

Original article

Skin test diagnosis of grass pollen allergy with a recombinant hybrid molecule

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Background: A recombinant hybrid molecule (HM) consisting of 4 major allergens from timothy grass (Phl p 1, 2, 5, and 6) was expressed in *Escherichia coli*, purified, and characterized regarding its immunologic properties.

Objective: We sought to determine whether the recombinant HM can be used for the diagnosis of grass pollen allergy by means of skin testing.

Methods: Skin prick testing was performed in 32 patients with grass pollen allergy and in 9 control individuals by using increasing concentrations (4, 12, 36, and 108 µg/mL) of the HM and using commercial grass pollen extract. Specific IgE reactivities against the HM, grass pollen extract, and a panel of purified grass pollen allergens (recombinant Phl p 1, 2, 5, 6, 7, 12, and 13 and natural Phl p 4) were measured by means of ELISA, and timothy grass pollen-specific IgE levels were determined by using ImmunoCAP.

Results: Grass pollen allergy was diagnosed in all patients by means of skin testing with the HM. No false-positive skin test responses were obtained in the control individuals. There was an excellent correlation between IgE levels obtained with the HM and natural grass pollen extract measured by means of ELISA ($r = 0.98$, $P < .0001$) and by means of ImmunoCAP ($r = 0.98$, $P < .0001$).

Conclusions: The recombinant HM permitted accurate and specific *in vivo* diagnosis of grass pollen allergy in all tested patients. It can be considered a well-defined tool for the diagnosis and perhaps for immunotherapy of grass pollen allergy.

Clinical implications: A recombinant HM can replace traditional allergen extracts for skin test-based diagnosis of

grass pollen allergy. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Allergy, grass pollen, recombinant hybrid allergen, skin testing, diagnosis

Grass pollens are one of the most important airborne allergen sources worldwide and can elicit severe forms of allergic manifestations, such as asthma.¹⁻⁴ Pollens from various grass species contain highly cross-reactive allergens that, depending on initial biochemical studies and on the frequencies of patients' IgE reactivities, have been assigned to certain groups. Thirteen allergens are present in grass pollens, and the main frequencies of reactivity are attributed to group 1 (>90%), group 2 (>60%), group 5 (<90%), and group 6 (76%).⁵ Certain allergen groups are not present in every grass species. For example, it has been shown that Bermuda grass pollen does not contain detectable amounts of group 2, group 5, or group 6 allergens.⁶ On the other hand, timothy and rye grass pollen contain the majority of IgE epitopes present in most grass species.⁶ For this reason, timothy grass pollen allergens have been characterized in great detail at the molecular level.⁷ Recombinant timothy grass pollen allergens mimicking the immunologic properties of natural extract-derived allergens have been produced.⁸ It has been shown that individual recombinant timothy grass pollen allergens can be used for reliable *in vitro* and *in vivo* diagnosis of grass pollen allergy and can replace traditional extracts.⁹⁻¹² A clinically successful immunotherapy trial performed with the major recombinant timothy grass pollen allergens Phl p 1, 2, 5, and 6 has recently been reported.¹³ The crude grass pollen extracts available represent complex and variable mixtures of allergenic and nonallergenic components in which certain allergens might be underrepresented or vary strongly as regards their amounts.¹⁴ Hence it might be desirable to replace traditional grass pollen extracts for diagnosis and therapy with recombinant allergens. To facilitate the diagnostic and therapeutic applications of recombinant allergens for grass pollen allergy, we engineered a recombinant hybrid molecule (HM) containing the majority of grass pollen-specific epitopes.^{15,16} We produced a recombinant HM consisting of the 4 major timothy grass pollen allergens Phl p 1, 2, 5, and 6 in *Escherichia coli* and characterized the purified molecule regarding its immunologic properties.¹⁶ *In vitro* IgE

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Abbreviation used

HM: Hybrid molecule

measurements indicated that the HM can be used for the diagnostic identification of patients with genuine grass pollen allergy. This result has been achieved because the HM was engineered to contain major grass pollen-specific marker allergens only, which, when used in combination, allowed accurate diagnosis of grass pollen allergy in different populations. It did not contain cross-reactive panallergens (ie, Phl p 7 and Phl p 12), which are present in several allergen sources unrelated to grass pollen.¹⁶

Here we report the results of a clinical study in which we investigated whether the recombinant HM can be used for the *in vivo* diagnosis of grass pollen allergy by means of skin testing.

