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Evaluation of allergenic potency by REAST inhibition. A new tool for the standardization of allergenic extracts

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Abstract

The potency of allergenic extracts can be determined in vitro by RAST inhibition, and this has become the preferred method for the standardization of allergens. A disadvantage of this technique is the impossibility of obtaining data about allergens bound to the solid phase, i.e., the counterpart of the inhibiting extract. The REAST (reverse enzyme allergosorbent test) is based on the capture of IgE by a specific antibody bound to microtiter wells, the reaction of captured IgE with biotinylated allergen and the development of a colour reaction by subsequent addition of streptavidin-peroxidase and chromogenic substrate. The addition of an allergen extract in a dose-response fashion competes with the biotinylated allergen and inhibits the test. In the present study REAST inhibition has been evaluated with *Dermatophagoides pteronyssinus*, *Parietaria judaica* and mixed grass pollen extracts. The correlation of REAST inhibition with RAST inhibition and both intra-assay and inter-assay reproducibility have been evaluated. REAST inhibition is a potentially valuable new tool for the standardization of allergenic extracts.

Key words: Reverse enzyme allergosorbent test inhibition; Radio-allergosorbent test inhibition; Allergen; Allergenic potency; IgE

Abbreviations: DER, house dust mite *Dermatophagoides pteronyssinus*; DMSO, dimethyl sulfoxide; GRASS, grass pollen mix; IHR, in-house reference preparation; IUIS, International Union of Immunological Societies; OD, optical density; PAR, *Parietaria judaica* pollen; PBS, phosphate buffered saline; PNU, protein nitrogen unit; RAST, radioallergosorbent test; REAST, reverse enzyme allergosorbent test; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMB, tetramethyl-benzidine; WHO, World Health Organization.

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1. Introduction

Allergenic active substances are proteins or glycoproteins with molecular weights ranging from 5 to 60 kDa. They are freely soluble in aqueous solutions and are usually eluted rapidly from source materials such as pollens, mites, animal epithelia, moulds, foodstuff, etc. Allergenic extracts consist in rather heterogeneous mixtures of allergens (substances able to induce allergic sensitization in humans and to react with IgE

specific antibodies), antigens (substances able to induce antibodies in animals and to react with them) and immunologically inert materials. Allergenic extracts are obtained by extraction of defatted source materials with buffers and by purification steps based on molecular weight exclusion (filtration on porous membranes or gel filtration) to remove small molecules which are immunologically irrelevant (for a review see Baer et al., 1988).

The standardization of allergenic extracts was initiated by Noon and Cantar (1911) who developed a simple method to express the concentration of allergenic extracts based on a weight/volume extraction ratio (Noon units). They arbitrarily stated that 1 g of pollen source material contained 1 000 000 units. Afterwards Stool et al. (1933) proposed protein nitrogen units (PNU) corresponding to 0.00001 mg of nitrogen. However, many proteins in allergenic extracts are allergenically inactive, i.e., unable to bind IgE. This is the reason why units based on protein content are completely independent of the allergenic activity of the extracts. As a consequence, allergenic products standardized on the basis of protein content were characterized by a poor lot-to-lot consistency and sub-optimal quality (Food and Drug Administration, 1985).

In 1967 the radioallergosorbent test (RAST) was developed for the detection of allergen-specific IgE antibodies (Wide et al., 1967). It is substantially a 'sandwich' assay, in which an allergen bound to a solid phase (cellulose disc, polystyrene bead, etc.) is reacted with a serum sample and then with affinity-purified ^{125} I-anti-human IgE. The bound radioactivity is measured and results interpolated on a standard curve.

This technology generated a new method for evaluating the potency of allergenic extracts, called RAST inhibition (Gleich et al., 1974; Yman, 1975; Foucard et al., 1976). This commences with the preincubation of aliquots of a positive serum pool with a series of dilutions of the allergenic extract under assay. The serum aliquots are then tested with the RAST procedure and the more potent the allergenic extract under assay, the higher the degree of inhibition observed. The results, expressed as percent of

inhibition, are plotted against the dilution curve, the 50% inhibiting concentration is calculated and compared to a reference extract.

