

Lack of correlation between bronchial late allergic reaction to *Dermatophagoides pteronyssinus* and in vitro immunoglobulin E reactivity to histamine-releasing factor derived from mononuclear cells

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Background: Activity of immunoglobulin (Ig)E-dependent histamine-releasing factor (HRF) is dependent on the IgE molecules bound to the surface of basophils. Sera capable of passively sensitizing basophils to release histamine to HRF were designated IgE⁺ sera. IgE⁺ and HRF have been suggested to play a role in late allergic reaction (LAR).

Objective: The working hypothesis was tested that IgE⁺ induces a LAR. Further, activity of HRF produced by mononuclear cells (HRF_{mn}) was compared with that of recombinant HRF p23.

Methods: Atopic patients (n = 82) were bronchially provoked with *Dermatophagoides pteronyssinus* extract and the change in forced expiratory volume in 1 second was monitored. A LAR was defined as forced expiratory volume in 1 second as percentage of baseline <80% 4 to 10 hours after allergen challenge. The presence of HRF-responsive IgE in serum was determined using basophils sensitized in vitro by serum.

Results: The presence of HRF_{mn}-responsive IgE (IgE^{mn+}) in serum was shown not be essential for a LAR: 63% of the patients with a LAR had no IgE^{mn+} in their serum. Further, 71% of patients with IgE^{mn+} did not have a LAR. HRF_{mn} and recombinant HRF p23 were not equivalent in the bioassay: serum of 38 of 82 atopic patients sensitized basophils to release histamine to HRF_{mn}, whereas this was found with serum of 1 of 82 patients to HRF p23.

Conclusions: The results do not support the hypothesis that IgE^{mn+} induces a LAR, but do not exclude the alternative hypothesis that HRFs are released *during* a LAR and contribute to asthma severity.

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INTRODUCTION

Immunoglobulin (Ig)E-dependent histamine-releasing factors (HRFs) release histamine from basophils only if a special type of IgE is present on the surface of these cells. Sera capable of passively sensitizing basophils to release histamine to HRF were designated IgE⁺ sera.¹ Therefore, by definition, sera that do not react to HRF were termed IgE[−]. IgE-dependent HRFs are produced by different cell types such as macrophages,² mononuclear cells,³ and platelets.⁴ Culture supernatants of these cells consist of a mixture of HRFs. Previous studies have shown that chemokines, which are responsible for the main IgE-independent activity, can be

removed by treatment with heparin-agarose.⁵ One IgE-dependent HRF has been cloned: p23⁶ also known as translationally controlled tumor protein.⁷

Two hypotheses to explain the reactivity of IgE⁺ are heterogeneity of IgE, or autoreactivity. The HRF-reactive part of IgE⁺ might be present in the constant regions of the IgE molecule. MacDonald et al⁶ speculated that IgE⁺ reactivity is dependent on the mode of glycosylation of IgE.¹ However, no carbohydrate differences were found.⁸ Because IgE-dependent HRF demonstrates a priming effect on anti-IgE-induced basophil histamine release,⁹ a second signal (for instance polymeric IgE) might be necessary to achieve basophil activation upon stimulation with HRF. We recently presented data that IgE reactivity to HRF produced by mononuclear cells (HRF_{mn}) was conferred to basophils by monomeric IgE.¹⁰ Alternatively, according to the autoreactive IgE hypothesis, IgE⁺ might be an HRF-specific IgE antibody, and HRF an autoallergen. HRF would exert its reactivity, similar to conventional allergens, by cross-linking IgE⁺ on the surface of basophils, which in turn induces the release of mediators. Valenta et al¹¹ detected autoreactive IgE in sera of

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patients with atopic dermatitis. IgE autoantigens were detected in extracts of several human cell types. Using cDNA libraries of human cells, several IgE autoantigens were identified.¹²⁻¹⁴ We recently showed that IgE autoantigen-containing extracts did not induce histamine release of appropriately sensitized basophils.¹⁵ Further, no significant association was found between IgE autoreactivity to blotted human proteins and IgE-dependent responsiveness to HRF_{mn}. Thus, IgE autoreactivity on Western blot and IgE-dependent responsiveness to HRF_{mn} are distinct entities that coexist in atopic sera. Our working hypothesis is that HRF_{mn}-responsive IgE (IgE^{mn+}) is autoreactive IgE.

