

Systemic effects of allergen exposure on blood basophil IL-13 secretion and FcεRIβ

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Background: Airway allergen challenge studies have shown the upregulation of cytokines in local airway tissues and distal effects on bone marrow precursors for eosinophils and basophils.

Objective: We investigated whether local intranasal allergen challenge alters the phenotype of circulating basophils to a primed state.

Methods: Ten subjects with allergic rhinitis were challenged with allergen by means of intranasal spray on 3 sequential days. Basophils were isolated from subjects before challenge and 3, 24, and 96 hours after the third allergen challenge. Basophils were compared before challenge and after the last allergen challenge for levels of FcεRIβ protein by means of Western blotting and for FcεRIβ mRNA expression by means of real-time PCR. Basophils were also compared with regard to spontaneous secretion of IL-4 and IL-13.

Results: Basophil FcεRIβ protein levels increased in 5 of 6 subjects after allergen challenge relative to before challenge. Likewise, basophil FcεRIβ mRNA levels increased a median of 2-fold after the last challenge relative to before challenge ($P = .007$, $n = 9$). IL-13 protein was detected in supernatants of 7 of 9 subjects' basophil-enriched cultures after the last challenge compared with 3 of 9 basophil-enriched cultures before challenge (median, 6.2 vs 0 pg/mL; $P = .058$). IL-4 was not detected in any culture supernatant.

Conclusion: Intranasal allergen challenge transiently activates circulating basophils by increasing expression of the FcεRIβ subunit and spontaneous IL-13 secretion. Because FcεRIβ is an amplifier of FcεRI-mediated responses and IL-13 is proinflammatory, these findings support a primed basophil functional state and demonstrate a systemic effect of local allergen challenge that could contribute in exacerbating allergic reactions. (*J Allergy Clin Immunol* 2004;114:768-74.)

Key words: IgE receptors, basophils, IL-13, allergen challenge, FcεRIβ

Abbreviation used

NGF: Nerve growth factor

Respiratory allergy is primarily characterized by local manifestations. There is increasing evidence, however, that once local reactions take place and local symptoms are induced, the ensuing inflammatory cascade assumes both a local and a systemic nature. The latter might then act to intensify the overall disease process. This belief, in fact, has prompted the identification and characterization of such systemic manifestations resulting from allergic disease, with an attempt to better understand the organ-specific, cellular, and molecular mechanisms involved.¹

Although not widely appreciated, the circulating basophil has long provided correlates of allergic disease, with many comparisons being linked to increased releasability of histamine.² Such evidence was first demonstrated in an early study showing that the histamine released by a patient's basophils challenged *in vitro* with ragweed allergen could predict the severity of the respiratory symptoms that the individual would experience during the ragweed season.³ After this observation, subsequent studies showed that the basophils of some asthmatic subjects spontaneously release histamine *in vitro* and that increased releasability could be demonstrated by using various stimuli, both physiologic and nonphysiologic.⁴⁻⁶ The spontaneous release of histamine *in vitro* has been particularly evident in children with food allergies.⁷ More recent studies have extended the concept of increased releasability to include cytokine secretion, showing that basophils from allergic subjects secrete greater levels of IL-13 in response to IL-3 or nerve growth factor (NGF) than do basophils obtained from nonallergic subjects.⁸ Finally, there is evidence that phenotypic markers, including adhesion- and activation-linked molecules, are also increased on the basophils of allergic subjects, with the most recent example being CD69, possibly through IL-3 exposure.⁹ From these studies, it seems evident that there is a systemic activation or priming of basophils in individuals presenting with clinical allergic disease.

In addition to enhancing CD69 surface expression and IL-13 secretion by basophils, it has recently been shown that *ex vivo* IL-3 exposure can selectively increase basophil expression of FcεRIβ and increase basophil sensitivity and mediator responses to allergen.¹⁰ It had

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been demonstrated that FcεRIβ functions as an amplifier of signal transduction pathways initiated through FcεRI¹¹ and also amplifies surface levels of IgE receptor expression.¹² Although basophil FcεRIβ levels have been shown to be variable among subjects,¹⁰ it is unknown whether *in vivo* allergen exposure can modulate the expression levels of FcεRIβ.