The RAST inhibition procedure represents an important tool for the control and standardization of allergenic extracts. At present it is performed by every commercial producer in order to determine allergenic potency and to ensure lot-to-lot consistency (Hoek and Kersten, 1988). The main technological problems of RAST inhibition are related to the solid phase. In fact no data are available about the quantity and the quality of allergens bound to the solid phase. These problems in standardizing reagents make RAST inhibition for allergenic potency analysis valid for in-house standardization of extracts, but not helpful for inter-laboratory standardization.

In a collaborative study to identify a suitable extract for the first WHO-IUIS International Standard of *Dermatophagoides pteronyssinus*, there were disagreements between the laboratories in their estimation of potency by RAST inhibition (Ford et al., 1985). Such disagreements have been attributed to qualitative differences between the extracts under assay and the allergens bound to RAST solid phases. Moreover, RAST results can be biased by allergen-specific IgG antibodies, which in many sera interfere by competition with IgE antibodies for the same allergenic determinants (Lynch et al., 1975; Zimmerman et al., 1980).

The aim of the present study was to develop a new method of determining allergenic potency of extracts, based on the competition between the allergen under assay and a corresponding biotinylated allergen preparation. This method, which we have called REAST inhibition, is a modification of reverse enzyme immunoassay (REAST) and is based on the capture of sample IgE by a specific anti-human IgE antibody, subsequent reaction with an enzyme-conjugated allergen and the specific substrate (Moneo et al., 1983; Cuevas et al., 1983; Dominguez et al., 1984; Beaulieu et al., 1986) or, alternatively, with a biotinylated allergen and further reaction with streptavidin-enzyme and the specific substrate (Plebani et al., 1986; Olivieri et al., 1993). The REAST procedure has previously been applied to the study of

allergenic cross-reactivity between dusts of Obeche and Ramin wood in patients with occupational asthma (Hinojsa et al., 1986). The present study was performed using the following important allergens: house dust mite *Dermatophagoides pteronyssinus*, *Parietaria judaica* and mixed grass pollens.

2. Materials and methods

2.1. Sera

Negative serum comprised a pool of at least 20 sera of non-atopic patients. Positive serum pools were prepared by mixing approx 100 sera from atopic patients having RAST values of class 3 or 4. Three separate pools were prepared, reactive against the 3 allergens under study. The sera were subdivided into small aliquots and kept at -20°C until used.

2.2. Allergenic extracts

The following allergens have been studied:
Dermatophagoides pteronyssinus house dust mite (DER)

Parietaria judaica pollen (PAR)
mixed grass pollens (GRASS)

The extracts for biotinylation were extracted in PBS at 5% weight/volume ratio, overnight at 4°C with continuous stirring. After centrifugation, the clear supernatants were filtered through Millipore $0.2\ \mu\text{m}$ membranes and then stored frozen until used.

The extracts submitted to analysis were bulk extracts used for the preparation of diagnostic and therapeutic products. They were extracted at an 8% weight/volume ratio and kept lyophilized until used.

The IHR (in-house reference) extracts were 2% weight/volume extracts in PBS, were dialysed against PBS, filtered through Millipore $0.2\ \mu\text{m}$ membranes, aliquoted in one-dose vials, lyophilized and stored at -20°C until used.

2.3. RAST inhibition reagents

Polystyrene beads, diameter 6.4 mm and with a rough surface were used as the solid support. The

allergens were bound to the beads according to a patented procedure, based on activation of the surface by glutaraldehyde (Italian Pat. IT 21108-83 and extensions). Solid supports coated with DER, PAR and GRASS were available as a part of a RAST kit (Sferikit, Laboratorio Farmaceutico Lofarma, Milan, Italy).

Goat ^{125}I -anti-human IgE antibody, ϵ -specific and affinity-purified grade, was obtained from a commercial source (Sferikit, Laboratorio Farmaceutico Lofarma, Milan, Italy). The washing solution was 0.15 M NaCl containing 0.4% Tween 20.

