

Allergic rhinitis to ragweed pollen

II. Modulation of histamine-releasing factor production by specific immunotherapy

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A number of cytokines, including histamine-releasing factors (HRFs), have a role to play in IgE-mediated asthma. However, the influence of HRF in allergic rhinitis without asthma remains to be revealed. This article presents a double-blind, placebo-controlled study on the role of HRF in ragweed-allergic rhinitis and its modulation by natural pollen exposure and specific immunotherapy (IT). Twenty-seven patients allergic to ragweed were randomly assigned to receive either preseasonal alum-precipitated aqueous extracts of ragweed or placebo. Before the onset of therapy and during the ragweed-pollen season, subjects were evaluated for each of the following: clinical scores, ragweed IgE and IgG antibody levels, and spontaneous and allergen-driven HRF production. Thirteen nonatopic volunteers were also studied in the same protocol. First, before the initiation of therapy, more HRF was produced by both unstimulated and ragweed-stimulated mononuclear cells (MNCs) of atopic subjects as compared to MNCs of nonatopic subjects. Second, MNCs of the placebo-treated group produced significantly more spontaneous and ragweed-specific HRF during the pollen season compared to the preseasonal values. Finally, specific IT not only improved the clinical manifestation of allergy but also prevented the seasonal rise of spontaneous and ragweed-driven HRF production, along with a well-known change in other immunologic parameters associated with successful IT. (J ALLERGY CLIN IMMUNOL 1992;89:87-94.)

Key words: Immunotherapy, rhinitis, HRF, basophil, releasability, histamine, ragweed, symptoms, IgE, IgG

The IgE-mediated mediator release by mast cells or basophils is clearly involved in the pathophysiology of allergic diseases.¹ In contrast, the differences in the profiles of anti-IgE or allergen-induced HR by leukocytes from different donors are not explained by differences in the amounts of serum IgE Abs alone. These observations suggest that an intrinsic defect at the basophil level itself or a modulatory influence of other factors is involved in the clinical manifestations of hypersensitivity reactions.

Abbreviations used

Ab:	Antibody
HRF:	Histamine-releasing factor
MNC:	Mononuclear cell
IT:	Immunotherapy
PNU:	Protein nitrogen unit
Con A:	Concanavalin A
HR:	Histamine release
AR:	Allergic rhinitis
PBS:	Phosphate-buffered saline
MW:	Molecular weight
HPLC:	High-performance liquid chromatography

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Recent investigations in mast cell activation are currently focusing on cytokines, particularly the HRF that induces or augments HR from basophils and mast cells and contributes to the late inflammatory reaction.²⁻⁶ HRF is generated by a variety of cells, including MNCs from atopic and nonatopic subjects, even in absence of stimulation. Several proteins with

MWs ranging from 15 to 90 kd were found,⁶⁻¹¹ but it is not clear yet whether these different HRF molecules are due to the heterogeneity of a single gene product or represent a common bioactivity shared by multiple proteins. Their role in the pathophysiology of allergic diseases remains unclear. Recent studies in grass-sensitive subjects with asthma have also documented an increase in the spontaneous and allergen-induced production of HRF by MNCs and the abrogation of the seasonal rise of HRF by IT.^{2-6, 12-15} Furthermore, HRF has been found in increased amounts in lavage fluids of patients with allergic asthma. Altogether, these data provided support for the clinical relevance of HRF in allergic asthma. However, there is as yet no firm evidence of its role in seasonal AR without asthma.

Therefore, the first aim of this study was assessing the role of spontaneous and allergen-driven HRF production on the pathophysiology of AR without asthma and its modulation by IT. Accordingly, a double-blind, placebo-controlled trial was performed, including 13 normal volunteers and 27 patients with AR to ragweed, who were randomly assigned to receive either preseasonal alum-precipitated extracts of ragweed or placebo before the pollen season. Preceding the initiation of therapy and during the ragweed-pollen season, the following parameters were evaluated for all volunteers: ragweed IgE and IgG Ab levels, symptom/medication scores, physician evaluation, and HRF production.

