

How far can we simplify in vitro diagnostics for grass pollen allergy?: A study with 17 whole pollen extracts and purified natural and recombinant major allergens

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Background: Current diagnostics for grass pollen allergy are composed of mixtures of pollen of different grass species. Their complex composition hampers accurate standardization. **Objective:** The aim of the study was to investigate whether mixtures of grass pollen extracts can be replaced by a single pollen species and whether a single pollen species can be replaced by a limited number of purified natural or recombinant major allergens.

Methods: Sera ($n = 800$) were selected on the basis of a general suspicion for inhalant allergy and tested in a RAST for IgE reactivity with pollen from 17 different grass species. Cross-reactivity of IgE responses was studied by means of RAST inhibition. Sera with positive test results for grass pollen were tested in a RAST for natural Lol p 1 and Lol p 5 and recombinant Phl p 1 and Phl p 5.

Results: Specific IgE antibodies against one or more of the 17 pollen species were detected in 209 of 800 sera (26.1%). The highest responses were observed against *Poa pratensis* followed by *Festuca rubra*, *Phleum pratense*, and *Dactylis glomerata*. IgE responses were clearly lower (approximately by a factor of 5) against only three species (*Phragmites communis*, *Cynodon dactylon*, and *Zea mays*). With the exception of a few low-responder sera, no sera were found to have negative test results to the high responder species and positive results to any of the other species. Sera with positive test results for grass pollen ($n = 154$) were tested with purified Lol p 1 and Lol p 5. IgE anti-Lol p 1 and Lol p 5 accounted for an average of $81\% \pm 7\%$ of total anti-grass pollen IgE. For 14 sera (all with low anti-grass pollen IgE titers), a RAST with purified allergens resulted in a false-negative diagnosis for grass pollen allergy. With recombinant Phl p 1 and Phl p 5, the mean IgE reactivity was $57\% \pm 6\%$ of the anti-grass pollen IgE response ($n = 141$), with 13 false-negative results.

Conclusion: One grass species is sufficient for in vitro diagnosis of grass pollen allergy. With purified natural Lol p 1 and Lol p 5, greater than 90% of grass-positive sera is detected. Around 80% of the IgE response to grass pollen is

directed to these major allergens. Recombinant allergens, produced in *Escherichia coli*, did not equal the IgE-binding capacity of their natural counterparts. (J Allergy Clin Immunol 1998;102:184-190.)

Key words: Grass pollen allergy, diagnostics, purified allergens, recombinant allergens, group 1 allergen, group 5 allergen

Both in vivo and in vitro diagnostics for grass pollen allergy are usually performed with grass pollen extracts from many different grass species. The same is true for application of grass pollen extracts in immunotherapy. The rationale behind this approach is to ensure that species-specific IgE-binding epitopes are represented as much as possible. It has been known for many years that a large share of IgE antibodies against grass pollen are cross-reactive among different grass species.¹⁻¹¹ By mixing different, largely cross-reactive species, any species-specific structure will be diluted in an excess of cross-reactive epitopes. It is therefore doubtful whether this approach results in sensitive detection of (or effective desensitization against) species-specific epitopes.

Over the past years, regulatory authorities have been increasing their demands with respect to standardization and quality control of allergen products for in vivo use. Defining the composition of mixtures of pollen extracts, each consisting of many different (glyco)proteins and polysaccharides, is an almost impossible task. The trend in allergen standardization is slowly moving from biologic standardization toward the measurement of major allergens.^{12,13} To facilitate accurate measurement of major allergens, mixing of different species should be avoided. Is it possible to replace the present generation of allergen products made from mixtures of grass pollen with products made from pollen from a single grass species? The first part of this study addresses this question from the perspective of in vitro diagnostics.

Simplification could even go a step further. The two most important major allergens in grass pollen are the group 1 and group 5 allergens. For group 1 allergens, a frequency of recognition by grass pollen-sensitive patients of 90% to 95% was reported.^{14,15} For group 5 allergens, this figure is around 80% to 90%.^{15,16} The second part of this study addresses the question of whether in vitro diagnostics for grass pollen allergy can be performed with a combination of purified group 1 and group

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Abbreviation used

BSA: Bovine serum albumin

5 allergens. This was investigated for both natural and recombinant molecules.

