

Variability of crossreactivity of IgE antibodies to group I and V allergens in eight grass pollen species

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Summary

Crossreactivity to *Dactylis glomerata*, *Festuca rubra*, *Phleum pratense*, *Anthoxanthum odoratum*, *Secale cereale*, *Zea mays*, and *Phragmites communis* of IgE antibodies against *Lol p I* or *Lol p V* was investigated by means of RAST-inhibition. Within a group of sera the degree of crossreactivity was demonstrated to be highly variable. Individual sera were not always equally crossreactive to all pollen species. A high degree of crossreactivity for Group I allergens did not necessarily implicate the same for Group V. Group I and Group V representatives were found to be present in all eight species. It was demonstrated that within this group of grass species significant quantitative and qualitative differences exist, with respect to Group I and Group V allergens. Species with a low phylogenetic affinity to *Lolium perenne*, like *Zea mays* and *Phragmites communis* showed a very low degree of reactivity, even when measured with the most crossreactive sera. A higher taxonomic relationship however, did not always implicate a closer antigenic resemblance. Antigenically both allergens from *Zea mays* are more similar to *Lol p I* and *Lol p V*, than the analogues in *Secale cereale*.

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Introduction

Crossreactivity of IgE antibodies to different grass pollen species has been the subject of several studies [1–5]. Usually RAST-inhibition is applied in this field of research. In most studies published so far IgE binding to immobilized whole grass pollen extracts was inhibited with various pollen extracts. In the present study we performed RAST-inhibition experiments with Sepharose-coupled purified *Lol p I* or *Lol p V* instead of whole grass pollen extract. The presence of these allergens throughout the *Gramineae* family is most extensively studied for Group I [6–12]. Studies on Group V were less comprehensive [13–15]. By performing RAST-inhibition experiments with Sepharose-coupled purified major allergens we aimed at excluding crossreactions that are not restricted to grass pollen. It is known that some crossreactions are much more widespread: between pollen allergens, food allergens and some allergens originating

from lower animals like the honey-bee. Both periodate-susceptible and periodate-resistant crossreacting structures have been described [16,17]. So far, we have not found evidence that antibodies to Group I or V allergens are crossreactive with non-*Gramineae*.

The presence of allergens crossreactive with *Lol p I* and *V* was investigated by RAST-inhibition with eight grass species. We have chosen the approach of performing these inhibition experiments with a group of individual sera as opposed to RAST-inhibition with a pool serum. Application of a pool serum is often thought to minimize problems caused by individual epitope differences among crossreactive allergens. However, IgE against epitopes of minor importance will be overlooked due to dilution in a pool serum.

Our aims with the RAST-inhibition experiments were: (i) to determine the approximate levels of allergens, crossreactive with *Lol p I* or *Lol p V* in seven grass species, relative to *Lolium perenne*; (ii) to get an impression of the degree of antigenic resemblance of the allergens within Group I or Group V; and (iii) to investigate whether there are significant individual differences in the degree of

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crossreactivity observed in a group of sera.

All obtained data were also considered in a phylogenetic perspective.

Materials and methods

Pollen extracts

Pollen were obtained from Diephuis Laboratories (Groningen, The Netherlands). Extracts were made from the following eight pollen species: *Lolium perenne* (Lp), *Dactylis glomerata* (Dg), *Festuca rubra* (Fr), *Phleum pratense* (Pp), *Secale cereale* (Sc), *Anthoxanthum odoratum* (Ao), *Zea mays* (Zm), and *Phragmites communis* (Pc). The pollen was extracted as previously described [15]. In short, pollen was extracted for 2 hr in distilled water (5% w/v). The pH was maintained at 8.5 by adjustment with NaOH (0.1 mol/l) every 15 min. After centrifugation, the supernatant was defatted with Freon TF, dialysed against distilled water and freeze-dried. The yield ranged from 3.6% to 5.7% of the dry weight of the crude pollen.