MATERIAL AND METHODS

Study groups

Twenty-seven ragweed-sensitive subjects, aged from 19 to 56 years, were included in this study after an informed consent was obtained. They were selected at the Allergy and Immunology Clinic of Le Centre Hospitalier de l'Université Laval according to the following characteristics: a clear-cut history of ragweed AR without asthma, a positive skin prick test to ragweed extracts (1/20 wt/vol of glycerinated extract; Omega Laboratories, Montreal, Canada), and significant titers (more than class 2) of antiragweed IgE Abs, as determined by RAST (Pharmacia, Piscataway, N.J.). None had any significant nonrelated diseases, and none was receiving previous specific IT. Thirteen nonatopic volunteers with no history of AR and with negative skin tests and negative RAST to common inhalants were also included in this study.

IT regimens

All atopic patients were first stratified into two groups by their skin sensitivity to ragweed as determined by end point titration. This procedure was done to obtain groups with even distribution of moderately and highly sensitive subjects. Then, patients underwent prick testing with serial threefold dilutions of ragweed extracts (10,000 PNU per

milliliter in sodium chloride solution containing human serum albumin [0.3 mg/ml] and phenol [5 mg/ml], Omega Laboratories), starting at a concentration of 10,000 PNU per milliliter. Fifteen minutes after injection, wheal reactions were measured, and the dilution (end point) eliciting a wheal reaction equal to that of the positive control (histamine, 1 mg/ml in buffer solution with 50% glycerine)¹⁶ was determined. Afterward, patients were randomly assigned to receive between May and July subcutaneous injections of either alum-precipitated extracts of ragweed (50, 100, 200, 500, 1000, 2000, 3000, 3000, and 3000 PNU) in 9 consecutive weeks, or placebo (solution of histamine phosphate [0.5 mg/ml] to which caramelized glucose was added to make it undistinguishable in color compared to that of treatment solution). Neither the physician administering the injections nor the patient was aware of the individual's therapeutic regimen. Patients receiving placebo or ragweed injections had only a local wheal reaction of <2 cm of induration. In one case only, a patient developed a late (6 hours) local reaction of 8 cm accompanied with dyspnea; then, an additional dose of 50 PNU was repeated before continuing the schedule.

Assessment of clinical status

During the pollen season (mid-August to mid-September), all patients were asked to complete daily symptom diary cards. Then, each patient was requested to evaluate his symptoms for the previous 12 hours at noon and bedtime. Separate ratings were allowed for sneezing, itchy nose, rhinorrhea, nasal obstruction, lacrimation, and/or itchy eyes according to the following scale¹⁷: 0, none: no symptoms evident; 1, mild: trivial, definitely present, but not bothersome; 2, moderate: bothersome, but not disabling or intolerable; 3, severe: disabling and/or interfering with daily activities and/or sleep.

Also the patients were asked to report the type and the amount of permitted medication (chlorpheniramine, 4 mg tablets; pseudoephedrine, 60 mg tablets; naphazoline hydrochloride, pheniramine maleate, and eyedrops). The numerical scores were totaled to derive a daily symptom score for each patient day. During the ragweed season, the patients were clinically evaluated twice at 14-day intervals according to the same criteria by an allergist unaware of the treatment received by the patients.

Before the onset of IT (May) and during the pollen season (September), immunologic parameters, including serum-specific IgE and IgG Ab levels and HRF production from MNCs, were assessed in each participant.

Ragweed-specific IgE and IgG Ab determination

Serum antiragweed IgE Ab levels were determined on each coded samples by radioimmunoassay, as previously described.¹⁸ Briefly, disks were first coated (500 ng per disk) with concentrated form of crude ragweed extract described above, saturated with ethanalamine, 0.9%, and then incubated with human serum for 3 hours at 37° C. Unbound immunoglobulins were removed by a wash with Tween

(0.01%)-PBS (0.1 mol/L, pH 7.2), and ^{125}I -labeled anti-IgE was added to disks and incubated for 6 hours at 37° C. After extensive washes with Tween-PBS, the radioactivity fixed on disks was counted in a gamma counter (LKB, Bromma, Sweden). Results were means of duplicate analyses and expressed in counts per minute.

