

## **An Immunoglobulin E-dependent Recombinant Histamine-releasing Factor Induces Interleukin-4 Secretion from Human Basophils**

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### **Summary**

A novel recombinant histamine-releasing factor (rHFR), which stimulates secretion from a subpopulation of human basophils that express a particular type of immunoglobulin E (IgE) or IgE<sup>+</sup>, was found to induce interleukin-4 (IL-4) production from cells isolated from these same donors. The secretion of IL-4 protein induced by rHFR significantly correlated with histamine release and the amount of protein generated, and the kinetics were identical to those caused by anti-IgE activation. Furthermore, the ability of rHFR to induce IL-4 protein production from cells not normally responsive to this protein was transferred by passive sensitization with plasma containing IgE<sup>+</sup> antibody. That this novel protein stimulates both mediator release and the secretion of IL-4 protein from human basophils suggests a prominent role for this molecule in allergic disease.

Human basophils release potent inflammatory mediators, such as histamine and leukotriene C<sub>4</sub>, in response to a variety of stimuli both related and unrelated to antigen cross-linking of IgE/receptor complexes. Work in our laboratory using antigen challenge models, and that in a variety of other laboratories using biopsy and autopsy material from asthmatics, suggests that basophils infiltrate the tissue and play an important role in the pathogenesis of chronic allergic lesions (1-4). This concept is supported by recent studies showing that basophils generate and secrete high levels of IL-4 (5-8), a proinflammatory cytokine having immunoregulatory properties that affect many different cell types that infiltrate during allergic inflammation. The secretion of IL-4 protein, however, unlike that described for histamine release, seems more dependent on signals generated in response to IgE-dependent stimulation (8), with little evidence thus far that other physiologic stimuli can similarly activate the basophil to induce secretion of this cytokine.

Among basophil secretagogues, a considerable amount of attention has been directed to a group of proteins collectively referred to as histamine releasing factors (HRFs). The HRFs are generated by a number of different cell types, including monocytes (9), B cells (10), platelets (11-13), and endothelial cells (10). HRFs are felt to play a role in the pathogenesis of chronic allergic inflammation, a concept supported by the fact that these substances can be recovered from cutaneous, bronchoalveolar, and nasal lavage fluids. Two types of HRFs have been described: those that induce histamine release in a reaction requiring cell surface IgE,

and those that operate independently of IgE antibody. The IgE-independent activity is linked to a growing list of recombinant cytokines, the chemokines, that induce histamine release from basophils in addition to having chemotactic activity for a variety of cells. Of the chemokines tested, monocyte chemotactic protein-1 (MCP-1) and MCP-3 are the most potent activators of basophils, inducing mediator release at nanomolar concentrations (14). The IgE-dependent HRFs, described as causing histamine release from cells expressing a particular type of IgE (designated as IgE<sup>+</sup>), had only been partially characterized (15). Recently, however, a cDNA clone that codes for a protein with a molecular weight of 23,000 daltons has been subcloned and expressed. This human recombinant protein, rp23, and its murine homologue, rp21, have the same IgE-dependent histamine-releasing activity as the previously described HRF found in vivo (16).

In the present study, we investigate the ability of these recombinant molecules to induce IL-4 secretion from human basophils, since IgE-dependent activation is an important stimulus for the generation of this cytokine message and protein. Our results show that rp21 and rp23 are equally effective at promoting the secretion of IL-4 protein from basophils that release histamine in response to these recombinant proteins, with production dependent on the expression of IgE<sup>+</sup>. The IgE-independent HRF and MCP-3, and the cytokine, interleukin-3 (IL-3), do not appear to have the same IL-4-inducing capabilities associated with rp23/rp21. We hypothesize that this IgE-dependent HRF plays a

key role in the pathogenesis of human allergic disease, causing both histamine release and IL-4 secretion from basophils in vivo.