2.4. RAST inhibition procedure

RAST inhibition for allergenic potency assays was performed according to the following procedure:

- (i) allergenic extracts for assay were serially diluted 3-fold in duplicate, in PBS, and $30\ \mu\text{l}$ of each dilution were transferred into 3 ml plastic tubes;
- (ii) $20\ \mu\text{l}$ of the positive serum were added to each tube, and the tubes vortexed for a short time; the positive control was prepared by mixing $30\ \mu\text{l}$ of PBS and $20\ \mu\text{l}$ of positive serum, the negative control by mixing $30\ \mu\text{l}$ of PBS and $20\ \mu\text{l}$ of negative serum; all the tubes were covered and incubated for 2 h with gentle stirring;
- (iii) $50\ \mu\text{l}$ of PBS and the solid support (allergen-coated bead) were added to each tube, the tubes covered and incubated for 18 h with gentle stirring;
- (iv) the liquid was aspirated from the tube with a vacuum pump and three washing cycles were performed;
- (v) $50\ \mu\text{l}$ of ^{125}I -anti IgE and $50\ \mu\text{l}$ of PBS were added to each tube, the tubes covered and incubated for 18 h (overnight) with gentle stirring;
- (vi) three washing cycles were performed as above and bound radioactivity was measured in a gamma-counter.

The calculation of relative potency was performed according to the following criteria (Food and Drug Administration, 1985):

- (i) The inhibition rate (% inhibition) was calculated according the following formula:

$$\frac{(\text{cpm positive control}) - (\text{cpm sample})}{(\text{cpm positive control}) - (\text{negative control})} \times 100$$

- (ii) linear regression line was calculated taking into account only data showing between 10% and 90% inhibition rate, and the assay was conventionally considered valid if correlation coefficient (r) was 0.9 or more;
- (iii) the dose (horizontal axis) corresponding to the 50% inhibition rate (vertical axis) was calculated and expressed as μl or fractions: this value was indicated as C_{50} .

The IHR extracts were run in each experiment as internal controls and a 'yard-stick' of allergenic potency.

2.5. REAST reagents

Microplates (eight well strips, flat bottom) were purchased from Nunc (Maxisorp, Microwell Module, Nunc, Denmark).

The goat anti-human IgE antibody, affinity-purified, used for the coating of wells was of the same batch as that used for RAST inhibition.

Allergens were extracted from the source materials (*Dermatophagoides pteronyssinus* mite bodies, *Parietaria judaica* and grass pollens) at 5% percent weight/volume ratios with PBS and dialysed against the same buffer.

Activated biotin (ϵ -caproylamido-biotin-*N*-hydroxy-succinimide ester), streptavidin-peroxidase conjugate and chromogenic substrate (TMB, tetramethyl-benzidine) containing H_2O_2 (Ultra-Blue) were purchased from SPA (Milan, Italy). Horseradish peroxidase enzymatic activity was 170 ± 30 U/ml and streptavidin activity was 3 ± 1 U/ml. According to the manufacturer's recommendations the conjugate was diluted 1/2000 in PBS containing 0.1% Tween 20. The reaction was stopped with 1 N HCl.

2.6. Preparation of anti-IgE coated strips

The goat anti-IgE antibody was pre-treated at pH 2.5 according to Ishikawa et al. (1980) in

order to increase the binding to the plastic surface. Each well was coated with $0.375 \mu\text{g}$ of antibody in $125 \mu\text{l}$ of 1 M PBS, pH 8.5. The strips were incubated overnight, then aspirated and washed once with $300 \mu\text{l}$ of 70 mM PBS, pH 7.9, containing 0.5% BSA and 0.05% sodium azide, dried and stored at 4°C in plastic bags.

2.7. Preparation of biotinylated allergens

Before biotinylation allergenic extracts were dialysed against 0.1 M NaHCO_3 pH 8.4, and the protein content was adjusted to 1 mg/ml as determined with the Bradford reagent (Bradford 1976), Bio-Rad protein assay (Bio-Rad, Milan, Italy).