METHODS

Subjects

Thirty-two patients with grass pollen allergy were included in the study on the basis of positive skin test results to a mixture of natural grass pollen extracts, including timothy, orchard grass, and rye grass (100 IR/mL; Stallergènes Laboratories, Antony, France) and of a clinical history of grass pollen allergy (rhinitis, conjunctivitis, and/or asthma during May and June). This group included 18 men and 14 women (mean age, 31.9 years). For control purposes, we also included 9 more individuals: 5 nonallergic subjects (mean age, 26.6 years) and 4 subjects allergic to aeroallergens other than grass pollen allergens (mean age, 30.4 years). Demographic (age and sex) and clinical (symptoms and sensitization to other respiratory allergens according to our usual panel¹⁷) data were recorded for each patient and are displayed in Table I. After informed consent was provided, skin tests were performed, and blood was collected for serum sampling. Ten of the 32 patients agreed to provide a complementary blood sample for histamine release tests. Only patients who had not received specific immunotherapy in the last 5 years were allowed to participate in the study. None of the patients was taking antihistaminic medication at the time of the study. Ethical approval for skin testing with recombinant allergens in human subjects was obtained from the "Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale" of Strasbourg.

Allergen extracts and purified allergens

The natural grass pollen extract used for comparison was a mixture of 3 different grass species: *Phleum pratense* (timothy grass), *Dactylis glomerata* (orchard grass), and *Lolium perenne* (rye grass; Stallergènes Laboratories). It was used at a concentration of 100 IR/mL, as recommended by the manufacturer. The contents of group 1, 2, and 5 allergens were measured by using ELISA assays.^{14,18} Recombinant Phl p 1, 2, 5, 6, 7, 12, and 13, as well as natural Phl p 4, used for the determinations of patients' IgE reactivity profiles were purified as previously described.¹⁹⁻²³ The recombinant HM consisting of Phl p 1, 2, 5, and 6 was expressed in *E coli* and purified as previously described.^{15,16}

Skin testing

Four 3-fold increasing concentrations (4, 12, 36, and 108 µg/mL) of the HM were applied. The lyophilized HM was reconstituted and

TABLE I. Demographic and clinical characterization of study subjects

Patient no.	Demographic and clinical data			
	Age (y)	Sex	Allergies	Symptoms
1	38	M	g, t	r, c, as
2	18	M	g	r, c, as
3	37	M	g	r, c, as
4	23	F	g, w	r, as
5	25	M	g, m	r, c, as
6	25	F	g, a	r, c
7	25	F	g, t, m	r, c
8	26	F	g, t, w	r, c
9	24	M	g, t	r
10	52	F	g, t	r, c
11	31	M	g, t, m	r, c
12	30	F	g, a	r, c, as
13	44	F	g, m, a	r, c, as
14	25	M	g, a	r, c, as
15	32	M	g, t	r
16	21	F	g, m	r
17	47	M	g, w, a	r, c
18	50	M	g, w, m	r, c
19	28	F	g, t, w, a, m	r, c
20	22	M	g, t	r, c
21	20	F	g, w	r, c
22	52	F	g, t, m	r, c, as
23	31	M	g, t, w	r, c
24	38	M	g, t	r, c
25	21	F	g, t, w	r, c
26	23	M	g, t	r, c
27	24	M	g, w, a	r, c
28	55	M	g, m	r, c
29	35	M	g, t, a	r, as
30	48	F	g	r, c
31	23	F	g, t, w, a, m	r, c, as
32	30	M	g, t	r, c
Demographic data for subjects allergic to allergen sources other than grass pollen				
31	M	m		r, a
33	F	a, m		r, c
24	M	t, m		r, c
40	F	t, co		r
Demographic data for nonallergic subjects				
28	F	0		
24	M	0		
25	F	0		
25	M	0		
31	M	0		

M, Male; g, grass pollen; t, tree pollen; r, rhinitis; c, conjunctivitis; as, asthma; F, female; w, weed pollen; m, mites; a, animals; co, cockroach.

diluted with 0.9% sodium chloride solution on the day of the test, as previously described.²⁴

