

ORIGINAL PAPERS

Evaluation of the Potency of Allergenic Extracts by Inhibition of IgG-Antibody Binding

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Abstract. A method is described for the *in vitro* potency evaluation of allergenic extracts by using their capacity of binding to specific human IgG antibodies. The results obtained with this inhibition-type enzyme immunoassay are compared with the analyses by the customary method of IgE antibody binding. A large series of allergenic protein fractions from the pollen of the Gramineae, *Olea europea*, *Parietaria judaica*, and from the dust mite *Dermatophagoides pteronyssinus* as well as from cat dander and peanuts were examined for inhibition of specific antibodies of both IgG and IgE isotypes. Potency evaluation by inhibition assays for IgE- and IgG-binding showed a significant correlation for the points of 50% inhibition ($r = 0.92$, $p = 0.0001$), but not for the slopes of the inhibition curves, i.e. the respective antibody avidities. Evidence is provided that the strict relationship between IgE- and IgG-inhibition by allergens could not be explained by possible cross-contaminations of the anti IgE- or anti IgG-reagents employed in the immunoassays. It is concluded that the inhibition of IgG-antibody binding presents a fast, reliable and low-cost alternative for the potency control of allergens used in clinical practice.

Introduction

Since the introduction by Noon in 1911 of extracts of natural substances for the injection treatment of human patients with allergic bronchial asthma, hayfever or rhinitis,¹ the standardization and potency assessment of these widely used allergenic vaccines has continued to be a problem. Over the years, it has become clear that of all the chemical and immunochemical methods proposed none will give a wholly reliable estimate of the *in vitro* allergenic potency.^{2,3} Since the mode of action of allergenic extracts during long-term hyposensitization or immunotherapy is still uncertain, the *in vivo* evaluation of potency and efficacy of allergens for therapeutic application has remained only partially successful.

It is generally accepted that there is one exception, viz: the method of binding to specific human antibodies of the IgE isotype, mostly in the variant of an *in vitro* inhibition assay. Results obtained with this so-called 'RAST-inhibition' method have in many cases been shown to correlate with data from diagnostic skin-testing.^{4,5} In consequence, recently elaborated regulations regarding *in vitro* allergenic potency control require evaluation of the IgE-binding potential of allergenic batch preparations.^{6,7} In all of these considerations an important point has never-

theless been consistently overlooked, namely that there exists a near-perfect and well-documented quantitative relationship in collections of sera from allergic people between the relative levels and epitope specificities of IgE- and IgG-class antibodies to most of the common allergens.^{8–15,20–22}

In view of the generally tight correlation between IgE- and IgG-antibodies to allergens in individual sera, the aim of the present study was to investigate whether this finding could be exploited to substitute *in vitro* IgE-based potency tests by immunoassays founded on the binding of specific IgG-class antibodies, which occur in much higher absolute levels and thus proceed at higher serum dilutions. Such an approach had already been recognized as promising by Leiferman *et al.*, using the technique of radio-immuno-precipitation of ragweed pollen allergen with specific IgE- or IgG-class antibodies.¹⁶

Materials and methods

Sera

Blood serum samples were obtained from various hospital centres in Spain and in The Netherlands. Patients with clinically manifest allergies were selected on the basis of the symptomatology, a positive (family) history of atopy, and positive wheal-and-

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flare reactions to skin tests with aqueous allergenic extracts (from C.B.F. LETI, S.A., Madrid, Spain or HAL Allergen Laboratories, Haarlem, The Netherlands, respectively). The sera were individually analysed for allergen-specific IgE-antibodies and were then organized into seven groups, viz: the sera of patients allergic to the pollen of the Gramineae, to the pollen of the trees *Olea europea* and *Betula alba*, the pollen of the weed *Parietaria judaica*, to house dust mites of the *Dermatophagoides* spp, or to cat skin epithelium. Sera from 20 subjects in each group were selected on the basis of high IgE-antibody titers, and equal volumes of each individual serum were mixed to form a pool. Pooled serum from adult patients with severe atopic dermatitis was used as a source of IgE antibodies to peanuts. All serum pools were stored at -20°C until use.