A 1 mg/ml solution of activated biotin (ϵ -caproylamido-biotin-*N*-hydroxy-succinimide ester) was prepared in DMSO (dimethyl sulfoxide, dried) just before the use, to prevent hydrolysis. Assuming conventionally for allergens a mean molecular weight of 20 kDa, each allergen was biotinylated at the following allergenic protein/biotin molar ratios: 1:10, 1:20, 1:40. To obtain the above-mentioned ratios, 227, 454 and $908 \mu\text{l}$ of biotin solution were added to 1 ml of pre-cooled ($+4^\circ\text{C}$) allergenic protein solution. The mixtures were stirred for 4 h at room temperature, and finally dialysed at $+4^\circ\text{C}$ against PBS 0.15 M pH 7.2 containing 0.05% sodium azide.

2.8. REAST inhibition procedure (see scheme illustrated in Fig. 1)

REAST inhibition was performed according to the following procedure:

- (i) the anti-IgE-coated strips were incubated with $100 \mu\text{l}$ of allergic serum (or negative serum for the negative control) and incubated for 90 min; three washing cycles with $300 \mu\text{l}$ of 0.15 M PBS pH 7.2 containing 0.05% Tween 20 (washer 812 SW1, SLT Labinstruments, Milan, Italy) were performed;
- (ii) the allergenic extract under assay and IHR extracts were serially diluted 3-fold in duplicate, in PBS; $50 \mu\text{l}$ of each dilution and 50

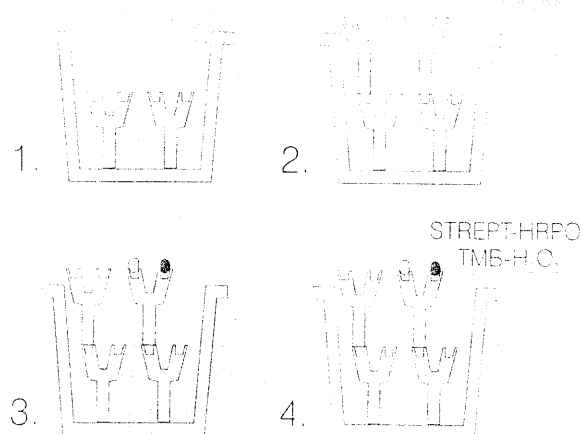


Fig. 1. Schematic outline of REAST inhibition reaction: (1) the anti-IgE antibody is bound to microtiter well; (2) IgE antibodies (both specific and non-specific) are captured; (3) specific IgE antibodies react with biotinylated allergen (●) and free inhibitor allergen (○); (4) biotinylated allergens are revealed by adding streptavidin-peroxidase and chromogenic substrate.

μl of the corresponding biotinylated allergen solution were pipetted into each well, and incubated for 60 min; three washing cycles were performed as above;

- (iii) 100 μl of streptavidin-peroxidase were added to each well and incubated for 30 min; five washing cycles were performed as above;
- (iv) 100 μl of chromogenic substrate (TMB-H₂O₂) were added and the strips incubated for 15 min;
- (v) the reaction was stopped by adding 100 μl of stopping solution (1 N HCl) and the OD at 450 nm was determined using a Spectra ELISA reader (SLT Lab Instruments, Milan, Italy).

The calculation of relative potency was performed according to the criteria described for RAST inhibition, calculating OD values instead of cpm. Before calculation of inhibition rates the values were corrected for blank OD (chromogenic substrate alone).

3. Results

The optimal biotinylation conditions (protein/biotin molar ratio and working dilution) of allergens, taking into account both positive signal

(allergic pool) and background (normal non-atopic sera) were found to be as follow: DER, 1/10 protein/biotin ratio, dilution 1/2000; PAR, 1/40 protein/biotin ratio, dilution 1/1000; GRASS, 1/20 protein/biotin ratio, dilution 1/750. These conditions were used in all the experiments.

The background value with normal sera was OD 0.040 after subtraction of blank OD (chromogenic substrate alone, which was 0.030). The positive values with pools of allergic sera (RAST class 3–4) were: OD 2.560 for DER, 2.650 for PAR and 3.300 for GRASS.