Serum-specific IgG Abs to ragweed were measured by ELISA with a modification of the technique previously described.¹⁹ Briefly, 96-well microtiter plates were coated with concentrated form of crude ragweed allergen described as above (100 ng per well), saturated with 10% bovine serum albumin, and then incubated with human serum for 2 hours at room temperature. After washes, alkaline-phosphatase-labeled antihuman IgG antiserum (Zymed Laboratories, San Francisco, Calif., 1:1000) was added for 1 hour before addition of 4-nitrophenyl phosphate (1 mg/ml). The reaction was stopped 1 hour later by the addition of sodium hydroxide (3 mol/L), and the absorbance was read at 405 nm with an automated spectrophotometer (Dynatech Laboratories, Chantilly, Va.).

HRF production

MNCs from atopic and nonatopic subjects were isolated by centrifugation over a Ficoll-Hypaque gradient. After washes with Hanks' balanced salt solution, the cells were resuspended at a concentration of $5 \times 10^6/\text{ml}$ in RPMI 1640 supplemented with L-glutamine, penicillin, and streptomycin. MNCs were incubated either alone, or with mitogen (Con A, 10 $\mu\text{g}/\text{ml}$), or with ragweed extracts (0.01, 0.1, and 1 $\mu\text{g}/\text{ml}$) for 4 hours at 37° C in 5% CO_2 . The cells were then carefully washed twice with Hanks' balanced salt solution, resuspended in culture medium alone, and incubated under the same conditions during the following 20 hours. The supernatants containing HRF were collected by centrifugation at 1000 rpm for 10 minutes. Cell viability, as assessed before and after culture by trypan blue exclusion, was >90%. To remove histamine, all culture supernatants were dialyzed against water and PBS at 4° C (MW cutoff of 3500, Spectrum Medical Industries, Los Angeles, Calif.) and stored at -20° C for basophil HR assay. In some experiments, MNCs were incubated with ^{125}I -labeled ragweed for 4 hours and then washed, and the radioactivity was counted in a gamma counter (LKB, Bromma, Sweden). Ragweed was iodinated with ^{125}I with Iodo-Gen or Iodo beads (Pierce Chemical Co., Rockford, Ill.). Free ^{125}I was separated from ^{125}I -labeled protein by passage through a Sephadex G-50 column. Specific activities were estimated according to the instructions of the company and were in the range of 5 to 12×10^6 cpm/ μg .

Basophil HR assay

The basophil HR experiments were performed as described in preceding articles.^{1, 18, 20} Briefly, heparinized blood was drawn from a single atopic donor. Leukocyte-enriched pellets containing basophils were obtained by a dextran sedimentation (Dextran T-500, Pharmacia) and resuspended at a final concentration of 3×10^6 cells per milliliter in cell-free supernatant containing HRF. After a 30-minute incubation at 37° C, the reaction was stopped by cooling, and

the supernatant was recovered for histamine determination with a single radioenzymatic assay.²¹ The results were expressed as percent of HR and calculated as follows:

$$\% \text{ HR} = (A - B)/T \times 100$$

where A equals HRF-induced HR from basophils, B equals buffer-induced HR from basophils, and T equals total histamine content of basophils.

Each experiment was performed in triplicate.

Gel filtration HPLC procedure

Concentrated crude supernatants of ragweed-stimulated MNCs were applied onto 75 by 600 mm TSK G2000 SW and TSK G4000 SW (Beckman Instruments, Inc., San Ramon, Calif.) gel filtration HPLC columns equilibrated with Tris buffer (25 mmol/L, pH 7.4) containing CaCl_2 (0.6 mmol/L) and MgCl_2 (1 mmol/L). The column was run at 0.2 ml/min, and 1 ml fractions were collected. Samples were then assayed for HR activity, as described above.

Statistical analysis

Results were expressed as means of percentage of $\text{HR} \pm \text{SEM}$. Results were analyzed via point-by-point comparisons with a least-significant difference procedure on the least-square means (LS Means procedure, SAS Proc GLM, Cary, N.C.),²² supplemented by a test for heterogeneity of slopes and common intercept. The effects of specific IgE Abs and HRF synthesis were analyzed by analysis of covariance procedure. Finally, correlations between various parameters were computed by Spearman's rank-correlation coefficient.