Protein determination

Protein was determined with BCA protein assay reagent (Pierce) according to the manufacturer's instructions [18]. BSA was used as protein standard. This resulted in the following protein contents expressed as milligrams of protein extracted from one gram pollen: 31 for Zm, 19 for Lp, 31 for Pc, 29 for Fr, 27 for Dg, 25 for Sc, 35 for Pp, and 21 for Ao.

Selection of sera

Sera were selected from sera submitted for serologic allergy tests, on the basis of >10% binding (of ¹²⁵I-labelled sheep anti-human IgE) in a RAST with a mixture of Sepharose-coupled Dg and Pp. Of these sera 23 scored ≥10% binding in a RAST for Lol p I and/or Lol p V and were used for RAST-inhibition assays. From this panel of 23 sera 18 were selected for RAST-inhibition assays for Lol p I and 13 for Lol p V.

Purification of allergens

Lol p I and Lol p V were purified as described earlier [15]. In short, Lol p I was purified by gel-filtration, hydrophobic-interaction chromatography and ion-exchange chromatography. Lol p V (earlier designated as 'Grass 4B1' [15]) was purified by affinity chromatography with monoclonal antibody 4B1.

Coupling of allergens to a solid phase

As solid phase we used CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Lol p I

(1.2 mg of protein) and Lol p V (365 µg of protein) were each coupled to 1 g of Sepharose. Protein was determined spectrophotometrically, extinction coefficients being 1.53 and 1.37 respectively. Of the dialysed and lyophilized Dg and Pp pollen extracts 5 mg (dry weight) were coupled to 1 g of Sepharose.

RAST and RAST-inhibition

The RAST for specific IgE to total pollen extract was performed with a mixture of Sepharose-coupled Dg and Pp pollen extract (0.25 mg Sepharose of each per test) and ¹²⁵I-labelled sheep antibodies to human IgE [16]. Per test 50 µl of serum was used. RAST-results are expressed as percentages of radioactivity bound to the solid phase. Specific IgE to Lol p I or V was measured in the same way, with Sepharose coupled Lol p I or V (0.25 mg per test). The same Sepharoses with purified allergens were used for the RAST-inhibition experiments. In this case however, the sera were diluted in 6% (w/v) HSA in order to obtain binding percentages in the range from 10% to 20%. Inhibition was performed by a 2 hr preincubation of the serum with different concentrations of the eight grass pollen extracts in PBS (between 0.1 µg/ml and 10 000 µg/ml) in a total volume of 100 µl. The uninhibited value was obtained with PBS. Different counting times were applied for each combination of serum and Sepharose. At the counting time used, 10 000 counts were found in the absence of inhibitor. The inhibited samples were counted for the same time. The background without serum or inhibitor was subtracted from the results. From the resulting inhibition curves the allergen concentrations needed for 50% inhibition and the slopes at this point of the curve were calculated by means of linear regression on logit-transformed data. The 50% inhibition values were used as measure for the inhibitory potencies. This was assumed to represent the best quantitative approximation, when inhibition curves are not parallel. In some cases the inhibitor concentrations used were not sufficient to approximate 50% inhibition. This was especially observed with Zm and Pc. These inhibitions were not taken into account with any further calculations.

Results

Contents of crossreactive allergen

RAST-inhibition assays were performed with Lol p I or V coupled to Sepharose as allergosorbent and whole-pollen extracts from eight grass species as inhibitor. From the inhibition curves the pollen concentrations needed for 50% inhibition were calculated. From these results sera with the highest degree of crossreactivity (i.e. sera for which 50% inhibition was reached with the lowest