## Materials and Methods

**Buffers and Media.** 10 × Pipes contained 250 mM Pipes, 110 mM NaCl, and 50 mM KCl, pH 7.4. A Pipes/albumin/glucose buffer (PAG) contained 0.1 10 × Pipes in addition to 0.003% human serum albumin (HSA) (Calbiochem-Bering Corp., La Jolla, CA) and 0.1% D-glucose. PAG-EDTA additionally contained 4 mM EDTA. Isotonic Percoll (referred to as 100% Percoll) consisted of 1 part 10 × Pipes and 9 parts Percoll (Pharmacia Biotech, Piscataway, NJ). Percoll solutions of 55% ( $d = 1.072$ ) and 65% ( $d = 1.083$ ) were made by mixing the appropriate amounts of 1 × Pipes and 100% Percoll. Conditioned IMDM (C-IMDM) consisted of IMDM containing 5% heat-inactivated (56°C for 30 min) FBS (Sigma Chemical Co., St. Louis, MO), 1 × nonessential amino acids, and 5 µg/ml gentamicin.

**Special Reagents.** Recombinant IL-3 (rhIL-3) was a kind gift from Dr. S. Gillis (Immunex Corporation, Seattle, WA). MCP-1 was purchased from Pepro Tech (Rocky Hill, NJ). Polyclonal anti-human IgE antibody was generated in a goat and affinity purified.

**Preparation of Recombinant HRF.** The cDNA for rp21 was kindly supplied by Drs. M. Koots and G. Brawerman of Tufts University (Boston, MA). Primers were designed, and the PCR product was expressed in *Escherichia coli* as a protein fused to glutathione S-transferase (GST) and affinity purified on immobilized glutathione. The same primers were used to generate the human rp23 from the U937 cell line. This was also expressed as a GST fusion protein and affinity purified. Since we have previously shown that both rp21 and rp23 induced similar IgE-dependent histamine release (16), we have used both of these recombinant proteins in this article. The concentration of recombinant HRF used in these studies was 8 µg/ml, or  $\sim 4 \times 10^{-7}$  M.

**Basophil Preparation.** Venous blood from consenting donors was anticoagulated with 10 mM EDTA. Whole blood was centrifuged at 300 g, and the leukocyte interface (buffy-coat) was aspirated and transferred to PAG-EDTA (1:1 vol/vol). Basophil-enriched suspensions were prepared by Percoll density centrifugation, as previously described (8). In brief, the diluted buffy-coat suspension was layered onto gradients consisting of 12 ml of 55% Percoll layered onto 12 ml of 65% Percoll in clear 50 ml polypropylene tubes. Percoll gradients were centrifuged at 700 g for 20 min at room temperature. Cells banding at the 55% Percoll interface and penetrating the top half of this layer were removed and discarded. Basophils were in the fraction consisting of the lower half of the 55% Percoll, the 65% interface, and the upper half of this layer. Cells in this fraction were washed twice in PAG-EDTA followed by a final wash in cold (4°C) PAG. Basophils were counted in Spiers-Levy chambers using Alcian blue (17). The purity of the basophils in the cell suspensions used for these studies ranged from 5% to 40%.

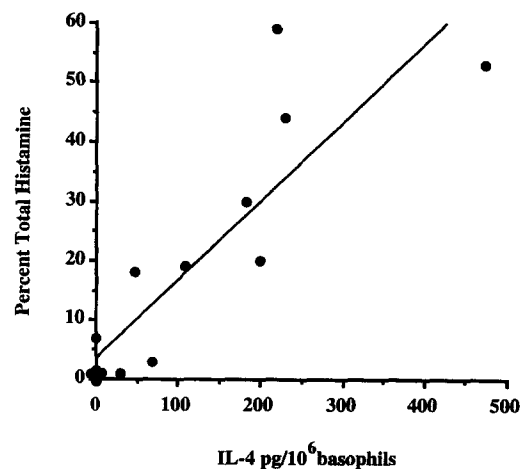
**Passive Sensitization of Basophils.** For some experiments, surface IgE was removed from a portion of Percoll-isolated basophils by lactic acid treatment (18). Basophils were then passively sensitized with IgE (2–8 µg/ml) from the serum of one of two patients responsive to IgE-dependent HRF (IgE<sup>+</sup>). For controls, a set of acid-treated cells were sensitized with an equal concentration of normal polyclonal IgE or myeloma IgE (IgE<sup>-</sup>) or were simply left unsensitized.