RESULTS

Characteristics of the study groups

Twenty-seven patients allergic to ragweed were randomly divided into two groups of comparable skin sensitivity to ragweed, 14 receiving placebo and 13 receiving ragweed-specific IT. Both groups were comparable in age, clinical history to ragweed, and pre-seasonal IgE Ab levels, as previously reported.¹ Thirteen nonatopic subjects were also included in this study.

Effects of IT on clinical responses, and IgE and IgG Ab levels

As previously reported,¹ daily symptom scores were significantly lower during the pollen season in the ragweed-treated patients than in patients receiving placebo. It was also demonstrated that the seasonal rise of IgE Ab production was effectively prevented by specific IT, whereas IgG Ab level was higher in patients receiving IT (data not presented).

Modulation of HRF production by specific IT

The production of HRF by unstimulated MNCs from atopic patients, studied before the pollen season

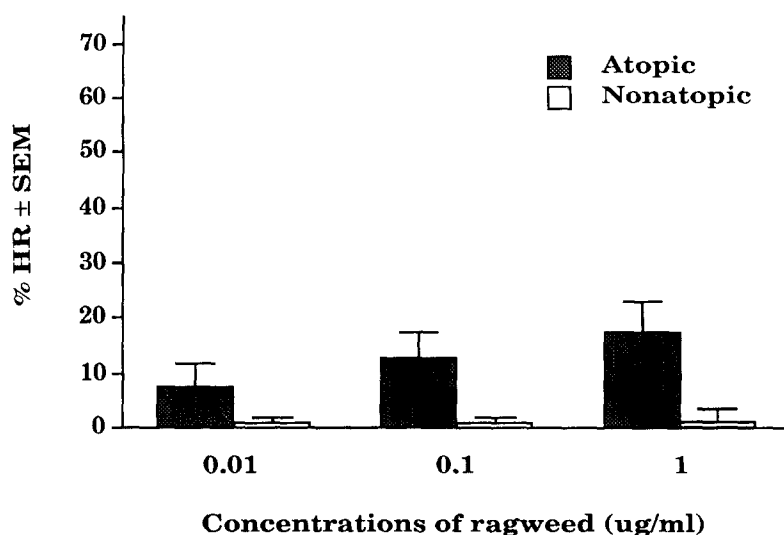


FIG. 1. Ragweed-driven HRF production in atopic and nonatopic subjects before desensitization protocol. Spontaneous HR levels have been subtracted from each mean value ($p < 0.02$).

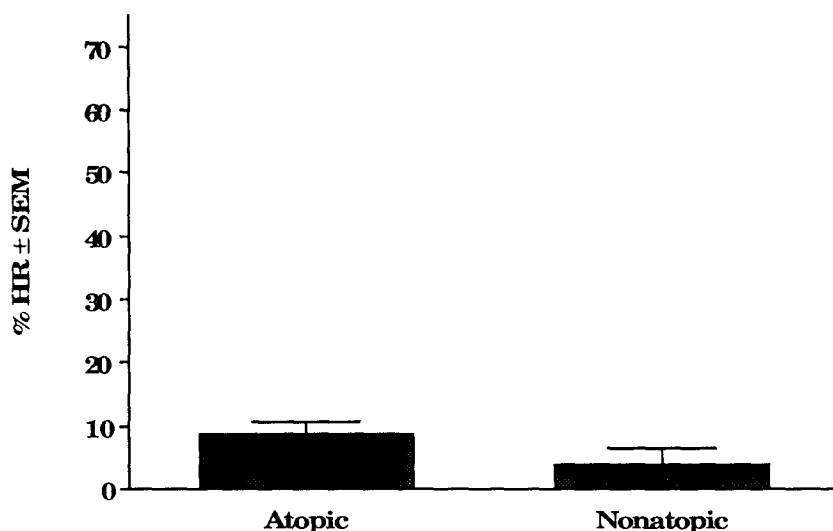


FIG. 2. Con A-driven HRF production by MNCs of untreated (*placebo*) and ragweed-treated subjects before and during the pollen season. Spontaneous HR levels have been subtracted from each mean value (p , not significant).