IgE-dependent HRFs and IgE⁺ have been suggested to play a role in the late and chronic allergic reaction.¹⁶ In many individuals, the acute allergic reaction is followed by a late allergic reaction (LAR) 4 to 11 hours later.^{17,18} As the LAR occurs without a renewed exposure to allergen, the activation of basophils during the late reaction¹⁹⁻²¹ might be induced by IgE-dependent HRF. This was supported by the observation that IgE-dependent HRF was present in late-phase skin blister fluids¹⁶ and late-phase nasal lavages.¹ Charlesworth et al²² hypothesized that HRFs and chemotactic factors are produced during the acute allergic reaction. Subsequently, after attraction of basophils to the allergic site by the chemotactic factors, basophils with IgE⁺ on their surface might be activated by HRFs causing a LAR. IgE-dependent HRF might also trigger other cells present during the LAR by binding to IgE molecules present on, for instance, eosinophils^{23,24} and monocytes,^{25,26} or by binding to an HRF-specific receptor on cells.²⁷

In the current study we investigated the hypothesis that IgE reactivity to HRF_{mn} causes a bronchial LAR. The association between IgE^{mn+} in serum and the presence of a LAR was analyzed in a well defined group of atopic patients, who participated in a bronchial provocation study.²⁸ Further, the activity of HRF_{mn} was compared with that to rHRF p23.

MATERIALS AND METHODS

This study was performed after written informed consent of the patients and was approved by the local ethical committee.

Human Subjects

Atopic patients with asthma. Forty-nine subjects with asthma were investigated (31 female; mean age 27.0 years, ranging from 18 to 42 years old). Diagnosis was based on history of episodic dyspnea with wheezing and reversible bronchus obstruction. All individuals had mild to moderate asthma diagnosed according to the Global Initiative for Asthma guidelines (score 1 to 3), and were positive to *Dermatophagoides pteronyssinus* by radioallergosorbent test (RAST) and skin prick test. The median total IgE value in serum was 247 IU/mL, ranging from 13 to 3,568.

Atopic patients without asthma. Twenty-six subjects with rhinitis were investigated (18 female; mean age 27.5 years, ranging from 19 to 37 years old). The patients had a history

of rhinorrhea, nasal blockade, sneezing, and pruritus, but no history of current or past episodes of dyspnea or wheezing. All patients were RAST- and skin prick test-positive to *D. pteronyssinus*. The median total IgE value in serum was 118 IU/mL, ranging from 23 to 1,994. Further, seven subjects without a history of allergic upper or lower airways disease were studied (3 female; mean age 24.7 years, ranging from 18 to 30). They were positive to *D. pteronyssinus* in RAST and skin prick test. The median total IgE in serum was 183 IU/mL, ranging from 42 to 488. This group is referred to as asymptomatic. The atopic nonasthma group consists of the allergic rhinitis patients and the asymptomatic subjects.

Control group. Fifteen subjects without a history of allergy or airways disease were used as control group (11 female; mean age 31.0 years, ranging from 20 to 50). They were skin prick test- and RAST-negative to the common inhalant allergens (house-dust mite, grass pollen, birch pollen, cat dander, dog dander; *Alternaria*, and *Aspergillus*).

Cell Donors

Buffy coats were derived from 1,000 mL blood (Hemonetics Plasma Collection System, Hemonetics Corporation, Braintree, MA) of donors from the Department of Plasmapheresis at the CLB.

Bronchial Allergen Provocation

The allergic subjects were provoked bronchially by *D. pteronyssinus* extract (ALK, Hørsholm, Denmark).²⁸ The bronchial allergen challenge and measurement of forced expiratory volume in 1 second (FEV₁) have been described in detail by Van der Veen et al.^{28,29}

All subjects were investigated in a stable phase of the disease and had a FEV₁ $\geq 70\%$ of predicted. All patients had to stop their medication of oral antihistamines, inhalation therapy with short-acting β_2 -agonists, and/or corticosteroids for 2 weeks, 12 hours, and 6 weeks, respectively, before allergen challenge. None of the patients had received immunotherapy with house-dust mite extract. They did not have a history of respiratory tract infection or obvious changes in allergen exposure for at least 6 weeks before allergen challenge.