**Culture Conditions.** Cultures were performed in C-IMDM with 96-well microtiter plates (Costar Corp., Cambridge, MA), as previously described (8). The number of basophils cultured ranged from 50,000 to 200,000 per well. For most experiments, the cells were added to culture wells in 125 µl of medium and brought to 37°C in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>) before adding an equal volume of medium (preequilibrated in the incubator) containing twice the final concentration of stimulus. After 1–6 h of incubation, cell-free culture supernatants were harvested and centrifuged at 150 g at 4°C, and the supernatant was removed for histamine and IL-4 protein analysis by automated fluorometry and ELISA, respectively (8). In some instances, IL-4 protein was measured by a commercial ultrasensitive ELISA (sensitivity, <1 pg/ml), according to the manufacturer's protocol (BioSource International, Camarillo, CA).

## Results

**Induction of IL-4 Secretion by Recombinant HRF Correlates with Histamine Release.** Basophils generate IL-4 in response to anti-IgE at concentrations nearly 10-fold less than those required for optimal histamine release (8). However, as shown in Fig. 1, the generation of IL-4 significantly correlated with maximal histamine release ( $r = 0.90$ ,  $p = 0.001$ ,  $n = 14$ ). Of the donors previously shown to release histamine in response to the native HRF, all generated detectable amounts of IL-4 when challenged with the recombinant HRF, with protein levels ranging from 40 to 470 pg per 10<sup>6</sup> basophils. In contrast, in five donors who did not release histamine in response to the native HRF, but who are known to secrete IL-4 in response to anti-IgE antibody, rp21 or rp23 failed to induce IL-4 generation.

**Time Course of IL-4 Secretion by Basophils Induced by Recombinant HRF.** Previous studies have shown that the native IgE-dependent HRF induces histamine release from



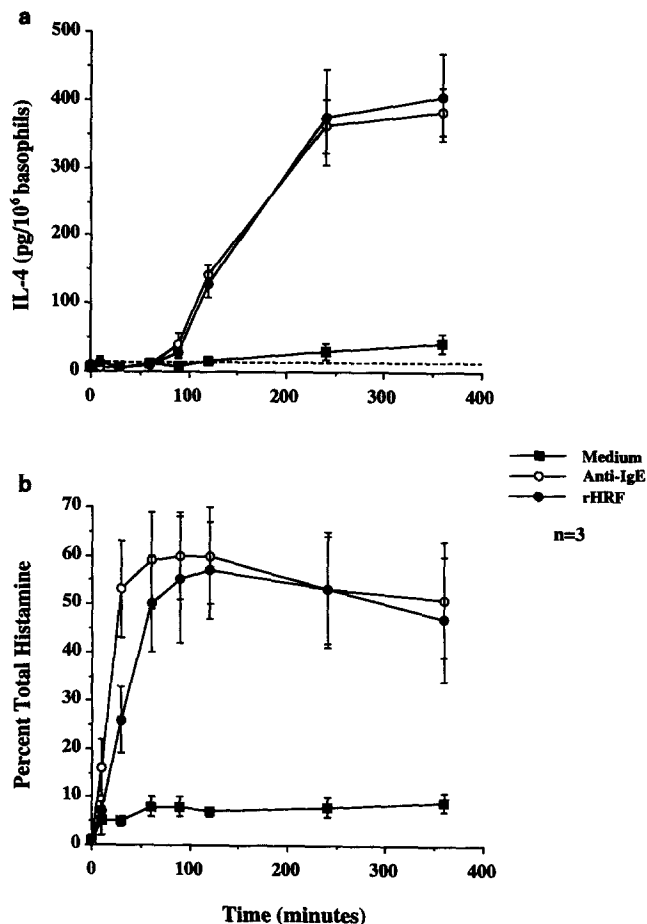
**Figure 1.** The secretion of IL-4 protein from basophils challenged with recombinant HRF significantly correlates with histamine release. Basophils from 14 donors were prepared as described in Materials and Methods. Cultures were challenged with rp21 (8 µg/ml) or rp23 (7.5 µg/ml) for 4 h before harvesting cell-free supernatants for histamine and IL-4 protein measurements. The secretion of IL-4 significantly correlated ( $r = 0.90$ ,  $p = 0.001$ ) with histamine release, as determined by Spearman's rank correlation coefficient.