and before initiation of IT, and from nonatopic subjects were first compared. Then, this spontaneous production of HRF was significantly higher in atopic than in nonatopic subjects ($8\% \pm 1.8\%$ versus $4.4\% \pm 1.4\%$; $p = 0.05$; data not presented). By subtracting the values of HRF of unstimulated cells from the values of ragweed-induced HRF, more ragweed-specific HRF was produced by MNCs from atopic patients than from nonatopic patients on stimulation with various concentrations of ragweed ex-

tracts in a dose-response fashion (Fig. 1). This phenomenon was not explained by free allergen carried over in the culture, since no radioactivity could be detected in the cell supernatants when the cells were first incubated with ^{125}I -labeled ragweed (data not presented). In contrast, in Fig. 2, Con A-driven HRF synthesis tended to be slightly higher in atopic patients than in normal volunteers ($7.5\% \pm 2\%$ versus $3.8\% \pm 2.5\%$), but this difference did not reach statistical significance.

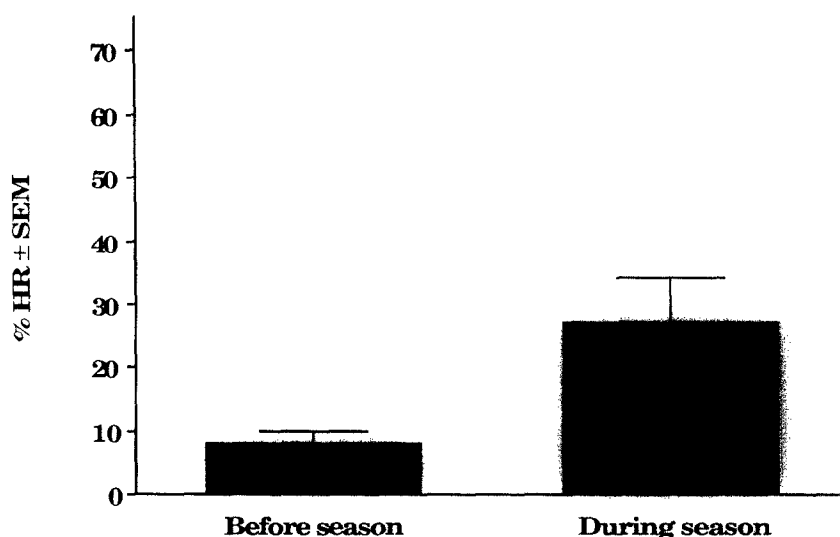


FIG. 3. Effect of natural exposure to pollen on the spontaneous HRF production in atopic subjects ($p = 0.05$).

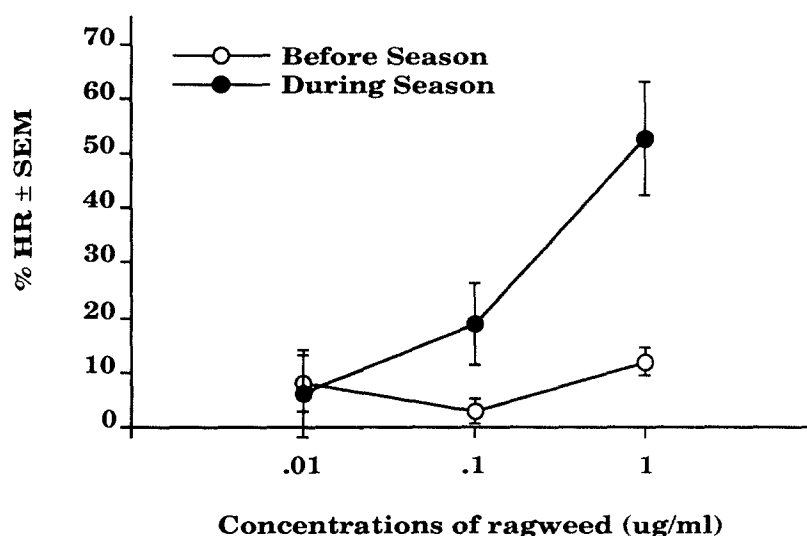


FIG. 4. Profile of ragweed-induced HRF by MNCs of atopic subjects before and during the pollen season ($p < 0.001$, heterogeneity of slopes). Spontaneous HR levels have been subtracted from each mean value.