Allergen challenge of nasal tissues have been shown to increase expression of mRNA for T_H2 cytokines, such as IL-3, IL-4, and IL-5¹³; however, the role that allergen exposure plays in the increased releasability, priming, or both of basophils is an issue that has not been fully addressed. Denburg¹⁴ has performed several studies showing that responses in the bone marrow follow allergen challenge, with changes resulting in the increased release of a common progenitor for both basophils and eosinophils. At least one study has also reported increased histamine release from blood basophils prepared from subjects 24 hours after undergoing experimental allergen challenge in the lung. However, only minor priming effects were observed, as indicated by thapsigargin-induced histamine release.¹⁵

Given the evidence for modification of basophil function through cytokines and *in vivo* evidence of heightened basophil releasability in allergic subjects, we investigated whether local allergen led to a systemic effect on circulating basophils. In the present study we explored the effect of allergen exposure using an intranasal allergen challenge protocol involving 3 consecutive-day challenges. Our results support a systemic manifestation of intranasal allergen exposure by showing that IL-13 is spontaneously secreted in basophil suspensions prepared 1 day after the third allergen challenge. As a separate measure of blood basophil activation, we also noted enhanced expression of mRNA and protein for the FcεRIβ subunit of the high-affinity IgE receptor complex. Taken together, our results support the hypothesis that intranasal allergen challenge leads to a systemic effect on circulating basophils that is consistent with a primed or activated basophil phenotype.

METHODS

Subjects

Human subjects with seasonal allergic rhinitis were enrolled in the allergen challenge protocol after informed consent was obtained, as approved by the Johns Hopkins Bayview Institutional Review Board. The subjects had seasonal rhinitis symptoms and were sensitive to either ragweed or timothy grass allergen (Greer Laboratories, Lenoir, NC), as determined by means of standard allergen puncture skin testing. Subjects underwent challenge outside of their allergen season and during a time period when their nasal symptom scores were at baseline. Venous blood was sampled before the instillation of nasal allergen and at time points subsequent to the last allergen challenge (Fig 1).

Allergen challenge protocol

Allergen application used escalating doses of 10, 100, and 1000 protein nitrogen units of total ragweed antigen (Greer Laboratories)

or 25, 250, and 2500 biologic allergen units of total timothy grass antigen (Greer Laboratories) sprayed intranasally in both nares for 3 sequential days. As a control, 3 subjects underwent nasal challenge with saline alone, as per the allergen challenge protocol. Blood sampling occurred on day 1 before the first nasal allergen challenge and at 3, 24, and 96 hours after the third allergen challenge. For simplicity, day 1 samples will be referred to as prechallenge samples, day 3 to 4 samples will be referred to as postchallenge samples, and day 7 samples will be referred to as resolution samples (Fig 1). The results reported for the postchallenge time point represent the peak value detected at either the 3- or 24-hour time points for the given assay.

Cell isolation

For experiments of FcεRIβ mRNA expression and cytokine secretion, basophil-enriched suspensions were prepared from venous blood by using a double Percoll (1.072/1.081 g/mL) density centrifugation protocol.¹⁶ All cell suspensions of greater than 5% basophils after density centrifugation were adjusted back down to 5% basophils by adding mononuclear cells (lymphocytes and monocytes) from the 1.072 g/mL Percoll interface. This was done to maintain an equal number of basophils and total cells, both for RNA extraction and for cytokine secretion (see below). Because the mononuclear cells floating on the 1.072 g/mL Percoll interface contained less than 0.5% basophils, they were also used as control cultures to assess IL-13 secretion for comparison with that produced in the basophil-enriched suspensions.