responsive basophils with kinetics comparable to those induced by anti-IgE stimulation (15). We compared the kinetics of IL-4 secretion by basophils stimulated with anti-IgE antibody or with the recombinant HRF. In Fig. 2, the time course of the generation of IL-4 induced by rp21 or rp23 was identical to that caused by anti-IgE activation (a). With both stimuli, protein was first detected after 1 h of culture, with levels peaking by 4 h; no additional increase was observed at 6 h. Also shown in this figure, for comparison, are the kinetics of histamine release (b). Although the responses induced by recombinant protein were rapid and generally peaked by 60–90 min, histamine release was slightly faster for anti-IgE stimulation, particularly at the earlier (<60 min) time points.

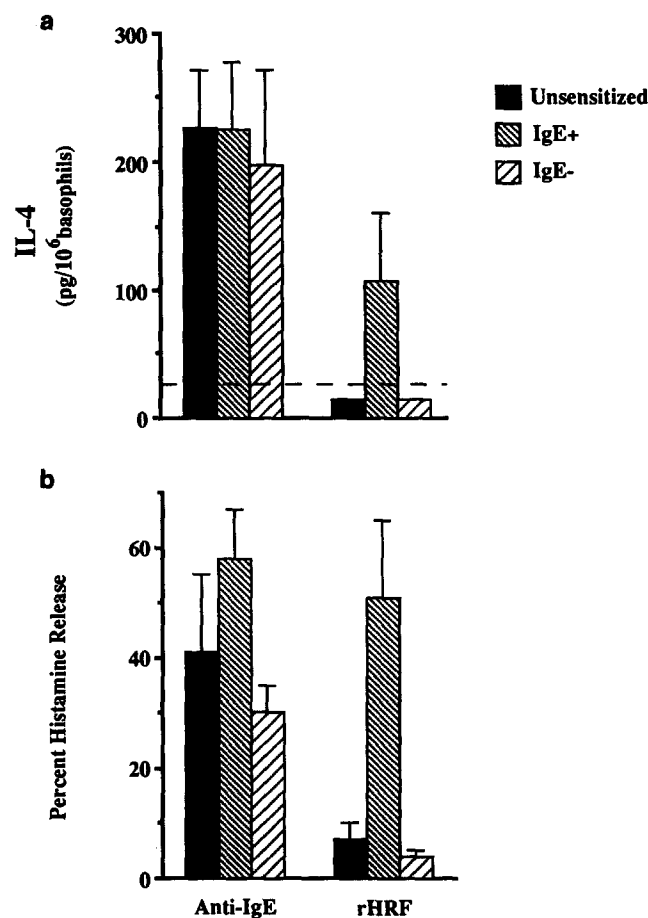
**Transfer of Releasability with Passive Sensitization.** For the next series of experiments, we isolated basophils from normal donors who did not release histamine, or secrete IL-4, in response to rp21 or rp23. A portion of these cells was left untreated; surface IgE was partially removed from the re-

maining cells by lactic acid treatment (see Materials and Methods). Half of the cells treated with lactic acid were passively sensitized with IgE from the serum of a patient responsive to the HRF in an attempt to transfer reactivity to normal cells. As a control, the other portion of the treated cells was passively sensitized with an equal concentration of polyclonal serum IgE from a normal patient, or with myeloma IgE. As shown in Fig. 3, cells left untreated and those passively sensitized with both forms of IgE secreted IL-4 in response to anti-IgE antibody (a). These cells also released histamine in response to anti-IgE (b). However, IL-4 protein and histamine were secreted in response to recombinant HRF only by cells that were passively sensitized with IgE<sup>+</sup>.

