilities why priming of symptoms did not occur. Although this model of an artificial allergy season may be more representative of a true allergy season than a single nasal instillation of aller-

Table 1 Summary of p values from Figs. 1 and 2

	Symptoms	IL-5	ECP	IFN- γ
Day 2 vs day 1	<0.0002	NS	<0.02	NS
Day 9 (active) vs day 1	<0.02	0.02	NS (0.08)	0.02
Day 9 (placebo) vs day 1	NS	NS	NS	NS
Day 9 (active) vs day 2	NS	<0.01	NS	0.02
Day 9 (placebo) vs day 2	<0.02	NS	NS	NS
Day 9 (active) vs day 9 (placebo)	0.02	NS	NS	NS

gen, it has limitations. Administration of moderate quantities of allergen only once a day may not have the same physiological effect of continuous or intermittent exposure to allergen encountered in the environment. It is possible that priming of symptoms may have been observed if subjects were required to administer allergen on more than one occasion per day. Furthermore, the level of initial symptoms chosen for determination of the dose of allergen could have been too high because the subjects all reached 40% of their maximal symptom score of 18. Perhaps a lower level of initial symptoms is necessary to see a clinically significant priming effect. Finally, allergen administration and sample collection typically took place in the morning when endogenous cortisol levels are at their peak and this may have adversely affected the results. Pipkorn *et al.* showed dampening of the priming effect in the late-phase inflammatory mediator response with administration of systemic glucocorticoids.¹⁰

CONCLUSIONS

Daily administration of moderate levels of allergen leads to a measurable immunologic response that may be a good model for a naturally occurring allergy season. Increasing nasal symptoms characteristic of priming were not observed although immunologic activation suggestive of priming was present.

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