Allergens

Dry pollen preparations were purchased from BioPol, Spokane, U.S.A. Most of the allergenic preparations employed in these studies consisted of the customary unfractionated non-dialysable portions (nominal cut-off 10 000 Daltons) of aqueous allergenic extracts manufactured by Laboratorios LETI (Barcelona, Spain) and stored in the lyophilized form as in-house reference standards. Prior to use, aliquots were weighed and dissolved in phosphate-buffered saline (PBS) pH 7.4 buffer. The group of the Gramineae consisted of extracts of the pollen of *Agrostis tenuis*, *Anthoxanthum odoratum*, *Cynodon dactylon*, *Dactylis glomerata*, *Holcus lanatus*, *Lolium perenne*, *Phleum pratense*, *Poa pratensis* and *Secale cereale*. As potent representatives of allergenic tree pollen, samples were chosen from *Olea europea* and *Betula alba*, while the pollen of the widespread Mediterranean species *Parietaria judaica* were used as representative of a highly sensitizing weed. Two allergenic extracts of the house dust mite *Dermatophagoides pteronyssinus* were employed, viz: the dialysed in-house reference extract of whole culture (Laboratorios C.B.F. LETI, Madrid), and the International Standard for *Dermatophagoides pteronyssinus* # 82/518 (purchased from NIBSC, Potters Bar, Herts, U.K.). The cat allergen preparation was a dialysed aqueous extract of cat dander supplied as the dried skin flakes separated by differential filtration of clipped cat hair and dander suspended in acetone (ARTU Biologicals NV, Lelystad, The Netherlands, lot nr 86L24/K1). An enriched allergenic protein product from ether-defatted unshelled peanuts was prepared by alcohol precipitation of whole aqueous extract. From the dialysed macro-

molecular material of *Phleum pratense* pollen four pooled subfractions were isolated, PP I through PP IV in descending order of molecular size, by chromatographic separation on a column of Sephadex G75 in phosphate-buffered saline (PBS). Finally, two chromatographic subfractions, DP I and DP II, were separated from the dialysed culture extract of *Dermatophagoides pteronyssinus* by molecular sieving through a Sephacryl S 300 column, whereby DP I comprised the pooled eluate fractions containing the major allergen *Der pI* as monitored by enzymatic conversion of the chromogenic tripeptide S-2288, while DP II represented the enzyme-depleted residue.¹⁷

Inhibition of IgE antibody binding

The method employed is based on an enzyme immunoassay in which excess of allergen is bound covalently to Whatman no. 1 paper discs by means of cyanogen bromide according to the method of Ceska et al.¹⁸ with some slight modifications. The allergen-coated discs were incubated with a series of sequential dilutions of the respective serum pools in order to establish the optimal dilution for performing IgE-inhibition assays (usually 1:4 to 1:8). The inhibition procedure was carried out in two steps. In the first step, a series of concentrations of soluble allergens were incubated in the wells of flat-bottom microtiter plates with the corresponding and appropriately diluted serum pool in a final volume of 100 μl for 3 h at room temperature. In the second stage, these preincubated allergen-serum mixtures were each reacted overnight with shaking at ambient temperature with allergen-coated discs in order to capture residual unbound specific IgE. After washing with PBS-Tween 20 (0.1%), 150 μl monoclonal anti-IgE (Ingenasa, Madrid, Spain) labelled with horse radish peroxidase (HRP) was added to the discs. After an additional 3 h at room temperature, the discs were washed repeatedly with PBS-Tween, whereupon the degree of specific IgE-binding was evaluated enzymatically for 30 minutes at room temperature by adding 100 μl 0.16 M sodium acetate buffer pH 6.0, containing 2 μl TMB (dissolved in DMSO) and 0.2 μl 1 M hydrogen peroxide per well. The reaction was stopped with 100 μl 2 N sulphuric acid and aliquots from each well were transferred to another microplate. The colour was finally read in a microplate reader at 450 nm.