In experiments involving FcεRIβ protein determination by means of Western blotting, basophils were further enriched by means of negative selection with a basophil isolation kit (Stem Cell, Vancouver, Canada) in conjunction with an LS columns and a Mini MACS magnet (Miltenyi, Auburn, Calif), as previously described.¹⁰ Basophil purities were greater than 50%, with an average purity of 80% ± 5% (mean ± SEM), as determined by means of Alcian blue staining.¹⁷

Western blotting

Total cell lysates from 5×10^5 basophils were loaded per time point (before allergen challenge and 24 hours after the third allergen challenge). Paired lysates for each subject were examined in the same blot as before.¹⁰ Immunoblotting used a rabbit polyclonal antibody anti-FcεRIβ (UBI, Saranac, NY) and anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Pharmacia Biotech, Uppsala, Sweden). Intensities of detected protein bands were measured with a Kodak DC120 digital camera and National Institutes of Health Image software.

RNA extraction–quantitative RT-PCR

Total RNA was extracted from 5×10^6 leukocytes, of which 5% (approximately 250,000) were basophils, as determined by using the RNazol method (Tel-Test, Inc, Friendswood, Tex). Previous studies support that basophils are the sole source of FcεRIβ mRNA in such leukocyte preparations.¹⁰ RNA samples were subjected to a 1-step reverse transcription and real-time PCR analysis with specific primers to the human FcεRIβ by using the Taq Man kit and a 7700 Prism thermocycler (Applied Biosystems, Foster City, Calif). Reaction conditions were as follows: RT at 48°C for 30 minutes followed by PCR amplification (95°C for 10 minutes; 40 cycles of 95°C for 15 seconds and 60°C for 1 minute). The number of cycles to reach an equivalent number of specific mRNA copies was determined. Pilot studies demonstrated that 2-fold dilutions of a preparation of FcεRIβ mRNA resulted in a 1.02 ± 0.15 difference in cycle number for

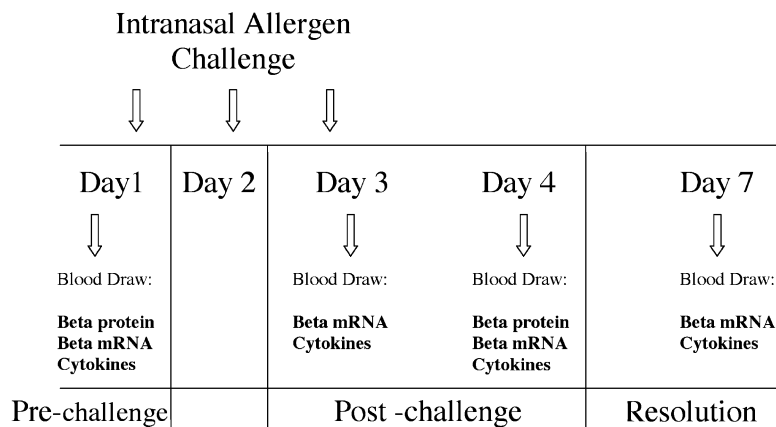


FIG 1. Protocol for intranasal allergen administration and blood sampling. Enrolled subjects underwent graded intranasal application of either ragweed or grass allergen on 3 sequential days outside of the normal allergen season. Blood sampling occurred at the indicated time points for basophil studies.

each 2-fold dilution. Primer sets were as follows: FcεRIβ, 5'-CCA-GGA-AGT-ATC-TTC-AGG-CAG-ACT-3' and 5'-TCA-AAA-CTG-TCA-GCC-ATG-TAT-GC-3'; internal reporter construct, 5'-FAM-TTG-AAG-TCG-GCC-TCA-TCC-CCA-CC-TAMRA-3'.