The natural grass pollen extract (Stallergènes Laboratories) and the recombinant HM preparations at 4, 12, 36, and 108 µg/mL were applied in duplicates on the volar side of the 2 forearms. The 4 increasing concentrations of the HM were applied from the wrist to the elbow in reverse order. The test sites were placed 3 cm apart to avoid false-positive results. The skin was then pricked with Prick-Lancets (Stallergènes Laboratories). A negative control test was performed with saline solution, and a positive control test was done with histamine (10 mg/mL). The tests were performed by the same

investigator. The wheal and the flare contours were outlined 15 minutes later with a skin-marking pen. The areas of the wheals and the flares were determined by means of digital planimetry (Adobe Photoshop; Adobe, San Jose, Calif) and analyzed by using National Institutes of Health Image software. For each allergen preparation, the mean value of the skin test reaction was calculated. Wheals with a mean diameter of greater than 3 mm, wheal areas of greater than 7 mm², or both were regarded as positive. Local late reactions and generalized reactions were also recorded.

Detection of allergen-specific antibodies

Sera were tested for IgE antibodies to the HM, purified allergens, and natural grass pollen extract by means of ELISA.¹⁶ Specific IgE levels of timothy grass pollen extract were measured by using the CAP assay (Phadia, Uppsala, Sweden).

Histamine release assays

Whole heparinized blood samples were obtained from 10 of the 32 patients with grass pollen allergy to perform histamine release tests. Whole blood samples were exposed to different concentrations of the HM and anti-IgE according to the method described by Tanizaki et al.²⁵ Ten milliliters of venous blood was drawn into a plastic syringe containing 1 mL of heparin (17 IU/mL). Aliquots of 250 µL of different concentrations of HM (dose range, 10⁻⁴ to 10 µg/mL) and anti-IgE (dose range, 10⁻⁴ to 10⁻³ dilutions; Immunotech, Beckmann Coulter, Marseille, France) were added to the test tubes containing 500 µL of whole blood diluted 1:4 in Tris buffer (10 mmol/L Tris, 136 mmol/L NaCl, 2.7 mmol/L KCl, 0.23 mmol/L MgCl₂, 1.8 mmol/L CaCl₂, and 5.5 mmol/L glucose, pH 7.3). The aliquots were then incubated for 30 minutes at 37°C. The reaction was stopped, and the cell-free supernatants were obtained by means of centrifugation (375g, 5 minutes, 4°C). Aliquots of 200 µL of the cell-free supernatants were used for histamine quantification in an enzyme-immunoassay (Immunotech, Beckmann Coulter). All experiments were performed in duplicates. Total histamine was measured after cell lysis by means of repeated thawing and freezing. The results were expressed as percentages of the total histamine content. The parameter used to describe the degree of basophil sensitivity was the lowest allergen concentration inducing 30% of total histamine release.

Statistical analysis

The logs of the wheal areas (means of double determinations) were plotted in dose-response curves as a function of log HM concentrations. The model $\log A = a + b \log C$ was used, where A denotes the mean of the 2 areas obtained in duplicate tests, a denotes the intercept with the y-axis, b denotes the slope of the dose-response relationship, and C denotes concentration. The individual slopes were calculated as previously described.^{26,27}

Correlation between different parameters was tested by using Spearman nonparametric tests with Visualstat Professional software (Visualstat 2005, Visualstat Computing Inc, Smyrna, Ga). The mean wheal areas obtained with the different concentrations of the HM and with the crude natural extract were compared by using the Wilcoxon nonparametric test with Visualstat Professional software.

RESULTS

The HM permits sensitive and specific diagnosis of grass pollen allergy by means of skin testing

Each of the patients with grass pollen allergy had positive reactions to the HM at 108 µg/mL, with a wheal