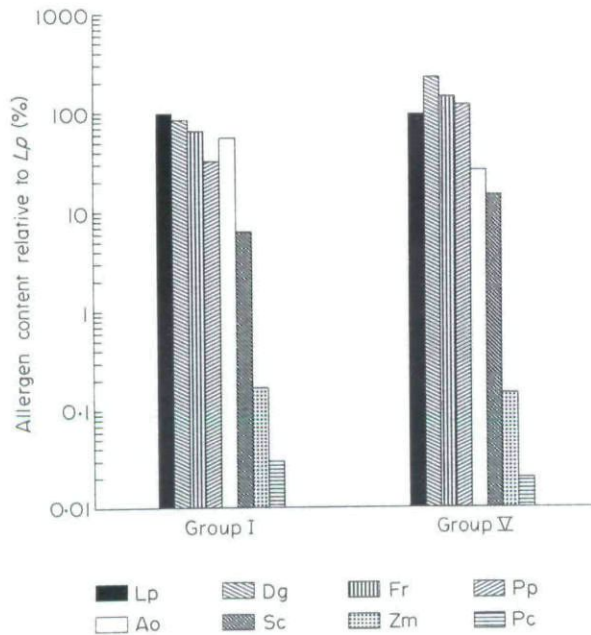


Fig. 1. Contents of crossreactive allergen relative to *Lp* (100%). The contents were calculated from the RAST-inhibition with those sera that showed the highest degree of crossreactivity for a combination of *Lol p* I or *Lol p* V and a whole-pollen extract.

concentration of a pollen extract) were selected. This was done for all 16 combinations of Sepharose and inhibitor, resulting in a maximum inhibitory potency for each pollen extract, within the given panel of sera. These maximum inhibitory potencies were taken as measure for the content of allergen, crossreactive with *Lol p* I or V, in each pollen species. Figure 1 shows these allergen contents relative to *Lp*. Both *Zm* and *Pc* had <0.2% of the potency of *Lp* with regard to both Group I and V allergen. These grass species do not belong to the subfamily *Festucoideae* in which the other six grasses are classified [19]. Within this subfamily the variation is much smaller. The largest differences were obtained with *Sc*, for which the potencies were 6% (Group I) and 15% (Group V) of *Lp*. This species, as well as *Ao* and *Pp* each belong to another tribe (*Hordeae*, *Phalarideae* and *Agrostideae*, respectively) than *Lp*, *Dg* and *Fr*, which are grouped in one tribe (*Festuceae*). The potencies found for *Ao*, *Pp*, *Fr* and *Dg* were within a factor 4 from *Lp*.

Variability of crossreactivity of IgE antibodies

In Fig. 2a an example of the inhibition curves for Group I is depicted with one serum. The inhibitor concentrations are expressed in arbitrary units of allergen, crossreactive with Group I. As 1 arbitrary unit the amount of Group I allergen present in 80 ng of lyophilized *Lp* extract was

chosen (0.1% of the highest inhibitor concentration used). The inhibitory potency, obtained with the most crossreactive serum for each of the seven pollen extracts (see Fig. 1) was used to calculate the amount of arbitrary units of crossreactive allergen in those extracts. This would mean that all inhibition curves coincide in the 50% inhibition point, when a serum is as crossreactive towards the seven pollen species as the most crossreactive sera (with respect to Group I). In Fig. 2a this is almost the case (around 6000 counts). In Fig. 2b the inhibition curves for Group V with another serum are shown. In this case 1 arbitrary unit is the amount of Group V allergen in 80 ng of lyophilized *Lp* extract. These curves illustrate that the degree of crossreactivity of a serum can clearly differ from the most crossreactive serum. The 50% inhibition point was reached with different amounts of Group V units. Figure 3a summarizes this type of variation within the panel of sera used in the inhibition assays for Group I. For each serum the inhibitory potencies of the grass pollen extracts relative to *Lp* were compared to the maximum potency (as calculated with the most crossreactive serum), and expressed as percentage of this maximum potency. Figure 3b shows the same for Group V. Especially in the case of Group I there was a high degree of variation in crossreactivity within the panel. Individual sera showed different crossreactivity patterns. Some sera were comparably crossreactive for all grass species, while others showed considerable differences. This is illustrated in Fig. 3 for both Group I and Group V, in each case with two sera. A high degree of crossreactivity to Group I (or Group V) allergen from one of the seven pollen species, did not always implicate the same for Group V (or Group I) allergen from the same pollen species. This is illustrated in Fig. 3 with one serum for *Sc*.