**Secretion of IL-4 Protein by Basophils Induced by Recombinant HRF versus That Caused by Other Secretagogues.** In a final series of experiments, we compared IL-4 secretion and hista-

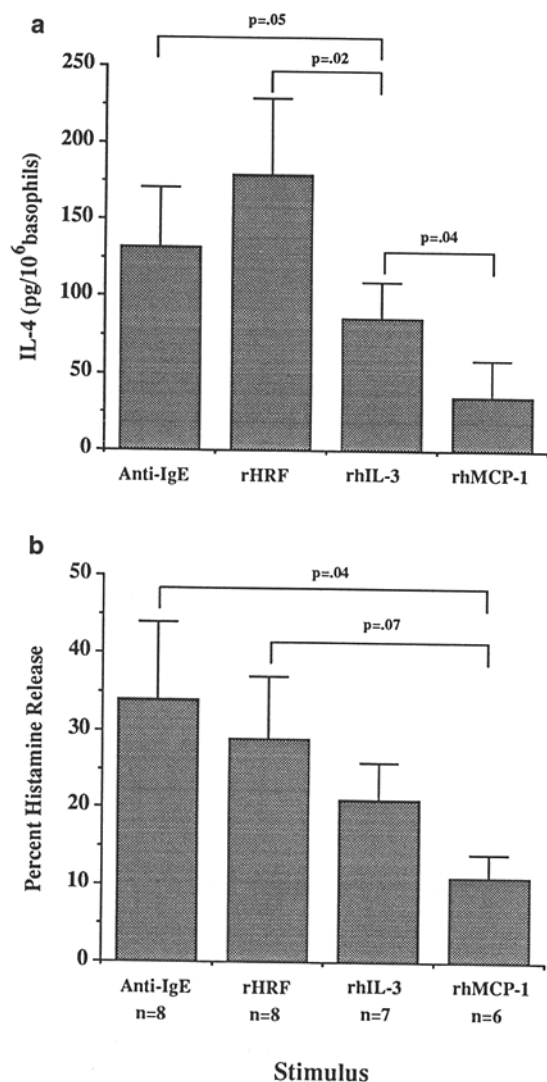


**Figure 2.** Time course of IL-4 protein secretion and histamine release induced by recombinant HRF. Basophils from three donors were prepared as described in Materials and Methods. Cell cultures were challenged with either rp21 (8  $\mu$ g/ml) or with rp23 (7.5  $\mu$ g/ml) for the indicated times, before harvesting cell-free supernatants for IL-4 protein (a) and histamine (b) measurements.



**Figure 3.** Basophil releasability to recombinant HRF protein transfers during passive sensitization with IgE<sup>+</sup>. Basophil suspensions were prepared from four donors not normally releasing to HRF. After removal of IgE from a portion of cells with lactic acid treatment (see Materials and Methods), basophils were passively sensitized with 2–8  $\mu$ g/ml of IgE from the plasma of one of two patients responsive to HRF (i.e., IgE<sup>+</sup>) or with an equal concentration of either myeloma or normal polyclonal IgE (i.e., IgE<sup>-</sup>). As an added control, a portion of cells from each suspension was left untreated (unsensitized). Cells were washed before culturing for 4 h with anti-IgE antibody (10 ng/ml) or with recombinant HRF (8  $\mu$ g/ml). Cell-free supernatants were analyzed for IL-4 protein (a) and histamine content (b).

mine release induced by anti-IgE, and by recombinant HRF, with that generated in response to the IgE-independent HRFs, including IL-3 and MCP-1. As shown in Fig. 4, rp21 or rp23 caused IL-4 secretion from basophils isolated from all donors who had reacted to the native HRF. Nearly identical amounts of protein were also secreted in response to an optimal concentration of anti-IgE antibody (a). However, the amounts of IL-4 induced by both anti-IgE (10 ng/ml) and recombinant HRF were statistically different from those released in response to rhIL-3 (1–100 ng/ml) ( $p = 0.05$  and  $p = 0.02$ , respectively). There was also significantly less IL-4 protein generated in response to MCP-1 ( $10^{-7}$ – $10^{-9}$  M) compared with the other stimuli.



**Figure 4.** Comparison of IL-4 protein secretion induced by recombinant HRF, anti-IgE, rhIL-3, and MCP-1. Basophil suspensions were prepared as described in Materials and Methods. Cell cultures were challenged with anti-IgE (10 ng/ml), recombinant HRF (8  $\mu$ g/ml, or  $\sim 4 \times 10^{-7}$  M), rhIL-3 (1–100 ng/ml), or MCP-1 ( $10^{-7}$ – $10^{-9}$  M). After a 4-h culture, cell-free supernatants were analyzed for IL-4 protein (a) and histamine content (b). Statistical analysis was performed with the Wilcoxon nonparametric method.