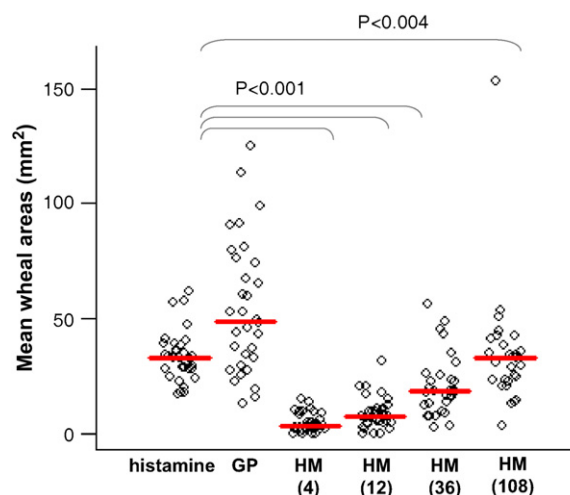


FIG 1. Dot-plot representation of the skin reactivities. Mean wheal areas (in square millimeters; y-axis) are displayed for the different concentrations (4, 12, 36, and 108 µg/mL) of the HM, histamine, and the natural grass pollen extract (GP; x-axis). Horizontal lines indicate median values. The natural grass pollen extract induced a significantly larger skin reaction than the HM for the 3 first concentrations ($P < .001$) and for the highest concentration ($P < .004$).

reaction greater than the threshold of positivity (wheal area, ≥ 7 mm²), whereas 31 (96.87%) of 32 patients reacted to 36 µg/mL of the HM. The percentage of positive skin prick test responses was lower at the first 2 concentrations tested. Nine (28%) patients with grass pollen allergy reacted at 4 µg/mL, and 18 (56.25%) reacted at 12 µg/mL.

The geometric mean wheal areas obtained with the different concentrations of HM are shown in Fig 1. In most cases the natural grass pollen extract induced a significantly larger skin reaction than the recombinant HM for each of the 4 concentrations ($P < .004$, Fig 1). With the crude grass pollen extract, the mean wheal area value was 54.48 mm², whereas with the highest concentration of the HM, it was 34.55 mm².

To study the dose-response relationship between recombinant HM concentrations and the areas of the skin prick tests, we calculated the regression line by using a model involving the log of the HM concentrations and the log of the mean wheal areas: a linear relationship among the 4 increasing concentrations of recombinant HM preparations and the mean wheal areas was established (Figs 2 and 3). Regarding the 32 patients, the mean slope was 0.630 (0.370-1.207). No positive test results were found in the 9 control patients, showing the high specificity of the test results obtained with the HM. Two patients with grass pollen allergy had late local reactions (ie, edema at the application sites) without systemic effects when tested with the 36 and 108 µg/mL concentrations of the HM 4 to 7 hours after the skin prick test. The wheal (mean diameter, 20 mm) was painful and persisted over 24 hours with the highest concentration (108 µg/mL).

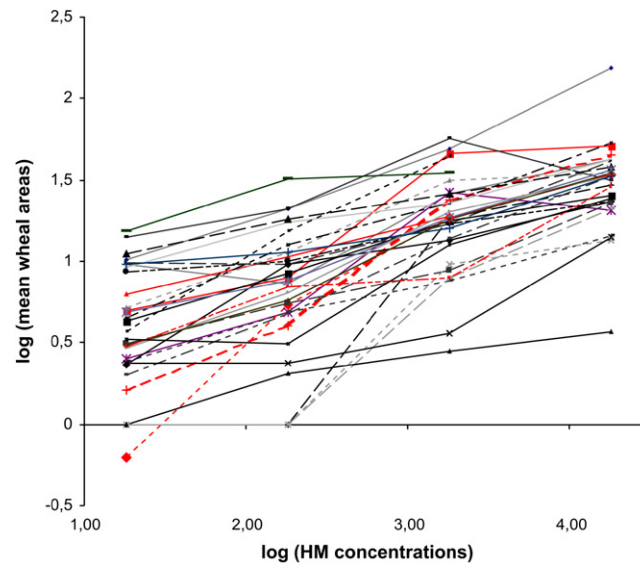


FIG 2. Dose-response curves of the log of the mean wheal areas (y-axis) obtained with the log of the 4 different concentrations of the HM (x-axis; $n = 32$). Three of the 32 patients were not tested at 108 $\mu\text{g/mL}$ because they had clearly positive test responses at 36 $\mu\text{g/mL}$. The calculation of the slope does not differ significantly with or without their results.

These local reactions disappeared gradually with oral corticosteroids.