Cytokine measurements

Both basophil-depleted (<0.5% purity) and basophil-enriched (5% purity) cell suspensions were cultured in 96-well microtiter wells at equal cell concentrations in 0.200 mL of Iscove modified Dulbecco medium containing 5% FCS, nonessential amino acids, and 10 μg/mL gentamicin. After 18 hours of incubation, the cell-free supernatants were harvested, frozen at -70°C, and then assayed by means of ELISA for both IL-13 (Immunotech, Marseille, France) and IL-4 (Biosource, Camarillo, Calif). All leukocyte culture supernatants collected for a given subject after each blood draw were assayed simultaneously to best control cytokine measurements. Limits of detection for the assays were less than 2 pg/mL supernatant. In addition, measurements of IL-3 in plasma were attempted by using an ELISA (Biosource).

RESULTS

Adverse events of intranasal allergen challenge

Ten subjects underwent the intranasal allergen challenge protocol, and 4 subjects experienced an adverse event possibly or probably related to the allergen challenge. Two subjects reported a sore throat after the first allergen challenge (1 subject had unilateral palatal swelling that resolved within 1 hour after treatment with oral decongestant), 1 subject reported a headache after the first and third allergen challenges that resolved with ibuprofen, and 1 subject reported a cough after the second allergen challenge without any evidence of shortness of breath or wheezing. In all cases the symptoms did not progress beyond the same day and resolved spontaneously or with over-the-counter medication.

Effect of intranasal allergen challenge on basophil FcεRIβ mRNA expression

We initiated these studies with the hypothesis that nasal allergen challenge would induce priming effects in blood basophils. Previously, we have shown that FcεRIβ mRNA expression levels are enhanced by cytokines that prime basophils for mediator release (ie, IL-3 and IL-5).^{10,18} We therefore assessed levels of FcεRIβ mRNA in enriched blood basophil samples obtained from subjects before challenge, after challenge, and at resolution, as indicated in Fig 1. Fig 2 shows that steady-state FcεRIβ mRNA levels were increased in all subjects after challenge relative to before challenge, with a return to near-baseline values at resolution. ANOVA including the 3 time points (before challenge, after challenge, and at resolution) was significant ($P = .0009$, Friedman test). The median fold increases in FcεRIβ mRNA relative to values before challenge were 2.15-fold after challenge versus 1.10-fold at resolution. The median PCR cycle number to reach equivalent mRNA copies at preallergen challenge was 27.32 compared with 25.63 cycles after challenge ($P = .007$) and 26.10 cycles at resolution ($P = .37$, Wilcoxon signed-rank test, all comparisons with values before challenge). In the 3 subjects who underwent nasal saline challenge, no increase in steady-state levels of FcεRIβ mRNA was noted (data not shown).

Effect of intranasal allergen challenge on basophil FcεRIβ protein levels

Next we examined whether repeated allergen exposure through the intranasal route also enhances FcεRIβ protein levels. In a subset of subjects ($n = 6$), levels of FcεRIβ protein were compared in enriched basophil lysates before challenge and 24 hours after the third allergen challenge. Fig 3 shows that in 5 of 6 allergen-challenged subjects, the level of FcεRIβ protein measured at day 4 was increased relative to the prechallenge baseline (mean fold increase, 208%).

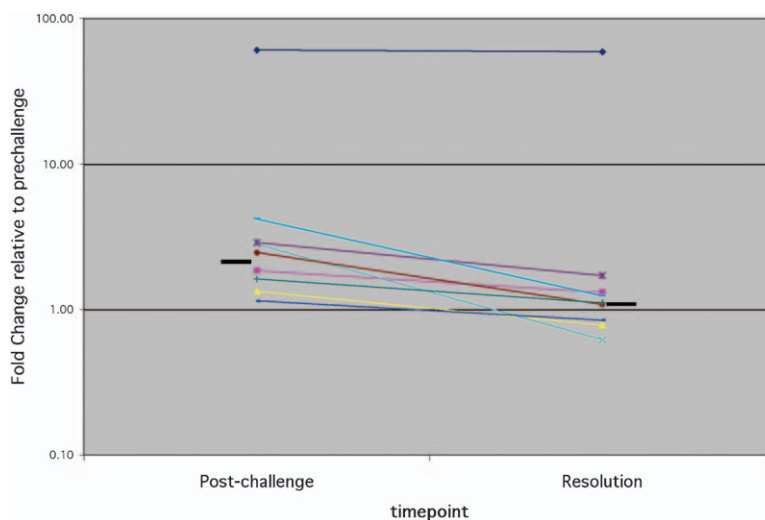


FIG 2. Effect of allergen challenge on the expression of FcεRIβ mRNA in blood basophils. FcεRIβ mRNA levels increased a median of 2-fold in postchallenge basophils relative to prechallenge basophils.