METHODS

Sera

From sera submitted for routine in vitro allergy diagnostics, a panel of 800 sera was selected on the basis of the following criteria: they were submitted for measurement of total IgE, measurement of specific IgE against the most common inhalant allergens, or both. In this way sera originated from subjects with a general suspicion of inhalant allergy.

A panel of sera ($n = 63$) from patients allergic to grass pollen living in areas with rye (*Secale cereale*) grass cultivation was kindly provided by Dr. F. Roossien (ARTU Biologicals, Lelystad, The Netherlands).

A panel of sera ($n = 240$) from subjects living in an area where Bermuda (*Cynodon dactylon*) grass is endemic was collected by the Bloodbank of Orlando, Florida. Sera were included when the following question was answered affirmatively: Do you know that you are allergic to grass pollen or do you suffer from hay fever symptoms during the spring and summer months?

Grass pollen extracts

Grass pollen from the following species were extracted as described previously¹⁵: *Poa pratensis*, *Festuca rubra*, *Phleum pratense*, *Dactylis glomerata*, *Arrhenatherum elatius*, *Lolium perenne*, *Holcus lanatus*, *Agrostis stolonifera*, *Anthoxanthum odoratum*, *Alopecurus pratensis*, *Secale cereale*, *Triticum vulgare*, *Hordeum vulgare*, *Avena sativa*, *Zea mays*, *Phragmites communis*, and *Cynodon dactylon*. Of each dialyzed and lyophilized extract, 100 mg (dry weight) was coupled to 5 gm of CNBr-activated Sepharose 4B (Pharmacia & Upjohn, Uppsala, Sweden) for application in RAST and RAST-inhibition assays.

Purified natural and recombinant allergens

Natural group 1 and group 5 allergens were purified from *L. perenne* grass pollen extract as described previously.¹⁵ Briefly, Lol p 1 was purified by hydrophobic interaction chromatography with Phenyl-Sepharose (Pharmacia & Upjohn) followed by gel filtration on Superdex 75 prep grade (Pharmacia & Upjohn). Lol p 5 was affinity-purified with monoclonal antibody 4B1. Purified recombinant Phl p 1 (rPhl p 1) and Phl p 5 (rPhl p 5), produced in *Escherichia coli*, were obtained from Biomay (Linz, Austria).^{8,17}

For application in RASTs, Lol p 1, Lol p 5, rPhl p 1, and rPhl p 5 were coupled to Sepharose at a concentration of 100 µg protein on 100 mg Sepharose. Protein was determined with BCA Protein Reagent (Pierce, Rockford, IL), with bovine serum albumin (BSA) as protein standard.

RAST/RAST inhibition

RASTs were performed as described previously.¹⁸ Briefly, serum (50 µl) was incubated overnight with 0.5 mg of Sepharose in a final volume of 300 µl phosphate-buffered saline/0.3% BSA/0.1% Tween-20. After washing away unbound serum components, radiolabeled sheep antibodies directed to human IgE were added. After overnight incubation and washing, bound radioactivity was measured. Results were expressed as RAST units (RU) calculated by means of a total IgE standard curve. This curve was obtained by incubating serial dilutions of a serum with known IgE content with Sepharose-coupled sheep antibodies

TABLE I. RAST values for 17 grass pollen species

	Mean ratio	95% CI
<i>F. rubra</i>	1.04	0.99-1.10
<i>Phl. pratense</i>	1.06	1.01-1.12
<i>D. glomerata</i>	1.06	1.01-1.11
<i>Ar. elatius</i>	1.18	1.12-1.24
<i>L. perenne</i>	1.19	1.14-1.25
<i>Hol. lanatus</i>	1.20	1.16-1.25
<i>Ag. stolonifera</i>	1.31	1.24-1.34
<i>Av. sativa</i>	1.39	1.30-1.49
<i>An. odoratum</i>	1.47	1.39-1.55
<i>Al. pratensis</i>	1.49	1.41-1.57
<i>T. vulgare</i>	1.65	1.53-1.78
<i>S. cereale</i>	1.71	1.61-1.81
<i>Hor. vulgare</i>	1.77	1.63-1.93
<i>Phr. communis</i>	5.45	4.65-6.38
<i>C. dactylon</i>	5.56	4.85-6.37
<i>Z. mays</i>	7.05	6.03-8.23

All RAST results were compared with responses to *Po. pratensis* (mean, 5.5 ± 0.8 RU/ml) in paired Student's *t* test. The mean ratios of the response to *Po. pratensis* and the 16 grasses are given. The 95% CIs of these ratios illustrate that the response to *Po. pratensis* is significantly higher than that to the other species, with the exception of *F. rubra*. The number of sera tested was 209, except for the cultivated grasses *Av. sativa*, *T. vulgare*, and *Hor. vulgare* ($n = 196$).

against human IgE instead of Sepharose-coupled allergen. A result of greater than 0.3 RU was regarded as positive.