The HM allows serologic diagnosis of grass pollen allergy and contains most of the IgE epitopes present in natural grass pollen extract

We found a significant correlation between the HM-specific IgE and natural grass pollen extract, specific IgEs measured by means of ELISA ($r = 0.98$, $P < .0001$), and also with timothy grass pollen-specific IgE measured by means of ImmunoCAP ($r = 0.98$, $P < .0001$, Fig 4). As previously reported, the HM also accounted for the majority of the IgE epitopes present in Phl p 1, 2, 5, and 6 (data not shown).¹⁶

Thirty-one of 32 sera of patients with grass pollen allergy contained specific IgE against HM, whereas 28 reacted positively against the natural grass pollen extract in the ELISA assays. Three of the 4 patients with negative specific IgE against the natural grass pollen extract were only sensitized to 2 grass pollen allergens at low level (<0.17 kUA/L), which is lower than the cutoff of the CAP system (ie, <0.35 kUA/L). They were all sensitized to Phl p 1 and also to Phl p 2, 4, or 5. One patient was monosensitized to Phl p 1. The geometric mean of HM-specific IgE levels was higher than the geometric mean of the natural grass pollen extract-specific IgE levels, although the respective highest and lowest specific IgE levels were not different. Furthermore, the analysis of sera by means of ELISA revealed that in all cases the sum of the levels of IgEs directed against recombinant timothy grass pollen allergens included in the HM was higher than

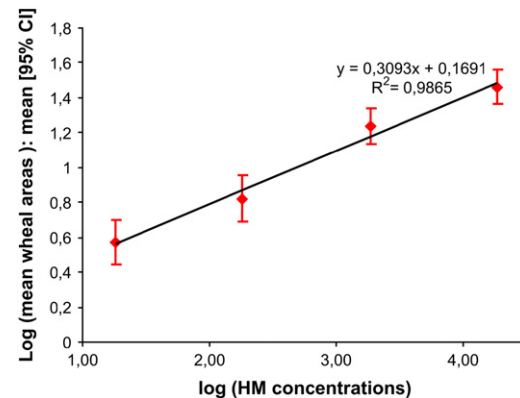


FIG 3. Dose-response curves of the log of the geometric mean wheal areas of the 32 patients and the 95% confidence limits (y-axis) obtained with the log of the 4 different concentrations of the HM (x-axis). The mean slope was 0.630 (0.370-1.207).

the natural grass pollen-specific IgE levels (data not shown). Fig 5 shows the specific IgE levels measured by means of ELISA for the HM, crude pollen extract, and the different grass pollen allergen molecules (Phl p 1, 2, 4-7, 12, and 13). The IgE-binding frequencies for rPhl p 1, 2, 4 to 7, 12, and 13 corresponded to those reported in previous studies.²⁸ Furthermore, we found complete concordance in the 32 patients with grass pollen allergy between positivity of skin prick test responses with the HM at 36 $\mu\text{g/mL}$ and HM-specific IgE detection. Considering the HM skin prick tests at 108 $\mu\text{g/mL}$, we had one discordance with the HM-specific IgE detection by means of ELISA (data not shown).

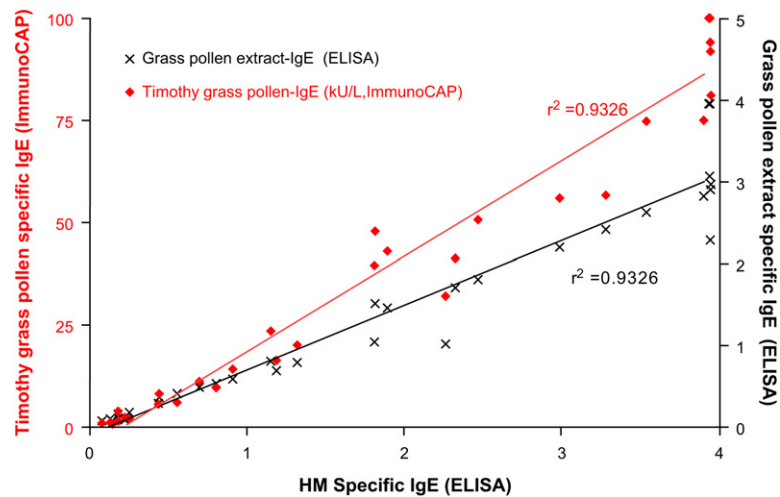


FIG 4. Correlation between HM-specific IgE levels (x-axis) and grass pollen extract-specific IgE levels (y-axis). *Right*, Grass pollen extract-specific IgE levels measured by means of ELISA. *Left*, Timothy grass pollen-specific IgE levels measured by means of ImmunoCAP (in kilounits per liter). A significant correlation ($r = 0.98$, $P < .0001$) was observed with both methods.