Doubling doses of allergen were inhaled with 10-minute intervals until a fall in FEV₁ $\geq 20\%$ relative to baseline FEV₁ occurred. If the FEV₁ dropped between 15 and 20%, the last dose of allergen was administered again. After the provocation, the FEV₁ was measured for 24 hours. The results of the response to allergen were expressed as percentage FEV₁ relative to baseline FEV₁. In the asymptomatic atopics, the allergic rhinitis patients, and the allergic asthma patients, the mean FEV₁ as percentage of baseline 4 to 10 hours after allergen provocation was $94.8\% \pm 6.6$ ($n = 7$, mean $\pm$ standard deviation [SD]), $87.3\% \pm 8.7$ ($n = 26$, mean $\pm$ SD), and $80.1\% \pm 14.2$ ($n = 49$, mean $\pm$ SD), respectively. A LAR was defined as a mean FEV₁ $< 80\%$ of baseline 4 to 10 hours after allergen challenge.

Total and Specific IgE

Sera from all subjects were tested to common inhalant allergens by RAST,³⁰ and the total IgE concentration was measured.³¹

Reference Plasma

HRF_{mn} reactive plasma 151 was defibrinated by recalcification and dialyzed. This plasma (total IgE: 1,600 IU/mL) was positive for IgE to the common inhalant allergens. One sensitizing unit was defined as the activity to HRF_{mn} found in 10 μ L of this serum.

Production of IgE-Dependent HRF_{mn}

IgE-dependent HRF_{mn} was prepared as described by Pasmans et al.³² Peripheral blood mononuclear cells obtained by leukapheresis were isolated by Percoll centrifugation (Pharmacia Fine Chemicals, Uppsala, Sweden) and elutriation. The cells ($5 \cdot 10^6$ /mL) were cultured for 18 hours at 37° C in RPMI containing 5% (vol/vol) heat-inactivated fetal calf serum, 50 μ M 2-mercaptoethanol, 100 IU/mL penicillin, 100 μ g/mL streptomycin, and 300 μ g/mL glutamine and were stimulated with 5.3 μ L streptokinase/streptodornase (Lederle Laboratories, Pearl River, NY) per milliliter culture supernatant. The culture supernatants were three times concentrated (YM3 membranes; Amicon, Lexington, MA). A previous study showed that the chemokine monocyte chemotactic protein is responsible for main IgE-independent histamine-releasing activity in the culture supernatants. These chemokines were removed by heparin-Sepharose (Pharmacia Biotech, Uppsala, Sweden).³² The culture supernatant was stirred overnight at 4° C with heparin-Sepharose (40 mg/mL culture supernatant), and after this the supernatant was separated from the Sepharose on a filter. The depleted preparation was further concentrated three times. The 11 most active HRF_{mn} preparations of 29 from different donors were pooled and further purified by size-exclusion chromatography (Sephadex G75; Pharmacia Biotech). The protein concentration of the HRF_{mn} preparation was 9.4 mg/mL. For control experiments a culture medium preparation containing streptokinase-streptodornase was prepared in the same way.

Recombinant HRF p23 Production.

Recombinant HRF p23 was produced as described previously.³³ IgE-dependent HRF isolated from supernatants from the human macrophage cell line U937 cell line was purified using Sephadex G75, MONO Q anion exchange, and repetitive Superdex chromatography. N-terminal sequencing revealed extensive homology to a mouse protein, p21, and its human homolog, p23. With primers based on the sequence of p21, human p23 cDNA was isolated from the U937 cell line and cloned in the PGEX-2T vector. Subsequently, rHRF p23 was subcloned using the restriction enzymes EcoR1 and BamH1, used in the *Escherichia coli* production, and the 524 basepair fragment was ligated into the baculovirus vector, pBlueBac III, which co-expresses β -galactosidase for color selection of successful recombination. As per the MAXBAC baculovirus system manufacturer's specifications (Invitrogen, San Diego, CA), plasmid DNA was transfected into Sf 9