The effects of our desensitization protocol on HRF production were next analyzed. First, as observed in Fig. 3, unstimulated MNCs of placebo-treated subjects produced more HRF during the pollen season compared to before the season ($27\% \pm 7.2\%$ versus $8\% \pm 1.8\%$, respectively; $p = 0.05$). In contrast, this seasonal rise of spontaneous HRF was partly prevented by specific IT ($26.7\% \pm 9.2\%$ versus $10.7\% \pm 3.4\%$; $p = 0.6$; data not presented). Second, when ragweed-specific HRF production was an-

alyzed (Fig. 4), MNCs of the placebo-treated group produced significantly more ragweed-specific HRF during the pollen season compared to the preseasonal values ($p < 0.001$, heterogeneity of slopes), especially at 0.1 ($18.9\% \pm 7.7\%$ versus $2.7\% \pm 1.4\%$; $p = 0.05$) and 1 $\mu\text{g/ml}$ ($52.8\% \pm 10.5\%$ versus $12\% \pm 2.7\%$; $p = 0.003$) of allergen. Moreover, no significant difference in Con A-specific HRF synthesis was found between the two groups (data not presented), which ensures the specificity of our findings.

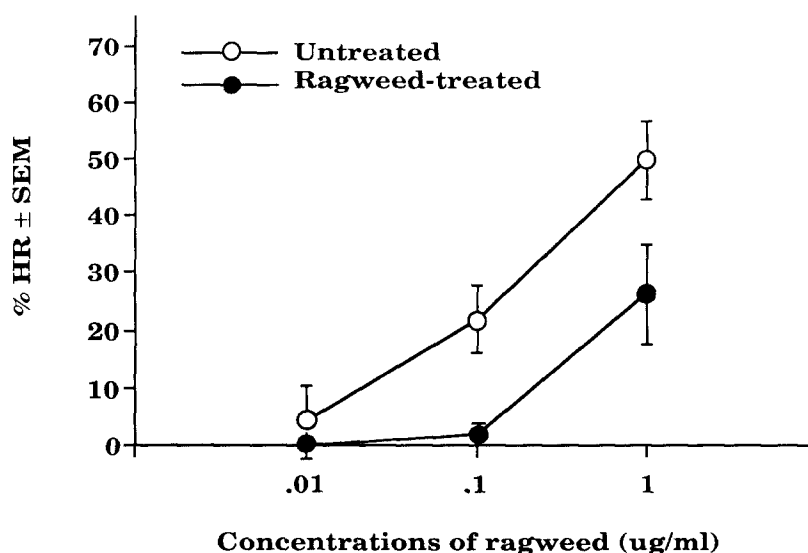


FIG. 5. Profile of ragweed-induced HRF by MNCs of untreated (*placebo*) and ragweed-treated subjects during the pollen season ($p < 0.005$, heterogeneity of slopes).

As illustrated in Fig. 5, specific IT significantly decreased the aforementioned seasonal rise of the ragweed-specific HRF synthesis. Indeed, less ragweed-induced HRF was produced during the pollen season in treated subjects than in untreated subjects, particularly at 0.1 ($2\% \pm 1.2\%$ versus $21.9\% \pm 6\%$; $p < 0.03$) and 1 $\mu\text{g/ml}$ ($26.2\% \pm 8.7\%$ versus $49.8\% \pm 6.7\%$; $p < 0.04$) of allergen. These data were studied with a regression-analysis procedure that selected out the possible discrepancy between groups before the pollen season. In addition, no significant difference was observed in Con A-induced HRF synthesis before and during the pollen season ($7.5\% \pm 2\%$ versus $10.6\% \pm 5\%$; p , not significant; data not presented).

MW of HRF

To demonstrate that the HRF molecules herein reported had MWs comparable to MWs described previously by other groups,⁶⁻¹¹ crude cell-free supernatant containing HRF was injected onto a gel filtration HPLC column, allowing a separation according to MW, and subsequently the HR activity of the peaks was measured as described above. The activity yield was mostly eluted at MW of about 30 kd (data not presented).