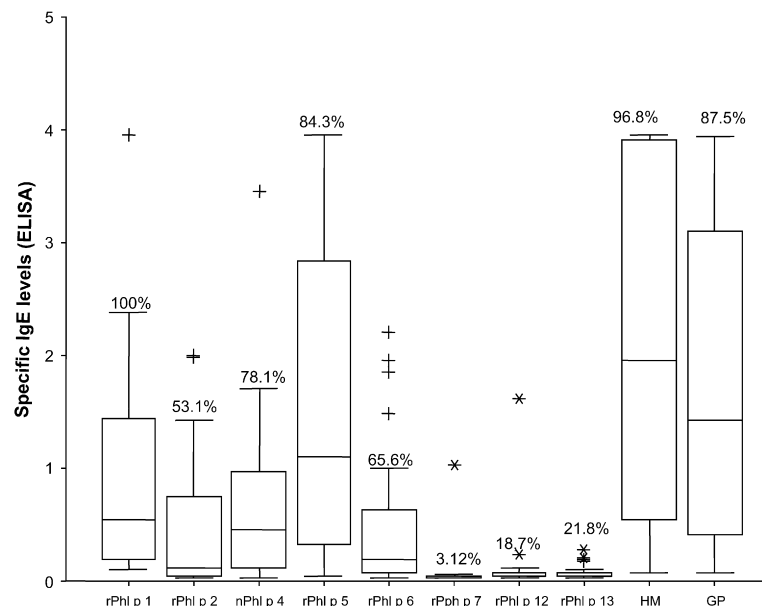


FIG 5. Box-plot representation of IgE levels. IgE levels (OD values) specific for individual grass pollen allergens, HM, and grass pollen extract (GP) are displayed for the 32 patients on the y-axis. Means and outliers (+), as well as the percentage of positive sera, are shown.

The HM induces specific and dose-dependent histamine release in basophils from patients with grass pollen allergy

Anti-IgE caused no histamine release in 3 of 10 subjects. Fig 6 shows the dose-dependent histamine release induced with the HM in 6 representative releasers. The seventh patient (patient 14), who released less than 25% of total histamine content with the HM and the anti-IgE, is not shown. The HM started to induce histamine release between 10^{-4} and 10^{-3} $\mu\text{g/mL}$, and a bell-shaped dose-response curve was observed.

DISCUSSION

We have recently constructed a recombinant HM combining the 4 most important timothy grass pollen allergens, Phl p 1, 2, 5, and 6. It has also been demonstrated that a mix consisting of these recombinant allergens allowed successful vaccination against grass pollen allergy.¹³ *In vitro* experiments have shown that the recombinant HM allowed accurate serologic diagnosis of grass pollen allergy.¹⁶ The aims of the study were to assess the diagnostic value of the new recombinant molecule