Antigenic variation within Group I and Group V

The RAST-inhibition experiments do not only demonstrate the variability on the serum side. When a high degree of variation in the percentages of the maximum inhibitory potency is found with a pollen extract, this points at antigenic differences of that pollen extract from *Lp* (see Fig. 3). For Group I the antigenic variation increased in the following order: *Dg*, *Fr*, *Pp*, *Zm*, *Ao* and *Sc*. To inhibit the binding of the least crossreactive serum to *Lol p* I with *Sc*, about 500 times more inhibitor was needed than for the most crossreactive serum. In the case of *Dg* and *Fr* this factor was only three. *Pp*, *Zm* and *Ao* had an intermediate position. The situation for *Pc* was more difficult to judge, because the inhibitor concentrations used often were not high enough to reach 50% inhibition. In a few cases we therefore applied higher concentrations (up to 100 mg/ml). These experiments

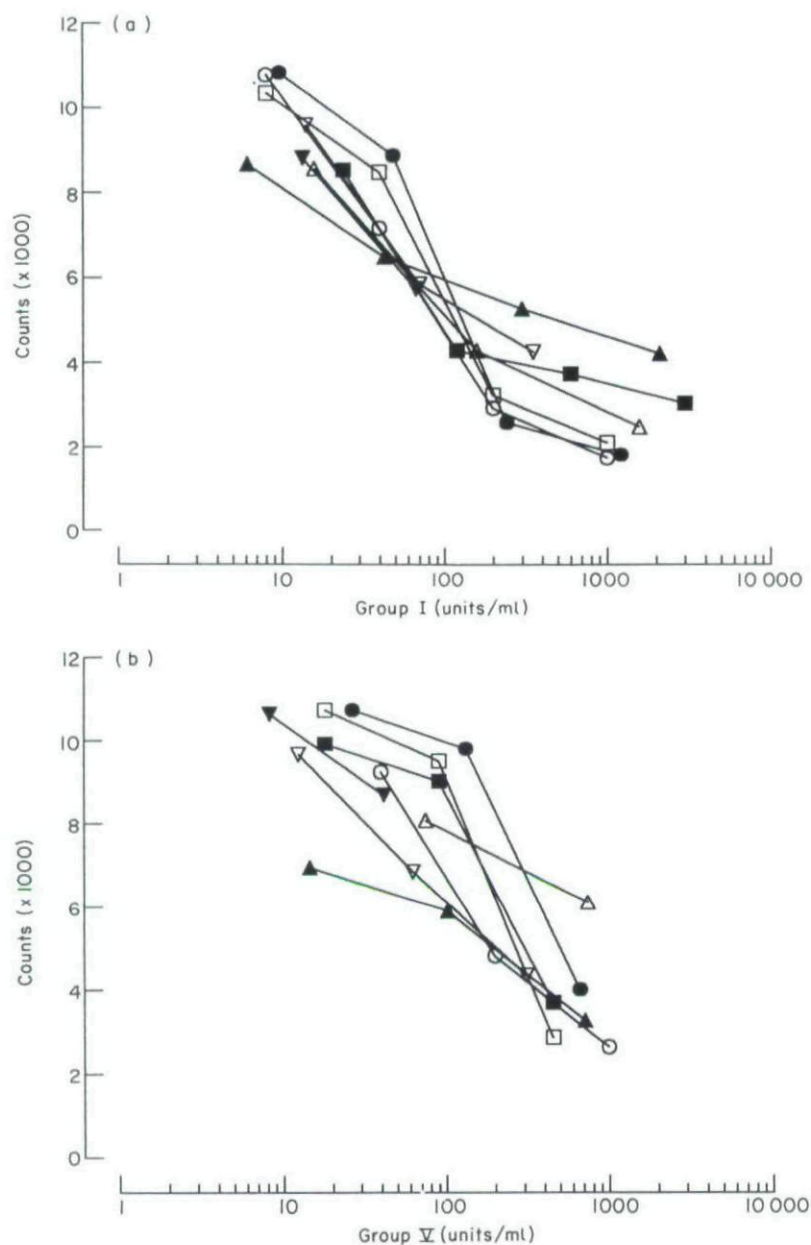


Fig. 2. Examples of inhibition curves for Group I (a) and Group V (b). The serum shown in (a) was the most crossreactive serum for *Dg* and *Fr* with respect to Group I allergen. It was used for calculating the maximum inhibitory potencies of these pollen species. The serum shown in (b) was applied for the same purpose in the case of *Sc* and *Zm*, with respect to Group V allergen. On the abscissa are arbitrary units of allergen in the pollen-extracts, calculated from the RAST-inhibition with the most crossreactive serum for each pollen species. One arbitrary unit of