Inhibition of IgG antibody binding

IgG-antibody binding was evaluated by enzyme immunoassay, using allergens coated physically to

the wells of microtiter plates. Allergenic preparations were dissolved at 0.01 mg/ml in sodium carbonate buffer pH 9.4–9.7 and incubated overnight at 37°C in aliquots of 150 µl per flat-bottom well of microtiter plates (Nunc-Immuno PolySorp). After a washing cycle the plates were stored at 4°C until use. For the inhibition assay, sequential allergen dilutions were preincubated for 3 h at ambient temperature with the optimal dilution of the serum pool previously established by titration (usually 1:20 to 1:100). The allergen-serum mixtures were then pipetted into the wells of the allergen-coated plates for capturing residual free IgG-antibody during a contact period of 30 min at room temperature. After a further series of washing cycles with Triton-X405 (0.04%, v/v), peroxidase-labelled monoclonal anti-IgG (Janssen Biochimica, Beerse, Belgium) was added and allowed to react for 30 min at room temperature. Plates were then washed with Triton-X405 solution, and the colour was developed for 30 minutes at room temperature by adding 100 µl 0.16 M sodium acetate buffer pH 6.0, containing 2 µl TMB (dissolved in DMSO) and 0.2 µl 1 M hydrogen peroxide. The reaction was then stopped with 100 µl 2 N sulphuric acid and the colour was read at 450 nm.

Specificity of revealing antibodies

In order to verify the purity of the monoclonal anti-IgE second antibody reagent an experiment was performed whereby monoclonal anti-IgG was coated overnight at ambient temperature to vinyl microtiter plates (Costar 96). After washing, the plates were then treated with purified polyclonal IgG protein or with IgG1 κ -chain paraprotein for 2 h at ambient temperature, after which unreacted sites were blocked for 1 h at room temperature with 1% bovine serum albumin (BSA). The plates were then incubated for 2 h at room temperature with peroxidase-labelled anti-IgE and finally developed as described above for the inhibition assays.

Expression of the results

For calculating the allergen concentration required for 50% fluid-phase inhibition of IgE or IgG binding under the conditions of assay, and for appraising the affinity (or 'avidity') of antibody binding, the results were plotted in double-logarithmic mode, whereby the equation for the linear regression line represents the derivation of the law of mass action for allergen-IgE binding: $\log [\text{fractional inhibition}] = k_1 \log [\text{allergen concentration}] + k_2$, in which k_1 represents the slope of the regression line and is directly

related to the affinity of allergen-antibody interaction. In the present work both the allergen potency for IgE and IgG binding, i.e. the points of 50% inhibition were calculated, as well as the relative values of k_1 as parameters for the binding affinities.¹⁹ Correlation coefficients r were calculated from linear regression analysis and in some cases also by rank order correlation according to Spearman (rS).

Results

Specificity of antibody reagents

For the determination of allergen-specific IgG antibody levels in immunoassay, human allergic sera are normally used in 1:20 to 1:100 dilution. Because the level of specific antibodies of the IgE isotype in human sera is much lower, serum samples for IgE determination by RAST must be at a higher working concentration, i.e. at dilutions of 1:2 to 1:8. It was therefore considered that, if the reported tight relationship between allergen-specific IgE- and IgG-antibody levels in direct assays with individual sera was due to contamination of the peroxidase-labelled antibody reagents, this argument would be valid only in the case of anti IgG contamination of the labelled anti IgE reagent, whereas a possible contamination of the anti IgG reagent with anti IgE would not affect the IgG-antibody readings. Based on this consideration, it was decided to check only the purity of the anti IgE -HRP antibody. For this purpose, a purified IgG κ -chain paraprotein and a purified polyclonal IgG preparation were separately incubated on microtiter plates precoated with monoclonal anti IgG. To verify possible nonspecific binding, the anti IgE-HRP was then incubated with the IgG-loaded plates. The data in Table 1 show that the anti IgE-HRP reagent failed to recognize homogeneous IgG on the wells, thus proving that the enzyme-labelled anti IgE reagent was indeed specific and uncontaminated with anti IgG.