For RAST inhibition, serum was preincubated for 1 hour with serial dilutions of inhibitor (allergen extract, 3.2 µg/ml to 2 mg/ml, or purified natural or recombinant allergen, 4 µg/ml to 0.5 mg/ml) before addition of Sepharose. For the uninhibited value, serum was preincubated with phosphate-buffered saline/0.3% BSA/0.1% Tween-20. Subsequent steps were identical to those described for the RAST. Results were expressed as percentage of the uninhibited value.

RESULTS

IgE responses to pollen from 17 different grass species

Of the 800 sera included in the study, 209 had a positive RAST (>0.3 RU/ml) result for one or more of 14 pollen species tested initially. The mean IgE responses decreased in the following order: *Po. pratensis*, *F. rubra*, *Phl. pratense*, *D. glomerata*, *Ar. elatius*, *L. perenne*, *Hol. lanatus*, *Ag. stolonifera*, *An. odoratum*, *Al. pratensis*, *S. cereale*, *Phr. communis*, *C. dactylon*, and *Z. mays* (Table I). Of these sera, 196 were subsequently tested on pollen of three additional cultivated grass species (*Av. sativa*, *T. vulgare*, and *Hor. vulgare*). In a paired Student's *t* test, the RAST values to *Po. pratensis* were shown to be significantly higher than to the other species, with the exception of *F. rubra* ($p = 0.073$). Spearman's rank correlation analysis revealed a close correlation between the IgE response to *Po. pratensis* and to the other 16 species (R_s [95% CI] ranging from 0.835 [0.789 to 0.872] to 0.992 [0.990 to 0.994]).

With a cut-off value of 0.3 RU/ml, 14 sera (6.7%) showed negative test results for *Po. pratensis* but positive

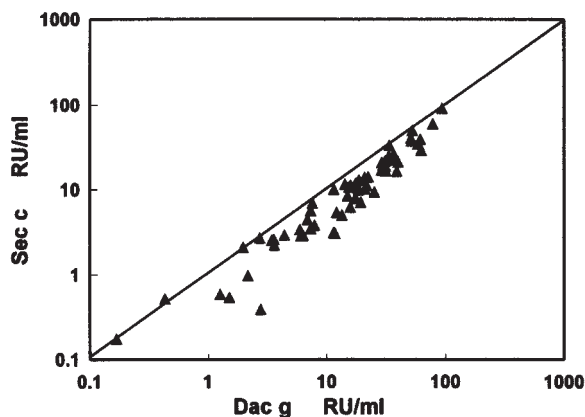


FIG. 1. High exposure to *S. cereale* (Sec c) pollen. RAST values ($n = 63$) for *S. cereale* pollen are plotted against those found for *D. glomerata* (Dac g) pollen. Results are expressed in RU/ml.

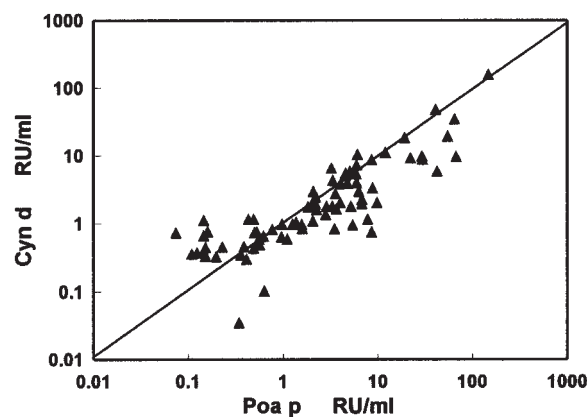


FIG. 2. High exposure to *C. dactylon* (Cyn d) pollen. RAST values ($n = 77$) for *C. dactylon* pollen are plotted against those found for *Po. pratensis* (Poa p) and *D. glomerata* pollen. Results are expressed in RU/ml.

results for one or more of the other species. For nine of these sera, all grass pollen RAST results were less than 0.5 RU/ml. The positive RAST results of the remaining five sera were, without exception, in the low range (<1.15 RU/ml). The number of false-negative results with single species-based grass pollen RASTs increased in the same order as the mean RAST values decreased.