allergen was the amount of allergen present in 80 ng of lyophilized *Lp* extract. This amount represents a certain inhibitory potency. For each other pollen species one arbitrary unit is the amount with the same inhibitory potency, when the most crossreactive serum (for each pollen species) is used. On the ordinate are counts bound (uninhibited binding: 10 000 counts). ○, *Lp*. ●, *Dg*. □, *Fr*. ■, *Pp*. △, *Ao*. ▲, *Sc*. ▽, *Zm*. ▼, *Pc*.

(data not shown) combined with the results shown in Fig. 3a suggest that the variation in case of *Pc* is smaller than for *Ao* and *Sc*. For Group V allergen it was difficult to indicate an order of increasing antigenic variation. The percentages (of the maximum potency) were ranging from 10% to 100% for all grass species, except with one serum for *Sc* (< 1%). For *Pc* the results were as difficult to judge as in the case of Group I.

Another way to look at the antigenic resemblance is to compare the slopes of the inhibition curves at 50%-

inhibition. For each serum the slopes were calculated relative to that obtained with *Lp*. A difference in slope indicates antigenic differences. In Fig. 4a the average slopes relative to *Lp* are shown for Group I, in Fig. 4b for Group V. In both cases *Sc* had the lowest slope. For *Zm* and *Pc* the slopes were lower than for *Lp*, although for *Pc* this was based on only a few allergen-serum combinations. *Dg* had a higher slope for Group I; for Group V it closely resembled *Lp*. The slopes in the case of *Fr* were almost equal to *Lp*. *Pp* had a slightly lower slope in both