Table 1. Mono-specificity of the antiserum reagents; for explanation see text

Immobilized Ab	Revealing Ab	OD (450 nm)
anti-IgG	anti-IgE-HRP	0.579
anti-IgG/BSA	anti-IgE-HRP	0.376
anti-IgG/BSA/IgG- κ	anti-IgE-HRP	0.419
anti-IgG/BSA/IgG- κ	anti-IgG-HRP	2.390
anti-IgG/BSA/IgG-pol	anti-IgE-HRP	0.393
anti-IgG/BSA/IgG-pol	anti-IgG-HRP	1.686

Dose-response curves for IgE and IgG binding by allergens

For all allergenic preparations listed in the Materials and Methods section dose-response inhibition curves were established for both IgE and IgG antibody binding, each time using the same pool serum for both assays. As an example, Fig. 1 shows the dose-response curves for specific IgE- and IgG-antibody binding by the allergenic extract of the dust mite *Dermatophagoides pteronyssinus*.

According to customary procedure,^{4,5} the potency of allergenic extracts is expressed as the quantity of allergen producing 50% inhibition of IgE antibody binding or, in this case also, of IgG antibody binding. In our mode of plotting, the usual requirement for parallel regression lines when comparing the IgE-binding potency of different extracts of a single allergen source would reflect itself in identical values for the slopes k_1 in the range of 30–70% inhibition. As shown in Figs 1 and 2, this condition is also being approached for the allergen-binding affinities of IgE as compared to IgG antibodies in the case of the *D. pteronyssinus* and *P. pratense* allergens. However, for the total group of different allergens investigated, a relationship between the binding affinities k_1 for specific IgE or IgG antibodies was not found ($n = 23$, $r = -0.03$, $P = 0.91$).

The precise concentrations for 50% inhibition varied for each allergen and for each antibody, whereby the IgG-values were invariably lower due to the

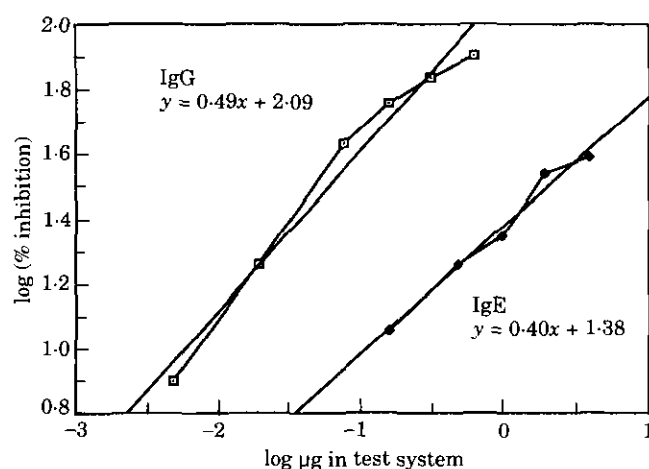


Figure 1. Double logarithmic plot of dose-dependent inhibition curves for binding of whole dialysed allergenic extract of cultures of *Dermatophagoides pteronyssinus* to specific IgE- and IgG-antibodies in human serum pool #[5]. The equations for linear regression are shown in the graph.

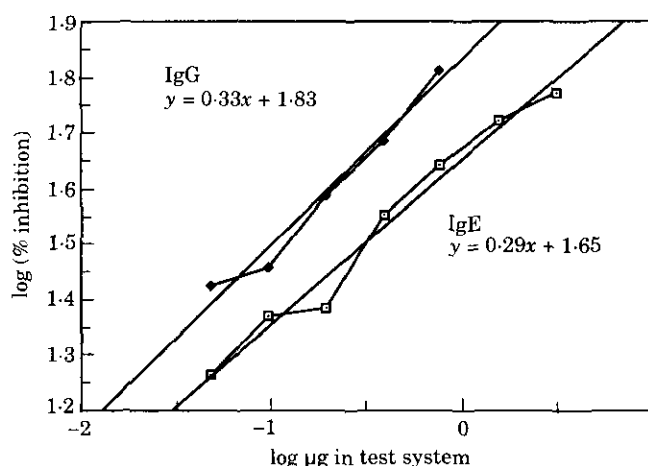


Figure 2. Double logarithmic plot of dose-dependent inhibition curves for binding of whole dialysed protein extract of the dried pollen of *Phleum pratense* to specific IgE- and IgG-antibodies in human serum pool #[1]. The equations for linear regression are shown in the graph.

greater sensitivity of the enzyme immunoassay (Figs 1 and 2).