DISCUSSION

The releasability of peripheral blood basophils and of mast cells from the skin and nasal mucosa of atopic patients after challenge with the relevant allergen is clearly greater than that of normal volunteers, and this

process depends on the amount of IgE Abs present on the cell surface.^{1, 23-26} However, there is much evidence that tends to suggest that the recently described HRF molecules have also a role to play in this phenomenon. Our study first revealed a higher spontaneous and ragweed-driven HRF production by patients with AR without asthma before natural exposure to pollen and when these patients were compared to normal volunteers. Then, to obtain reproducible data, the HRF activity was performed by use of basophils of a single atopic donor and on a single occasion. It has been suggested that the mechanism of action of HRF-induced HR is an IgE-dependent process and that this response could differ, depending on the source of IgE used. Therefore, it is expected that different numbers would have been obtained with different donors, but we can reasonably expect that the overall trend should be in the same direction and the same magnitude.

These results combined to the previous studies by Alan and Rozniecki¹⁰ and Alam et al.,^{10, 12, 13} demonstrating that lymphocytes from grass-sensitive patients with asthma produced spontaneously more HRF in vitro, suggest that elevated HRF production may be a marker of atopy in addition to the increased IgE Ab synthesis. Moreover, Alam et al.¹⁴ additionally indicated that the spontaneous HRF production by lymphocytes correlated with the severity of asthma, as assessed by metacholine bronchial challenge. In contrast, we determined in our study a higher spontaneous HRF production in patients studied before the season when they were asymptomatic. These data suggest that elevated spontaneous HRF production had

no direct reference to the disease activity in rhinitis and that this is quite different from what is reported in the activity in asthma. However, during the pollen season, both spontaneous and ragweed-induced HRF production was increased, whereas no seasonal effect of Con A-stimulated HRF was observed. These data underline the fact that natural pollen exposure induces a state of nasal hyperreactivity not only through IgE Ab synthesis but also by the modulation of the leukocyte HR through cytokine production. These cytokines could be the HRF itself or the recently described factor referred to as histamine-releasing inhibitory factor, presumably interleukin-8,^{27,29} which inhibits the action of HRF and which can be lacking in atopic patients. Furthermore, in our study limited to patients with rhinitis, the magnitude of HRF or the antiragweed IgE Ab levels had no bearing on the intensity of seasonal symptoms.

In contrast, we also demonstrated that specific IT prevents the seasonal rise of ragweed-induced HRF synthesis. The specificity of our results is ensured by the lack of seasonal effect on Con A-HRF synthesis. Along the same lines, Kuna et al.¹⁵ suggested recently that clinical improvement of grass-sensitive patients with seasonal asthma by specific IT correlated with the abrogation of seasonal rise of spontaneous and antigen-driven HRF production by MNCs. The authors then compared the preseasonal values to values obtained during the season, and their data suggested that HRF might be involved in the pathogenesis of pollen-induced asthma. But, their study is weakened by the fact that one concentration of grass allergen rather than a dose-response curve had been used and that the preseasonal values of grass-induced HRF production had been compared to the spontaneous HRF production rather than to grass-induced HRF obtained during the pollen season. Therefore, we believe that our results provide clearer indications that HRF is relevant to pollen allergy, as evidenced by the demonstration of significant difference in HRF production between normal volunteers and atopic patients and its modulation by pollen exposure and desensitization. However, in our study, in which all patients were benefitted from the preseasonal IT, we found no correlation between both the spontaneous and allergen-specific HRF synthesis and the degree of alleviation of clinical symptoms, whereas in a previous study,¹ we reported a significant correlation between clinical symptoms observed during the pollen season and the combination of IgE Ab level and the percentage of basophil histamine releasability by antigen. This lack of correlation could be explained by the too small sample size or by the fact that HRF is measured by a biologic assay. Indeed, the absence of quantitative

measurements of HRF makes it almost impossible to correctly demonstrate clinical correlations.

Although HRF has been reported to be derived from a variety of cell sources, it has not been purified from any of these sources. Preliminary data suggest that MW of HRF fractionated on gel filtration HPLC columns was estimated to be 30 kd. Additional research to characterize and to quantify HRF is in progress. Direct quantitation of these cytokines will allow us to determine precisely if they contribute to determining the severity of symptoms in AR or if they are a secondary phenomenon related to the degree of atopic sensitivity. Altogether, our observations suggest that the presence of HRF is an important feature of allergic diseases, as evidenced by the difference in its *in vitro* production between normal and atopic patients and its modulation by natural pollen exposure and specific IT.

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