Cross-reactivity of anti-grass pollen IgE

To confirm the cross-reactive nature of anti-grass pollen IgE, RAST inhibition was performed. The RAST for pollen of 14 species (*Phr. communis*, *C. dactylon*, and *Z. mays* not included) was inhibited with *Po. pratensis* and *D. glomerata* extract, as well as the respective homologous extracts. This analysis was performed for two sera (A and B) with a clear positive RAST for the 14 grass pollen (>10 RU/ml). In both cases, approximately 90% inhibition was reached at a concentration of 2 mg/ml of pollen extract (data not shown). For serum A, the mean of the quantities of *Po. pratensis* (59 ± 12 μ g/ml) and *D. glomerata* (63 ± 14 μ g/ml) needed for 50% inhibition was more than 2 times lower ($p < 0.002$) than that of the homologous inhibitors (134 ± 14 μ g/ml). For serum B, no significant difference ($p > 0.2$) was found between *Po. pratensis* (45 ± 11 μ g/ml) and the homologous inhibitors (35 ± 5 μ g/ml). *D. glomerata* (8 ± 2 μ g/ml) was, however, 4.5 times more potent as an inhibitor ($p < 0.001$). The degree of cross-reactivity between *Po. pratensis* and *D. glomerata* or *Phl. pratense* was investigated with 13 sera. In *D. glomerata* and *Phl. pratense* RAST inhibitions, *Po. pratensis* was at least as efficient as an inhibitor as both homologous extracts (data not shown).

For the low-responder species (*Phr. communis*, *C. dactylon*, and *Z. mays*), RAST inhibitions were also performed. Again, highly allergenic species like *D. glomerata* turned out to be more potent inhibitors than the homologous extracts (data not shown). Two sera with higher RAST values for either *S. cereale* or *C. dactylon*

(compared with *Po. pratensis* and *D. glomerata*) were also analyzed by RAST inhibition. Although the homologous extracts were more potent inhibitors, both *Po. pratensis* and *D. glomerata* inhibited both RASTs, with 60% to 80% inhibition at 2 mg/ml (data not shown).

Influence of high exposure to pollen of *S. cereale* or *C. dactylon*

IgE responses to pollen of cultivated grasses (e.g., *S. cereale*) and of grasses not growing in the Netherlands (e.g., *C. dactylon*) are lower than those to abundant grass species like *Po. pratensis* and *D. glomerata*, and they are largely cross-reactive to the latter species. We sought to determine if this is also true when exposure to these low IgE-binding pollens is higher. A panel of 63 sera from patients allergic to grass pollen living in the vicinity of rye (*S. cereale*) cultivation was tested in a RAST for *S. cereale* and for *D. glomerata*. The mean ratio (95% CI in a Student's t test) of the RAST values to both grass species (*D. glomerata* and *S. cereale*) was 1.7 (1.5 to 1.9). Responses to both species closely correlated (R_s [95% CI] = 0.970 [0.950 to 0.982]). No sera were found that reacted significantly stronger to *S. cereale* than to *D. glomerata* (Fig. 1). The same comparison was made between responses to both species for the group of 209 sera with normal pollen exposure. The overall picture was virtually identical: the mean ratio *D. glomerata*/*S. cereale* was 1.6 (1.5 to 1.7) and the R_s was 0.978 (0.971 to 0.983).

A panel of 240 sera from subjects exposed to Bermuda grass (*C. dactylon*) pollen was tested in a RAST for *C. dactylon*, *Po. pratensis*, *Phl. pratense*, and *D. glomerata*. The percentages of positive RAST results (>0.3 RU/ml) were 32%, 29%, 28%, and 29%, respectively. IgE responses to *Po. pratensis* and *D. glomerata* were 1.2 (95% CI: 1.0 to 1.5) times higher than those to *C. dactylon*. There was no significant difference between the RAST results for *Phl. pratense* and *C. dactylon* (*Phl. pratense*/*C. dactylon*, 1.2; 95% CI, 0.9 to 1.4). When the mean ratios (95% CI) *Po. pratensis*/*C. dactylon*, *D. glom-*