cells for viral isolation and amplification, and subsequently High Five insect cells were used for protein production. The insect cells were grown commercially on a large scale in serum free media (Paragon Biotech, Baltimore, MD). The cell pellet from 14 L of insect cells was dissolved in 400 mL of NBB buffer (20 mmol/L sodium phosphate, 500 mmol/L sodium chloride, pH 7.8) to which 400 μ L of PharMingen Inhibitor Cocktail (PharMingen, San Diego, CA) was added. The suspension was freeze-thawed twice and centrifuged at 9,000 rpm for 20 minutes. Purification of the supernatant, which contained the rHRF p23 protein, was accomplished by two-step column chromatography. First, the supernatant was mixed with De-52 (Whatman, Maidstone, England) in 0.02M Tris buffer, pH 8. The flowthrough was screened for the presence of rHRF p23 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using a polyclonal anti-HRF antibody generated against the recombinant material produced in *E. coli*. Positive fractions were concentrated and placed on a Sephadex G75 (Pharmacia, Piscataway, NJ) column in physiologic Pipes buffer. Again, SDS-PAGE and Western blotting were used to screen the column fractions. Fractions were pooled and concentrated, and the bioactivity was confirmed using the basophil histamine release assay. Protein concentration of rHRF p23 was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) and was found to be 160 μ g/mL. rHRF p23 was found to contain a single band on SDS-PAGE and was judged to be >95% pure.

Histamine Release Bioassay

The histamine release assay was performed as described previously.^{10,34} In short, after removal of IgE from surface of basophils by lactic acid buffer, pH 3.9, the cells were sensitized³⁵ by incubation (37° C, 90 minutes) with the sensitization mixture containing 150 μ L of human serum, 4 mmol/L EDTA, and 10 μ g/mL heparin in a total volume of 1 mL. The same amount of serum was used for sensitization, irrespective of the total IgE. Incubation (2.5×10^6 cells, 37° C, 60 minutes) was performed in 1 mmol/L CaCl₂ in a final volume of 350 μ L. Incubation with rHRF p23 was performed in 5 mmol/L CaCl₂ in a final volume of 100 μ L. The histamine release reaction was stopped by the addition of ice-cold 0.9% NaCl.

Histamine was determined by fluorometric analysis as described by Siraganian.³⁶ Histamine release was calculated as percentage of the total histamine, determined by lysis of the cells by perchloric acid.

Calculation of Levels HRF-Responsive IgE

Histamine release to HRF_{mn} (threefold dilutions, final protein concentration: 20 to 537 μ g/mL) of basophils sensitized by serum to be measured was compared with that of basophils sensitized by plasma 151 (100 sensitizing units to HRF_{mn} per milliliter). IgE^{mn+} sera were also tested for reactivity to the culture medium control in the presence of a saturating concentration of recombinant interleukin-3 (600 pM; Pepro Tech

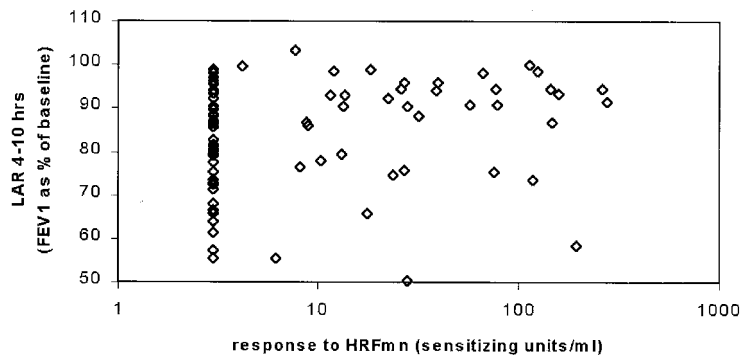


Figure 2. $\text{IgE}^{\text{mn}+}$ and the LAR in atopic patients. The presence of $\text{IgE}^{\text{mn}+}$ was determined using basophils sensitized in vitro by serum. The patients were bronchially provoked with *D. pteronyssinus* extract and the change in FEV_1 was monitored.