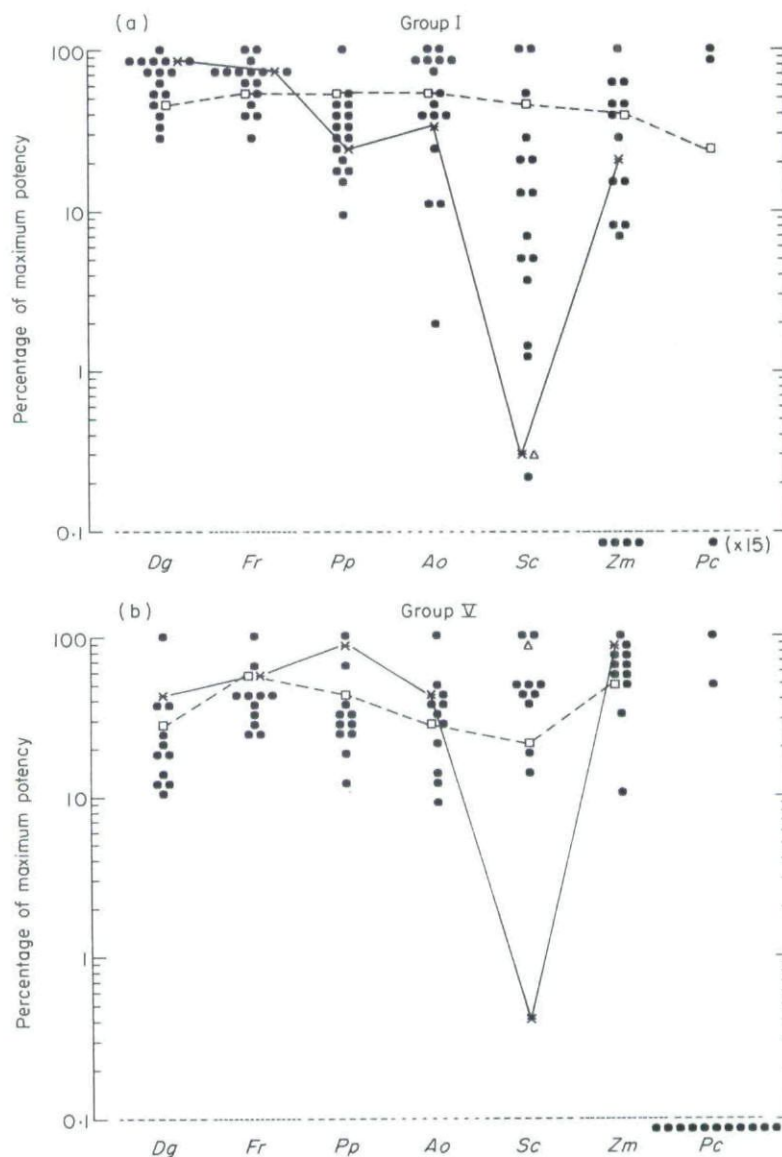


Fig. 3. Inhibitory potencies relative to *Lp* as found for individual sera, expressed as percentage of the maximum inhibitory potency. The maximum inhibitory potency was calculated with the most crossreactive serum for each pollen species. The variability of the inhibitory potencies found in this way, is shown for Group I in (a) and for Group V in (b). In both cases one serum is selected that was comparably crossreactive to all grass species ($\square$), and one to illustrate the opposite (*). The identical symbols used in (a) and (b) do not implicate identical sera. In case of *Sc* one serum is shown (Δ) that is highly crossreactive for one allergen group (Group V) and not for the other (Group I).

cases. This was also the case for *Ao* in combination with Group I. For Group V however, it had a much lower slope.

Discussion

We studied crossreactivity of IgE antibodies to eight grass pollen species. By means of RAST-inhibition, analogues of *Lol p* I and V were demonstrated in pollen of seven grass species. For almost all sera, >95% inhibition could be reached with all extracts. An exception to this was inhibition with *Zm* and *Pc*. Inhibitor concentrations needed for complete inhibition were in this case too high (> 50 mg/ml) for extensive application. With a few sera

we demonstrated that >95% inhibition could be obtained at these higher concentrations. In these cases we have to be aware of the fact that possible minor contamination with other pollen species could interfere. With the approach used exact quantification of *Lol p* I and V analogues is not possible. For this purpose purified or recombinant allergens from all species would be needed. It was demonstrated that a decrease in phylogenetic affinity to *Lolium perenne* is accompanied by a decrease in inhibitory potencies. The total potencies of the *Festuceae* (*Lp*, *Dg* and *Fr*) are the highest, closely followed by *Pp* and *Ao*. *Sc* clearly has a lower potency. The decrease in potency is paralleled by an increase in antigenic variation, when both allergen groups are taken together. The contents of crossreactive allergens found