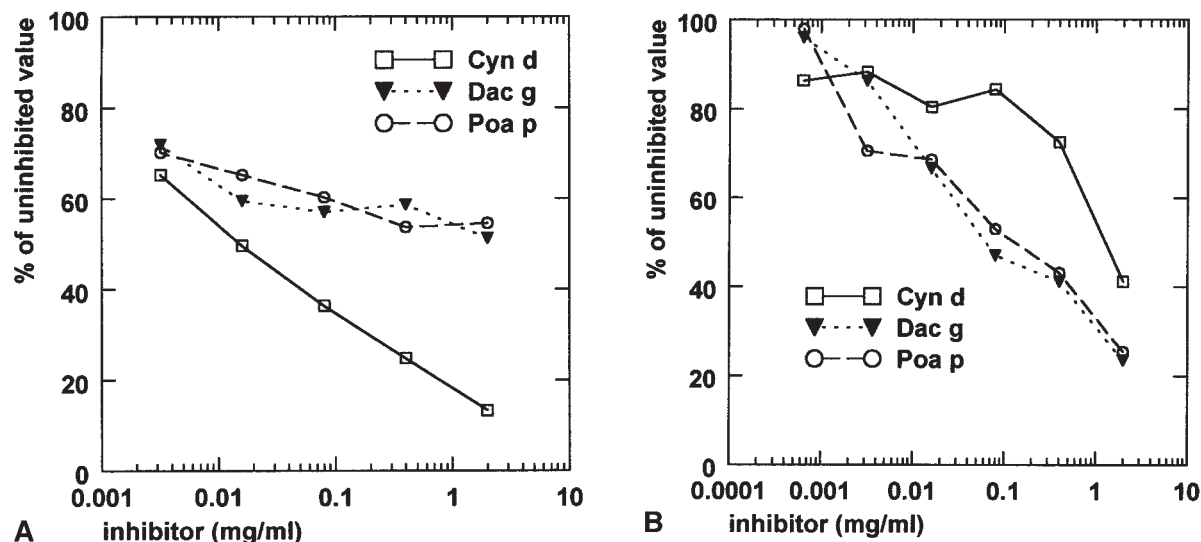


FIG. 3. *C. dactylon* (Cyn D) RAST inhibition. *C. dactylon* RAST was inhibited with *C. dactylon*, *D. glomerata* (Dac g), and *Po. pratensis* (Poa p). RAST values of two sera for *C. dactylon*, *D. glomerata*, and *Po. pratensis*, respectively, were 157.8, 162.1, and 147.5 RU/ml (A) and 5.5, 5.3, and 5.9 RU/ml (B). Results are expressed as percent of uninhibited value.

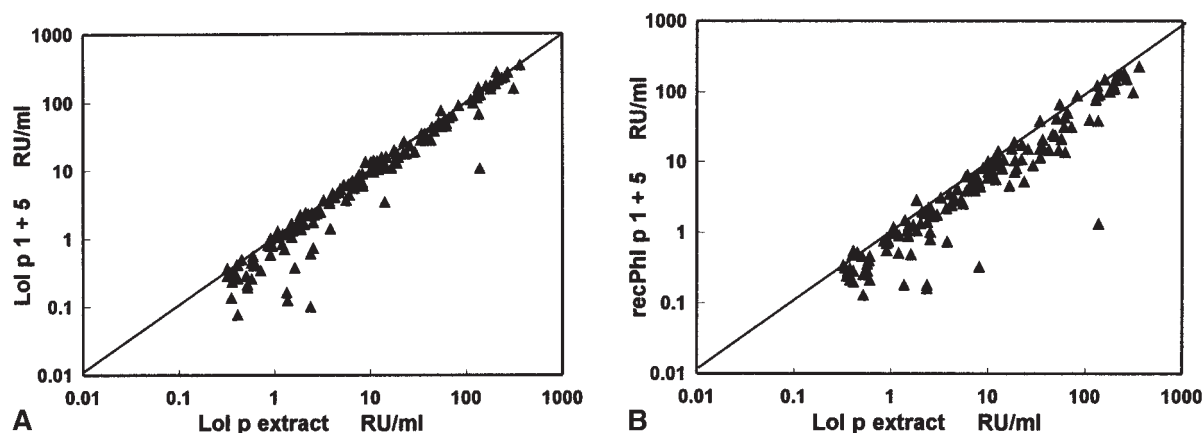


FIG. 4. RAST with purified group 1 and 5 allergens A, RAST results ($n = 154$) for natural Lol p 1 and Lol p 5 were combined and plotted against RAST for *L. perenne* (Lol p) extract. B, RAST results ($n = 141$) for recombinant (rec) Phl p 1 and Phl p 5 were combined and plotted against RAST for *L. perenne* extract. Results are expressed in RU/ml.