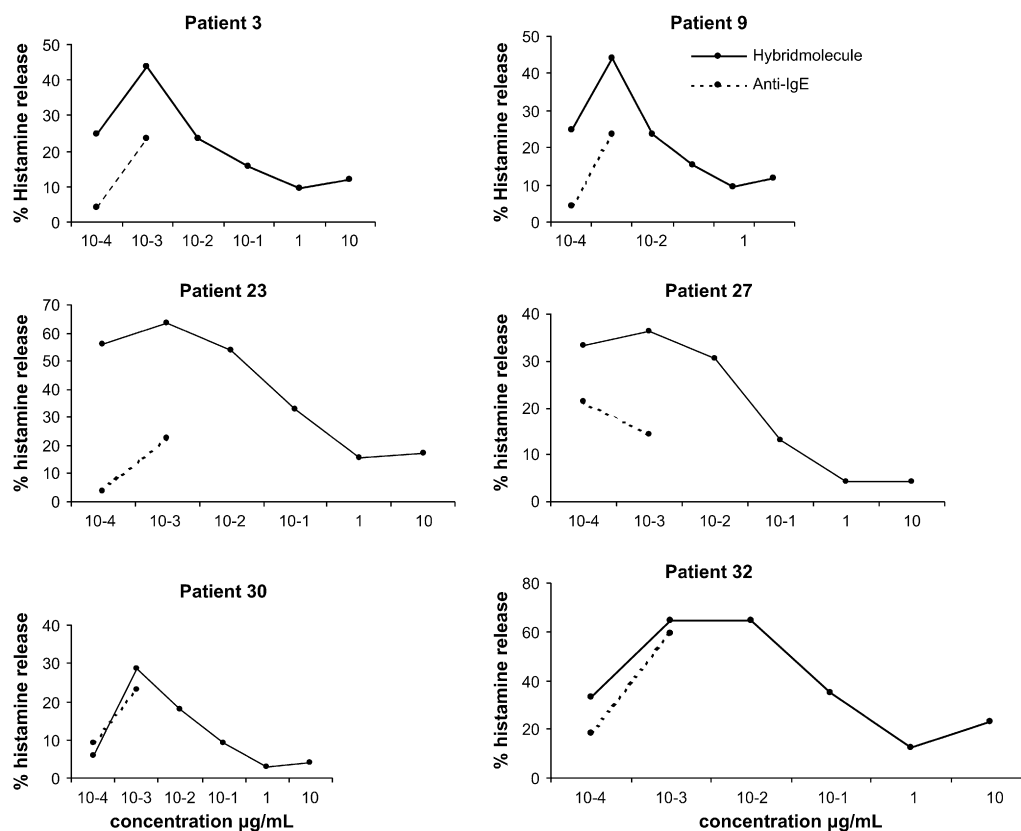


FIG 6. Induction of basophil histamine release with the HM. Basophils from 6 representative patients were exposed to increasing concentrations of the HM or anti-IgE (x-axis). The percentages of total histamine release are shown on the y-axis for each of the patients.

for skin test–based diagnosis of grass pollen allergy to study its safety profile and to define appropriate concentrations for diagnostic testing. The studies initially performed were only focused on *in vitro* investigations unable to identify the concentrations to be used for cutaneous tests with absolute certainty. It has been shown that with recombinant allergens, the concordance is generally good between *in vivo* and *in vitro* tests, although quantitatively, the correlations are not better than those found with crude extracts.¹⁷

The skin test results were comparable with those obtained with a natural allergen extract made out of pollens from 3 different grass species. Our results demonstrated that the recombinant HM induced dose-dependent skin reactions in each of the patients with grass pollen allergy but not in nonallergic individuals and not in allergic patients without grass pollen allergy. With a concentration of 36 µg/mL, we observed no important secondary local side effects, and all but 1 patient had been diagnosed. In fact, this patient had low specific IgE levels against rPhl p 1 only, and his symptoms lingered from spring to autumn, without any peak during the grass pollen season. At the moment, we are not able to define the HM's optimal concentration inducing a positive skin test response without an uncomfortable late reaction. A larger number of patients and concentrations should be tested to give

more precise dose recommendations. The most likely explanations for the stronger induction of skin reactivity by the natural allergen extract compared with the HM is the higher concentration of group 2 allergens (105.3 µg/mL allergens determined by means of ELISA) in the extract and possibly the presence of grass pollen allergens not included in the HM. Group 2 grass pollen allergens are indeed strong elicitors of cutaneous and nasal inflammation¹² and are also recognized by a high percentage of patients with grass pollen allergy. The absence of Phl p 7, 12, and 13 might have been responsible for the lower activity of the HM, but only few patients were sensitized against these allergens, and when they were, the allergen-specific IgE levels were low (Fig 5). Phl p 4 was frequently recognized, but several studies indicate that the biologic activity of Phl p 4 is very low.^{23,29}

To sum up, the HM allowed accurate diagnosis of grass pollen allergy and contained most of the IgE epitopes present in the grass pollen extract. It has important advantages over crude grass pollen extracts because it represents a well-defined molecule that can be manufactured under highly reproducible pharmaceutical conditions.

This HM could be considered a suitable candidate for *in vitro* and *in vivo* diagnosis of grass pollen allergy. Skin testing with the HM has 2 advantages at least. First, testing for the most important grass pollen–derived IgE epitopes

can be performed by using one molecule only. Second, the HM contains grass pollen-specific epitopes only, thus avoiding false-positive test reactions in patients sensitized to cross-reactive allergen molecules. The HM will also be particularly suitable for the desensitization treatment of patients with a matching IgE profile.⁸

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