However, with the exception of anti-IgE antibody versus MCP-1, there were no significant differences ( $p \leq 0.05$ ) among the percentages of histamine released, indicating that all the secretagogues were active (b).

## Discussion

Several studies have shown that basophils infiltrate allergic lesions in the nose, lung, and skin in response to administered antigen (1–3) in a reaction referred to as the late phase response. Lavage samples taken from similar reaction sites have also been shown to cause IgE-dependent histamine release from basophils in vitro (15, 19). We have recently reported the subcloning and characterization of an HRF thought to represent a unique cytokine, showing no homology with any known interleukin, chemokine, allergen, or antigen, yet having the same IgE dependency as that found in vivo (16). In the present study, both human (rp23) and murine (rp21) recombinant forms of this protein were also found to induce the secretion of IL-4 protein from basophils, with levels identical to those caused by anti-IgE and peaking by 4–6 h, a time fitting with the course of the late phase response. The activity of IL-4 protein has been linked to several immunoregulatory functions important in allergic inflammation, including the synthesis of IgE by B cells (20), adherence and selective transmigration of eosinophils on endothelium (21, 22), and the development of Th2 lymphocytes (23). The fact that the IgE-dependent HRF stimulates basophils to produce IL-4 underscores its importance in allergic disease. In this context, it has been shown that children with food sensitivity and severe atopic dermatitis have lymphocytes that generate an IgE-dependent HRF and have basophils that spontaneously release histamine, with both activities disappearing when the relative food is withdrawn (24). It is not clear at this time whether the HRF described in these children is identical to the recombinant proteins tested in the present study or is a member of a family of IgE-dependent HRFs.

In this manuscript, we present evidence that a significant correlation exists between histamine release and cytokine secretion induced by rHRF, and that the kinetics of IL-4 release are identical to those induced by anti-IgE antibody. As originally described for native HRF, basophil releasability to rHRF is very much dependent on the expression of a particular type of IgE (designated as IgE<sup>+</sup>), which is almost entirely restricted to atopic subjects (15). Cells not normally responsive to the IgE-dependent HRF, when passively sensitized with serum containing IgE<sup>+</sup>, do, upon activation with recombinant protein, secrete both histamine and IL-4 protein (see Fig. 3). The exact nature for the interaction between the HRF and IgE antibodies is not yet fully understood, but has sparked a number of hypotheses. First, the possibility that recombinant HRF represents a novel allergen or antigen cannot be absolutely excluded at this time, despite the fact that it shows no homology with any known allergen or antigen. We have reported that PBMCs, from donors both responsive and unresponsive to HRF,

express mRNA for this protein (as determined by reverse transcription-PCR). Thus, it seems that a protein as ubiquitous as HRF, if truly acting as an allergen, may, in fact, represent an autoantigen. In this instance, allergic inflammation might then be considered an autoimmune disorder. Second, an early thought suggested that differences in post-translational modifications of IgE antibody might be responsible for IgE heterogeneity, with HRF possibly binding to glycosylated species (25). While this hypothesis has not been proven incorrect, it seems unlikely in light of evidence showing that there appear to be no lectin-binding sites in the recombinant HRF. However, there is now good evidence for alternative splicing within the  $\epsilon$  DNA locus, which results in several different species of IgE mRNA

(26). Therefore, as a third possibility, it seems that the IgE-dependent HRF could preferentially bind to one or more of the antibody proteins derived from these transcripts. In collaboration with Dr. A. Saxon, we are currently testing this hypothesis. Finally, the IgE-dependent HRF may function by binding to a cell surface receptor that is part of, or closely associated with, Fc $\epsilon$ RI, such that the binding of HRF to basophils also expressing sufficient levels of IgE<sup>+</sup> triggers signals resulting in the release of histamine and the secretion of IL-4 protein. Whichever one of these hypotheses is correct, the fact that his novel protein causes both the release of potent inflammatory mediators and the secretion of IL-4 protein from basophils suggests that it plays a significant role in allergic inflammation.

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