erata/C. dactylon, and *Phl. pratense/C. dactylon* were compared for the *C. dactylon*-exposed and nonexposed population, a significant difference was observed ($p < 0.001$). Exposure resulted in 4 to 5 times lower ratios. IgE responses to *C. dactylon* and the three other species demonstrated a strong correlation ($R_s > 0.865$). This is illustrated in Fig. 2 for *Po. pratensis*.

Cross-reactivity was studied by RAST inhibition. One serum with a low positive RAST result to *C. dactylon* and a negative RAST result to the other species was tested in a *C. dactylon* RAST inhibition. *Po. pratensis* and *D. glomerata* did not inhibit the RAST (data not shown). A similar picture was observed for a serum with RAST values 1.7 to 1.9 times lower to *Po. pratensis*, *D. glomerata*, and *Phl. pratense* than to *C. dactylon* (data

not shown). When sera with similar reactivities to *C. dactylon* as to the other three species were tested, a heterogeneous picture was observed. In some cases, *C. dactylon* was a more potent inhibitor of the *C. dactylon* RAST than *Po. pratensis* and *D. glomerata*, and complete inhibition was not always reached with the latter species (Fig. 3, A). For other sera, however, *Po. pratensis* and *D. glomerata* were more potent inhibitors (Fig. 3, B). This was also the case for sera with a lower RAST value for *C. dactylon* than for the other three species.

IgE reactivity to purified Lol p 1 and Lol p 5

IgE reactivity to purified Lol p 1 and Lol p 5 was tested in a RAST for 154 sera with a positive RAST result to

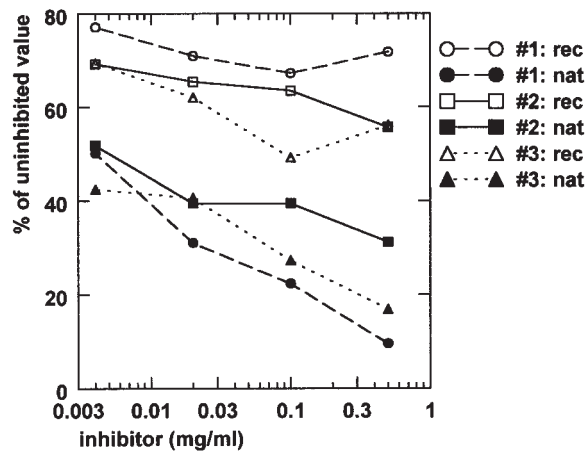


FIG. 5. Lol p 1 RAST inhibition. Lol p 1 RAST was inhibited with rPhl p 1 (rec) and with *Phl. pratense* extract (nat) for three sera (#1, #2, and #3). Results are expressed as percent of uninhibited value. Uninhibited RAST values were 7.1, 5.5, and 6.3 RU/ml, respectively (serum dilution, 1:5).

L. perenne extract. Lol p 1-specific IgE was demonstrated in 129 of 154 sera (83.8%), and Lol p 5-specific IgE was demonstrated in 96 of 154 sera (62.3%). The combination of both purified allergens resulted in 140 of 154 (90.9%) positive results. The mean ($\pm$ SEM) RAST for *L. perenne* of the remaining 14 negative sera was 0.7 ± 0.2 RU/ml, with three values greater than 1.0 RU/ml (2.4, 1.4, and 1.3 RU/ml). The correlation between RAST values for *L. perenne* and those for Lol p 1, Lol p 5, or Lol p 1 plus Lol p 5 was analyzed by Spearman's rank correlation coefficient. The R_s values (95% CI) were 0.895 (0.858 to 0.922), 0.851 (0.801 to 0.890), and 0.975 (0.966 to 0.982), respectively (Fig. 4, A). The mean (95% CI) of the individual ratios of IgE anti-Lol p 1 plus Lol p 5/IgE anti-*L. perenne* was 0.81 (0.74 to 0.88), indicating that IgE anti-Lol p 1 plus Lol p 5 accounted for 81% of the total IgE response to *L. perenne* pollen.