Potency ratios for IgE and IgG inhibition

To decide whether the values for allergen potency as determined by 50% inhibition of IgE- or IgG- antibody binding were interrelated, 23 different allergen preparations were examined, using their corresponding human serum pools. Potencies were compared by correlation analysis and rank correlation. The results for the total group are shown in Fig. 3, which demonstrates a strong relation between the IgE- and IgG-binding potencies [$n = 23$, $r = 0.92$, $P < 0.0001$; $r(S) = 0.91$; $P < 0.0001$] for a selection of allergens frequently used in allergy practice.

Although the ratio of IgE- and IgG-binding potencies among different allergens under the conditions of assay clearly represents a constant value, the numerical allergen dose for achieving half-inhibition is serum-dependent, as is the slope of the IgE- or IgG-inhibition curves. This merely underlines that the epitope-specificities and binding affinities for allergen of the antibody populations differ among patients; such differences level out when employing serum pools. These characteristics are independent of the relative or absolute antibody concentrations. The level of IgG antibodies in human allergic sera normally requires dilutions of 1:20 up to 1:100 for use in immunoassays. Consequently, potency evaluations for allergens by 50% inhibition of specific IgG in the working dilutions likewise are independent of the absolute antibody concentration.

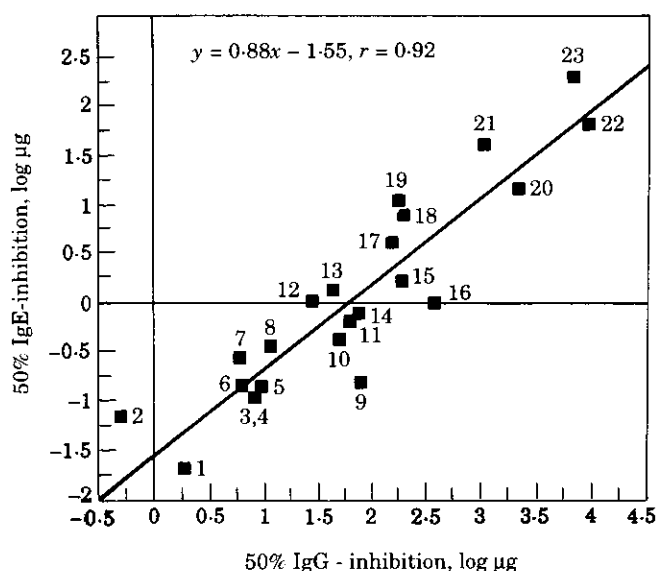


Figure 3. Scatter diagram showing the distribution of the potencies for 50% inhibition of IgE- and IgG- binding from the corresponding specific serum pools for the allergen preparations: (1) *Holcus lanatus* In-House Standard, (2) *Phleum pratense* whole protein In-House Standard, (3) *Lolium perenne* In-House Standard, (4) Peanut allergenic trypsin-inhibitor, (5) *Olea europea* In-House Standard, (6) *Dactylis glomerata* In-House Standard, (7) *Poa pratensis* In-House Standard, (8) *Cynodon dactylon* In-House Standard, (9) *Phleum pratense*, batch # 85G22 total protein, (10) *Agrostis tenuis* In-House Standard, (11) *Anthoxanthum odoratum* In-House Standard, (12) *Parietaria judaica* In-House Standard, (13) *Dermatophagoides pteronyssinus* In-House Standard, (14) *Phleum pratense* batch # 85G22—column fraction PP II, (15) Cat dander total proteins, (16) *Phleum pratense* batch # 85G22—column fraction PP I, (17) *Dermatophagoides pteronyssinus* WHO-IUIS International Standard #82/518, (18) *Secale cereale* In-House Standard, (19) *Dermatophagoides pteronyssinus*, dialysed whole culture extract, (20) *Dermatophagoides pteronyssinus*, enzyme fraction DP I mainly containing Der pI, (21) *Phleum pratense* batch # 85G22—column fraction PP III, (22) *Dermatophagoides pteronyssinus*, Der pI-depleted proteins DP II, (23) *Phleum pratense* batch # 85G22, column fraction PP IV.