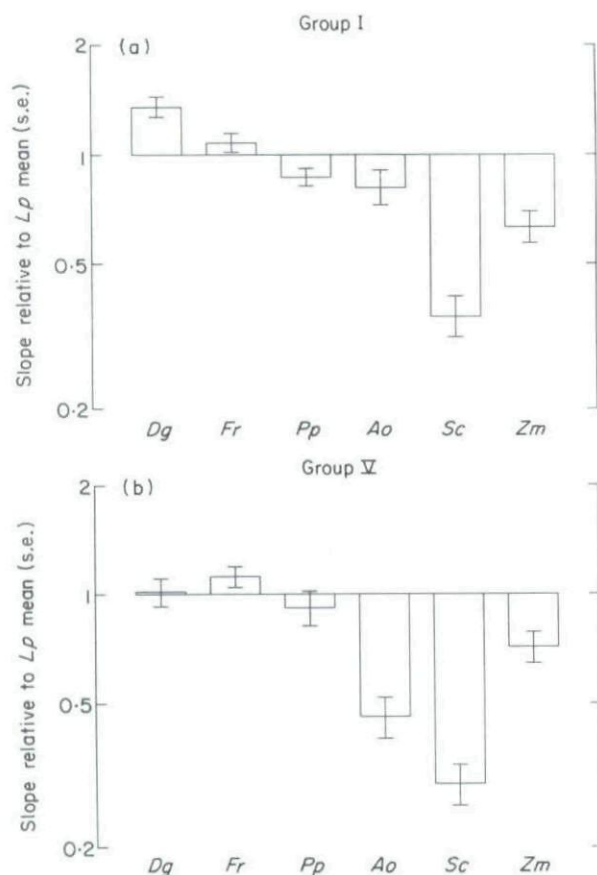


Fig. 4. The average slopes of the RAST-inhibition curves relative to *Lp* are shown. In each bar the standard error of the mean (s.e.m.) is given. The number of observations is 18 for Group I (except for *Zm*: 16) and 13 for Group V. *Pc* is left out because the number of observations was < 5. The average slopes in case of Group I allergen are shown in (a), for Group V in (b). A divergence from 1 implicates antigenic differences from *Lp*.

for *Zm* and *Pc* are between 35 and 150 times lower than for *Sc*, but in terms of antigenic structure both *Zm* and *Pc* seem closer to *Lp* than *Sc*.

It is difficult to determine to what extent the apparent quantitative differences can be explained by qualitative differences. Antigenic differences were traced in two ways: (i) differences in slopes of the inhibition curves and (ii) variation of the inhibitory potency of pollen extracts for the binding of different sera to *Lol p* I and V. For both parameters *Sc* gave the most divergent values relative to *Lp*. Antigenic disparity between the *Lp*-components and its analogues in the other species can cause underestimation of allergen contents. The results shown in Fig. 1 should therefore be interpreted as minimum values. The maximum inhibitory potencies of *Zm* and *Pc* are between 45 and 1000 times lower than those of *Sc*. At the same

time, *Sc* structurally seems less related to *Lp*. Both above mentioned parameters for detecting qualitative differences were for *Zm* and *Pc* more similar to *Lp* than for *Sc*. For this reason, it seems likely that absolute quantities of Group I and V equivalents in *Zm* and *Pc* are very small, and that this caused their low inhibitory potencies (besides antigenic differences from *Lp*). Recently Matthiesen *et al.* reported similar results for Group I and V allergen from *Pc* by means of CIE [20]. For the other species it is more difficult to judge the relative importance of qualitative versus quantitative differences.

Within the group of sera tested a high degree of variability was observed with respect to crossreactivity of IgE antibodies. This was illustrated most strikingly in RAST-inhibition experiments with Sepharose-coupled *Lol p* I as solid phase and *Sc* as inhibitor. The amount of *Sc* extract needed for 50% inhibition (relative to the amount of *Lolium perenne* extract needed for 50% inhibition) was very diverse within this group of 18 sera. Between the most and least crossreactive serum this amount differed with a factor 500. When a serum was shown to be highly crossreactive for one grass species, this did not implicate the same for all the other species. A significant correlation between the degree of crossreactivity for Group I and for Group V allergens could also not be demonstrated (see Fig. 3). This means that no evidence was found for a division of sera in a group that tended to be crossreactive in general and a group that did not. In fact a remarkably heterogeneous picture emerged.

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