IgE reactivity to purified recombinant Phl p 1 and Phl p 5

IgE antibodies against rPhl p 1 and rPhl p 5 were determined in a RAST for 141 of 154 sera tested on purified Lol p 1 and Lol p 5. Specific IgE against the recombinant allergens was demonstrated in 114 of 141 (80.9%) and 85 of 141 (60.3%) sera, respectively. With the combination of rPhl p 1 and rPhl p 5, 128 of 141 sera (90.8%) showed a positive RAST result. The mean RAST result ($\pm$ SEM) to *L. perenne* of the remaining 13 sera was 0.8 ± 0.2 RU/ml. As in the case of the purified natural allergens, only three of these sera showed a grass pollen RAST result greater than 1.0 RU/ml (2.4, 2.4, and 1.4 RU/ml). The correlation between the RAST for *L. perenne* and for rPhl p 1, rPhl p 5, or rPhl p 1 plus rPhl p 5 was analyzed by Spearman's rank correlation coefficient. The R_s values (95% CI) were 0.860 (0.810 to 0.898), 0.771 (0.694 to 0.830), and 0.946

(0.925 to 0.961), respectively (Fig. 4, B). The mean (95% CI) of the individual ratios of IgE anti-rPhl p 1 plus rPhl p 5/IgE anti-*L. perenne* was 0.58 (0.53 to 0.65).

The IgE responses to rPhl p 1 and rPhl p 5 closely correlated with those of their natural counterparts from *L. perenne* (R_s [95% CI], 0.955 (0.937 to 0.969) and 0.985 (0.977 to 0.990), respectively). Nevertheless, the RAST for the natural allergens was significantly higher than that for the recombinant versions: 1.7 times ($p < 0.001$ by paired t test) for group 1 allergen and 1.3 times ($p < 0.001$) for group 5 allergen. Natural and recombinant group 1 allergens were compared in a Lol p 1 RAST inhibition. As the source of natural Phl p 1, total *Phl. pratense* extract was used. Three sera with a higher RAST to Lol p 1 than to rPhl p 1 (1.7 to 3.3 times) were tested (Fig. 5). On a protein basis, the recombinant allergen was at least 10 times weaker as an inhibitor than the natural extract. Because only a fraction of the total protein (5% to 10%) is accounted for by Phl p 1, the difference in inhibitory potency is more likely to be around a factor of 100.

DISCUSSION

This study has shown that pollen from a single grass species is sufficient to perform reliable in vitro diagnosis of grass pollen allergy. Eligible candidates are predominantly Festuceae, such as *Po. pratensis*, *F. rubra*, and *D. glomerata*.¹⁹ An alternative would be *Phl. pratense*, an Agrostideae. Application of *Po. pratensis* resulted in the lowest number (14 of 209) of false-negative results, the majority being borderline positive results for species like *D. glomerata* or *Phl. pratense*. It is a widespread belief that inclusion of different species in a mixture can obviate these (minor) problems. Such mixtures usually contain between four to 10 different species and are used in a similar biologic potency as single species-based diagnostics. This implies that any (rare) species-specific structure is diluted four to 10 times, thereby possibly ending up under the detection limit. This study does not deny the existence of species-specific epitopes but has convincingly demonstrated their scarcity. The only guarantee to detect all possible specific structures would be to test all specific species separately and not hidden in largely cross-reactive mixtures. Because the clinical relevance of such an approach is doubtful, it is probably not worth the trouble and costs.

Exposure levels are most likely an important factor in determining the potency of pollen needed to sensitize atopic individuals. The species with high IgE-binding capacity all belong to abundantly growing grasses.²⁰ Does high exposure to normally less abundant species result in higher or more specific IgE responses? This was investigated in two panels of sera from subjects with clearly different patterns of exposure, compared with the normal Dutch population. High exposure to *S. cereale* did not result in significant differences. However, exposure to *C. dactylon* pollen, a grass species not growing in The Netherlands, had a significant effect. The ratio of IgE responses to different pollen clearly changed in favor of *C. dactylon*.