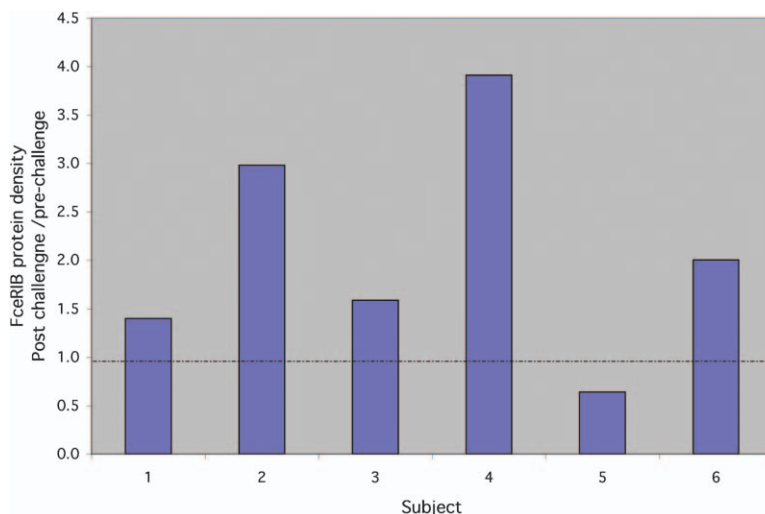


FIG 3. The effect of allergen challenge on the expression of FcεRIβ protein expression in blood basophils. Purified blood basophils from 6 subjects were obtained before challenge and after challenge (24 hours after the third allergen challenge). The densities of detected FcεRIβ protein bands after challenge are expressed relative to the prechallenge value. As shown, 5 of 6 donors had increased FcεRIβ protein expression after allergen challenge relative to baseline. The mean fold increase was 208% (n = 6; range, 64% to 391%).

Effect of intranasal allergen challenge on basophil cytokine secretion

Basophils are known to secrete large quantities of IL-4 and IL-13 *in vitro* after IgE-mediated stimulation.^{16,19,20} These cells also readily secrete IL-13 in response to several IgE-independent stimuli.^{21,22} In airway allergen challenge models, recruited basophils present in bronchoalveolar lavage fluid and in the mucosa have been shown to secrete IL-4.^{23,24} As a result, we proceeded to address whether basophil-enriched cell suspensions rapidly prepared from peripheral blood by means of density centrifugation would spontaneously produce these cytokines *ex vivo* after repeated intranasal allergen challenge. For these

experiments, cell cultures were incubated for 18 hours without any additional stimulation. As shown in Fig 4, basophil-enriched suspensions from only 3 of the 9 subjects secreted detectable IL-13 when evaluated before challenge (2 ± 1.2 pg/mL [mean $\pm$ SEM]; median, 0 pg/mL; n = 9). These levels increased, however, in 2 of these 3 subjects and became measurable in the supernatants from 5 others when evaluated at peak IL-13 secretion after allergen challenge (10.8 ± 5.3 pg/mL [mean $\pm$ SEM]; median, 6.2 pg/mL; $P = .058$, Wilcoxon signed-rank test in comparison with before levels challenge). ANOVA including the 3 time points (before challenge, after challenge, and at resolution) was significant ($P = .025$,