IgE in this serum was highest of all atopic nonasthma patients (1,994 IU/mL). Patients with a comparable IgE level were present in the asthma group, but no reactivity to rHRF p23 was found in their serum.

DISCUSSION

Our results indicated that IgE reactivity to recombinant HRF p23⁶ is less common than to HRF_{mn} . Only 1 of the 82 sera from atopic patients sensitized basophils to respond to rHRF p23. The serum of this rhinitis patient responded also to HRF_{mn} . The low response rate to HRF p23 is unlikely attributable to a suboptimal dose of HRF p23 compared with HRF_{mn} , as this patient did not have the most potent serum in respect to IgE reactivity to HRF_{mn} . Additionally, rHRF p23 could not be detected (<8 ng/mL) in an HRF_{mn} preparation as measured by an HRF p23-specific enzyme-linked immunoadsorbent assay (data not shown). These results indicate that HRF_{mn} and rHRF p23 are not the same factor. However, given the different methods to obtain these preparations, and the possibility that the recombinant molecule may not be properly posttranslationally modified, we can not exclude that the activity of HRF_{mn} is attributable to HRF p23.

We confirmed that $\text{IgE}^{\text{mn}+}$ is associated with atopic sensitization. $\text{IgE}^{\text{mn}+}$ was present in serum of 42% of atopic nonasthma patients and in 49% of mild or moderate asthma patients, but not in sera of 15 controls. The prevalence of $\text{IgE}^{\text{mn}+}$ was shown to be associated with a high total serum IgE . However, $\text{IgE}^{\text{mn}+}$ sera with a low total IgE and sera without $\text{IgE}^{\text{mn}+}$ with high total IgE were found. Thus, a high IgE concentration is not essential for IgE reactivity to HRF_{mn} . An association between $\text{IgE}^{\text{mn}+}$ and atopy has been shown previously by our group.⁵ In a previous study we reported that 44% of patients with allergic asthma ($n = 18$), 11% of patients with nonallergic asthma ($n = 19$), 29% of patients with allergic rhinitis ($n = 17$), and none of the control patients ($n = 19$) had $\text{IgE}^{\text{mn}+}$ in their serum. We now confirmed these results in the present patient group.

IgE -dependent HRF and IgE^+ were supposed to play a role in the LAR.^{37,38} The hypothesis was that HRFs produced during the early allergic reaction activate IgE^+ -containing

cells, which in turn induce the LAR. This hypothesis was based on the observation that both basophils and IgE -dependent HRFs were present during the LAR.^{1,16} However, our results indicate that $\text{IgE}^{\text{mn}+}$ is *not* essential for a LAR: 63% of the patients with a LAR had no $\text{IgE}^{\text{mn}+}$ in their serum. Further, of the patients with $\text{IgE}^{\text{mn}+}$ in their serum, 71% had no LAR. Our results also showed that $\text{IgE}^{\text{mn}+}$ did not contribute to the drop in FEV_1 during the LAR.

Our results are seemingly in contrast with results previously described in which IgE^+ is found in patients with severe forms of atopy. It was shown that the presence of $\text{IgE}^{\text{mn}+}$ in serum of intrinsic and extrinsic asthmatic patients was associated with severity of the disease.⁵ Further, mononuclear cells from children with atopic dermatitis and food hypersensitivity produced IgE -dependent HRF_{mn} , which declined with allergen avoidance and clinical remission of the skin disorder.³⁹ However, in the current study we showed that $\text{IgE}^{\text{mn}+}$ does not induce a LAR. $\text{IgE}^{\text{mn}+}$ and the tendency to develop a LAR might be independent risk factors for severe asthma. Because HRF has been found in late-phase fluids,¹⁶ our results are compatible with an alternative hypothesis: HRF_{mn} is produced *during* the late or chronic allergic reaction, and the subsequent IgE reaction effects the severity of the allergic reaction.

CONCLUSION

Our results prove that IgE reactivity to HRF_{mn} is not required for the induction of a LAR as previously thought. However, the results do not exclude the possibility that HRF is produced/released during a late-phase reaction and that it exerts effect during this reaction. To verify this hypothesis, the prevalence of $\text{IgE}^{\text{mn}+}$ in serum of an additional group of severe asthmatic patients is currently being investigated.

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