Nevertheless, *D. glomerata* and *Po. pratensis* were suitable extracts to detect the majority of the *C. dactylon*-positive sera. With *D. glomerata*, 10 of 80 sera showed false-negative results, but the mean ($\pm$ SEM) RAST value for *C. dactylon* was very low at 0.38 ± 0.05 RU/ml. With *Po. pratensis*, these figures were 11 of 80 and 0.54 ± 0.08 RU/ml. Spearman's rank correlation analysis revealed strong correlation between IgE responses to *Po. pratensis* or *D. glomerata* on the one hand and to *C. dactylon* on the other hand. This observation does not imply that all IgE responses were completely cross-reactive. In RAST-inhibition assays, some sera with similar RAST results for *C. dactylon* as for *D. glomerata* and *Po. pratensis* showed only partial cross-reactivity. This suggests that the observed close correlation is not explained completely by cross-sensitization but also by cosensitization.

The increased knowledge on major allergens, and the molecular cloning of most of these, has opened the way toward the development of diagnostics and therapeutics on the basis of purified natural or recombinant major allergens.^{16,21} Their application would facilitate standardization and increase batch-to-batch consistency. Several reports have addressed the question of whether a limited number of (recombinant) major allergens can replace allergen extracts for diagnostics.²¹⁻²⁸ Most of these studies have focused on immunoblotting, but some have focused on skin testing. Equivalence of natural and recombinant allergens was often studied by immunoblot inhibition, a technique that does not facilitate quantitative comparison. Therefore in this study RAST and RAST inhibition was used to investigate the potential of purified natural and recombinant allergens. With a combination of purified Lol p 1 and Lol p 5, greater than 90% of all *L. perenne*-positive sera were detected. The sera with false-negative results were, without exception, low responders. The purified allergens bound approximately 80% of all *L. perenne*-specific IgE. It can of course not be ruled out that in individual cases the *L. perenne* solid phase did not reach the point of saturation for certain low-affinity interactions. A single false-negative result and seven sera with a significantly lower (1.5 to 10 times) RAST for the purified allergens were analyzed on immunoblot (data not shown). Four sera (including the sera with the false-negative result) did not detect any protein. The remaining four sera predominantly reacted to Lol p 1 and Lol p 5. No clear binding to other allergens (e.g., groups 2, 3, and 4) was detected. The combination of group 1 and 5 allergens is sufficient to make a diagnosis for the vast majority of patients with positive reactions to grass pollen. Because about 80% of all anti-grass pollen IgE is directed to these major allergens, immunotherapy might be efficient with a limited combination of purified allergens. To test this approach, a double-blind, placebo-controlled clinical trial was recently performed in collaboration with HAL Allergen Laboratories, using a combination of purified Dac g 1, Dac g 5, and Dac g 11 (analysis in progress).

The IgE-binding capacity of the recombinant allergens Phl p 1 and Phl p 5 was significantly lower than

that of natural Lol p 1 and Lol p 5. This was especially true for group 1 allergen. Both allergens were expressed in *E. coli*.^{8,17} This implies that the machinery for posttranslational modifications is very limited. Group 1 allergens from grass pollen are glycosylated proteins with six cysteines, probably forming disulfide bridges.²⁹ The observed difference between natural and recombinant group 1 allergens might be explained by the lack of glycosylation and incorrect folding in *E. coli*. Recently, it was shown that group 1 allergens share homology with, and are functionally active as, expansins.³⁰ *E. coli*-derived recombinant Lol p 1 turned out to be inactive, again suggesting incorrect folding. For application of recombinant allergens in diagnostics, use of eukaryotic expression systems with appropriate possibilities for posttranslational modifications, such as the yeast *P. pastoris* or transgenic plants, is to be preferred. Whether correct folding is essential for application in immunotherapy is still an open question.

In summary, this study has demonstrated that diagnostics for grass pollen allergy can be simplified by replacing mixtures by a single species (e.g., *Po. pratensis* or *D. glomerata*). Purified group 1 and group 5 allergens cover greater than 90% of all sera with positive results to grass pollen. The advantage of such a well-defined diagnostic tool should be carefully weighed against missing a small number of low-responder sera. Recombinant allergens for diagnostic purposes should preferably be produced in eukaryotic expression systems for achieving correct folding. The assumption that the use of purified (recombinant) allergens from grass pollen for immunotherapy is unattractive because of the broad range of allergenic grasses, and the large number of different allergens is refuted by the data presented here. Only two purified allergens cover approximately 80% of all anti-grass pollen IgE.

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