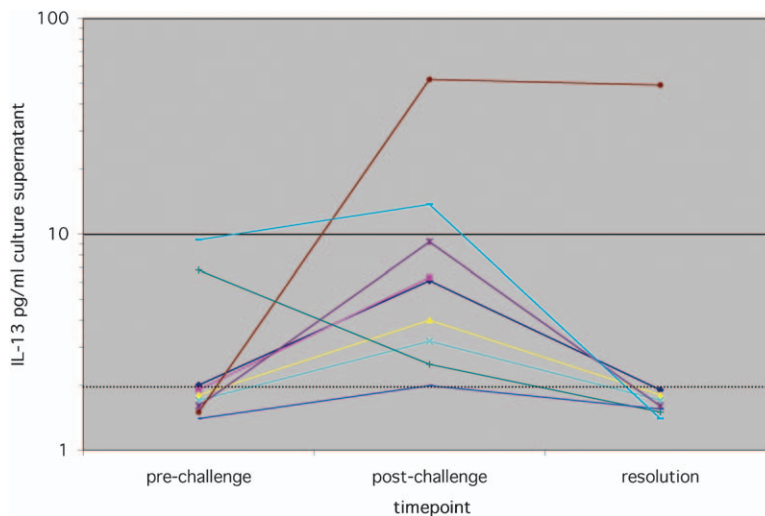


FIG 4. Spontaneous production of IL-13 by *ex vivo* basophil-enriched cultures before and after allergen challenge. Basophil-enriched cell suspensions prepared from peripheral blood of subjects at the cytokine time points in Fig 1 were cultured *ex vivo* for 18 hours without any additional stimulation. Supernatants were assayed for IL-13 protein by means of ELISA (dashed line indicates limits of detection, 2 pg/mL IL-13). As shown, mean IL-13 protein levels increased an average of 5-fold after challenge and returned to near baseline by the resolution time point (Friedman ANOVA: $P = .025$; $P = .058$ by Wilcoxon signed-rank test comparing prechallenge with postchallenge values).

Friedman test). Overall, IL-13 levels increased in 7 of the 9 basophil-enriched cultures. Cells from only one subject showed decreased levels of IL-13 after the allergen challenges, whereas those of another failed to secrete any measurable amounts of this cytokine at any time point. By the resolution time point, cells from only 1 of the 9 subjects showed evidence of IL-13 secretion. In contrast to the basophil-enriched suspensions, only 1 of the 9 basophil-depleted suspensions had measurable amounts of IL-13 before challenge, and the levels of this cytokine did not increase in these cultures after repeated allergen challenge (data not shown). Unlike IL-13, IL-4 was not detected in any of the culture supernatants from either the basophil-enriched or basophil-depleted cell preparations (data not shown). Furthermore, no spontaneous IL-13 secretion was noted from the basophil-enriched suspensions prepared from subjects undergoing saline challenges at any time point (data not shown).

DISCUSSION

Numerous studies, both recent and in the past, support the concept that basophils of allergic individuals show a primed or activated phenotype.²⁻⁸ The results from this study support this belief and, more significantly, add to the growing thought that such priming effects are a result of systemic immunologic manifestations arising after local allergen exposure. We found that circulating basophils obtained from subjects with allergic rhinitis undergoing 3 days of sequential intranasal allergen challenge show signs of *in vivo* priming that are not evident at baseline (ie, before experimental allergen challenge) or in subjects

undergoing similar challenge with saline alone. Rather than focusing on increases within the progenitor pool, as demonstrated by Denburg,¹⁴ we choose to investigate mature basophils for phenotypic, as well as functional, changes. In particular, our results show that repeated allergen challenge induced an average 2-fold increase in both protein and mRNA for FcεRIβ of the high-affinity IgE receptor. Functional changes were further demonstrated by the detection of increased IL-13 protein spontaneously secreted in these basophil-enriched cultures. Such changes in FcεRIβ and IL-13 were observed during the day after the third allergen challenge but were not correlated ($r = 0.31$, $P < .05$), with the levels of both returning to near-baseline values within 4 days. Overall, these novel observations signify that increased FcεRIβ expression and IL-13 secretion are independent markers of activated basophils that can be induced systemically with local allergen exposure.