Discussion

The results of this investigation reaffirm and extend previous observations on the close relationship between the relative levels of anti allergen IgE and IgG antibodies previously established under standard assay conditions in the blood serum of large groups of allergic individuals.⁹⁻¹⁵ For routine diagnostic purposes and for most of the common allergens the determination of specific IgG antibodies may therefore replace the more cumbersome and expen-

sive assays for specific IgE antibodies. In both kinds of diagnostic assay, the extent of antibody binding is quantitatively assessed using a fixed dose of (immobilized) allergen and a pre-determined single serum dilution. Since IgG anti allergen antibodies occur in every individual allergic serum at much higher levels than specific IgE antibodies, diagnostic tests based on IgG therefore require less serum and only insignificant amounts of allergens.

The present study has extended these observations in that a very close correlation was also found between the potencies of a variety of allergenic preparations established by inhibition of allergen-specific IgE and IgG antibody binding, using a single pre-dilution of pooled human allergic sera and a variable range of allergen concentrations. These results are in complete agreement with the data obtained by Leiferman *et al.*¹⁶ using the method of radioimmunoprecipitation, who also found that the potencies of various (ragweed pollen) allergen extracts established by IgG and IgE antibody binding were strongly correlated ($r(S) = 0.96$, $P < 0.001$). The strict correlation between the allergenic potencies established in the present work by (50%) IgE and IgG inhibition (Fig. 3) provides a solid basis for the proposal to employ IgG-inhibition for the control of allergenic potency. In view of the high levels of allergen-specific IgG-antibodies relative to IgE-antibodies in human allergic sera, such IgG-inhibition assays may easily be carried out on allergen-coated microtiter plates. By contrast, specific IgE-antibody fixation by enzyme immunoassay on allergen-coated microtiter wells usually remains too low for reliable quantitation. The observation that the allergen potencies established by IgE- and IgG-inhibition correlate quite well even when using two different assay formats (RAST-versus EIA-inhibition) and even when using pools of sera strengthens the conclusion that IgG-antibody binding by itself represents a valuable parameter for the *in vitro* assessment of allergenic potencies.

So far, we have found only one single exception to the constant ratio for IgE- and IgG-established potencies among allergens, i.e. in the case of the pollen proteins of the white birch, *Betula alba*, where the point of 50% IgE-inhibition in the RAST-system was established at the expected low value of 0.28 µg, whereas 50% IgG-inhibition with the same serum pool could only be achieved at the excessively high allergen dose of 341 µg. One possible explanation for this discrepancy may be that the polystyrene surface of the customary microtiter plates fails to physically adsorb, or prevents the expression of, the proper antibody-binding structural determinants; capture of specific IgE-

antibodies by birch pollen allergen-coated plates likewise failed completely. This point needs further elaboration and underlines the necessity to investigate the IgG versus IgE binding phenomenon for each individual allergen before deciding on the best practical method of potency evaluation.

Several investigators working with individual allergen molecules have shown that the corresponding IgE- and IgG-antibodies combine with exactly the same epitopes.²⁰⁻²² Dose-response curves for allergen interaction with both antibody isotypes in this situation should therefore run parallel, and the k_1 values from the regression lines for IgE- and IgG-binding should be identical. While for some individual allergen preparations this condition is nearly fulfilled (Figs 1 and 2), in the present work we did not find it to be true for the whole group of allergenic preparations, nor was this to be expected in view of the different antibody specificities against distinct allergens. Clinically applied allergen preparations, furthermore, represent highly complex mixtures of unfractionated dialysed extracts, while an extra complication arises on considering that the precise distribution of the IgG-subclasses IgG1 → 4 is known not to be the same for different allergens. These disturbances occasionally show themselves in individual sera by rather irregular inhibition patterns for allergen-IgG interaction, sometimes even displaying prozone effects precluding the correct assessment of regression lines and affinity constants. However, such problems most often do not intervene in the establishment of the points of 50% inhibition. Potency evaluation of the common allergenic extracts by *in vitro* IgG-inhibition may therefore represent a valid new modality for batch-to-batch control.

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