Although we can only hypothesize as to the factors responsible for these apparent priming effects on basophils, there is *in vitro* evidence to suspect that specific cytokines are involved. Most relevant, we have previously demonstrated that *in vitro* culture with IL-3 greatly enhances the expression of FcεRIβ, both at the protein and mRNA level.¹⁰ These same effects are also seen with IL-5, although to a lesser degree, but not with NGF.¹⁸ Likewise, we have also shown that IL-3, even when used alone, is a potent stimulant of IL-13 in basophils, but not of IL-4 or histamine.²² In fact, cells from allergic subjects secrete higher levels of IL-13 in response to either IL-3 or NGF compared with basophils from nonallergic subjects.⁸ Several other *in vitro* studies would also lead one to suspect that IL-3 plays a central role in regulating basophil

responses *in vivo*. For example, it is well known that this cytokine primes basophils for IgE-mediated mediator release and cytokine secretion. Moreover, basophils normally unresponsive to IgE cross-linking stimuli (ie, the nonreleaser phenotype) become responsive after several days in culture with this cytokine presumably by upregulating the expression of syk tyrosine kinase.^{25,26} IL-3 is also critical for basophil development from precursor cells and possesses antiapoptotic activity for mature cells.²⁷ Although some of these basophil-modulating effects are also seen with other cytokines that have been observed in active allergic subjects (eg, GM-CSF,²⁸ IL-5,¹⁵ and NGF²⁹), they are generally less potent and versatile compared with IL-3.²¹

Nonetheless, despite the plethora of *in vitro* data supporting a priming role for IL-3 in basophil responses, we were unable to detect this cytokine in any of the plasma samples collected at each time point after allergen challenge (data not shown). Furthermore, IL-3 mRNA was not detected in any of the mononuclear cell suspensions also prepared (data not shown). Although this could suggest that IL-3 is playing less of a role in the allergen-induced priming effect, we currently believe that its absence is more of a technical issue. For example, it has been our experience that current ELISAs are incapable of detecting IL-3 in serum or plasma, even after spiking such specimens with known amounts of recombinant IL-3 protein. Additional measures might be necessary for detecting this cytokine in blood.

Finally, it is also intriguing to think that the alterations in FcεRIβ resulting from repeated allergen exposure could somehow account for the spontaneous production of IL-13 observed in the basophil cultures. In studies using transfectant rodent cell lines, FcεRIβ has been shown to amplify early downstream IgE-signaling events¹¹ and to enhance FcεRI cell-surface expression.¹² Thus given these functions, it seems reasonable to speculate that regulation of FcεRIβ expression in mast cells and basophils holds implications for cellular responsiveness to allergen-triggered events. Nonetheless, it is important to note that we found no evidence of IL-4 production in this study (data not shown). However, our previous *in vitro* work would indicate that FcεRI-mediated activation plays a critical role in the production of this cytokine from basophils, whereas IL-13 is further induced through IgE-independent mechanisms.²² Therefore it seems unlikely that IL-4 would go undetected if alterations in FcεRIβ accounted for the IL-13 spontaneously secreted in this study. It seems more plausible, in light of the information discussed above, that the increased expression of FcεRIβ is of little consequence to the IL-13 produced but rather that these are independent observations being driven by a common factor or cytokine. Likewise, the possibility exists that IL-13 increased FcεRIβ expression in an autocrine fashion; however, the lack of a strong correlation between these 2 elements argues against this effect. Furthermore, we have not seen enhanced FcεRIβ expression (mRNA or protein) in basophils cultured with IL-13.

In conclusion, this study has shown that local allergen challenge, repeatedly administered through the nose, is capable of inducing systemic effects on circulating basophils, causing these cells to increase their expression of FcεRIβ and to spontaneously secrete IL-13. In that FcεRIβ is considered an amplifier of FcεRI-mediated responses and that IL-13 is an important T_H2-like cytokine linked to IgE synthesis by B cells, these priming effects on basophils could play a critical role in exacerbating allergic reactions.

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