For each allergen three separate REAST inhibition experiments were performed on three consecutive days, using in each run IHR extracts as the internal control. The dose-response curves obtained in the three separate experiments are shown in Figs. 2, 3 and 4 respectively for the allergens GRASS, PAR and DER. For each curve, there are from five to eight experimental points in the interval between the 10% and 90% inhibition rates, which is the range of acceptance of data according to the Food and Drug Administration (1985). Correlation coefficients (r) were very high, being in all the experiments greater than 0.98.

The results of the RAST inhibition analyses of the three sample-extracts under study are reported in Fig. 5 and in Table 1 the 50% inhibiting doses (C_{50}) observed in REAST inhibition and RAST inhibition are shown. The C_{50} values were higher for REAST inhibition than for RAST inhibition in the case of the PAR sample-extract (0.029 μl against 0.0043 μl), whereas for DER (0.228 μl against 0.295 μl) and GRASS (0.054 μl against 0.060 μl) the C_{50} values were similar.

The 50% inhibiting doses (C_{50}) observed in the three REAST inhibition experiments (inter-assay variations) are shown in Table 2, and means and standard deviations calculated for the three assays are also reported. Analyses were performed both on sample-extracts under assay and on IHR extracts. We also calculated the IHR/extract ratio, which in RAST inhibition is the basis for calculations of total allergenic potency. The standard deviation values of the sample-extracts assayed were 24.5%, 35.2% and 17.0% for DER, PAR and GRASS respectively. When related to

IHR (IHR/sample-extract ratio) the standard deviations of the three assays decreased respectively to 12.2%, 20.6% and 10.6%.

4. Discussion

The importance of determining the total allergenic potency of allergenic extracts for stand-

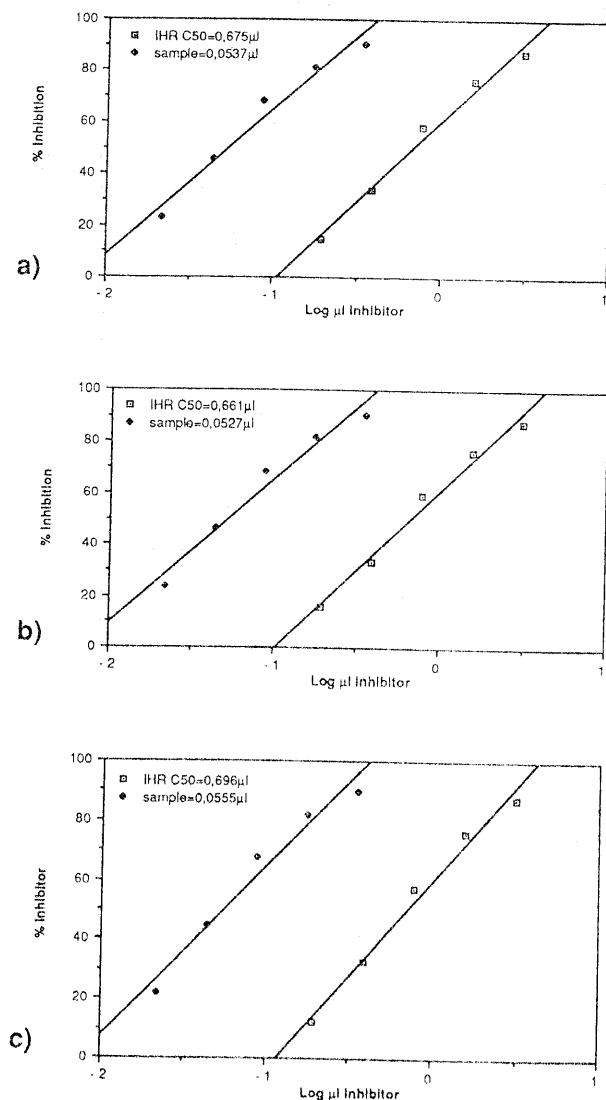


Fig. 2. REAST inhibition. Three separate (a, b, c) analyses of GRASS sample in comparison to GRASS IHR reference preparation. Regression lines and C_{50} values, corresponding to 50% inhibiting doses, are reported. OD of uninhibited serum was 3.300

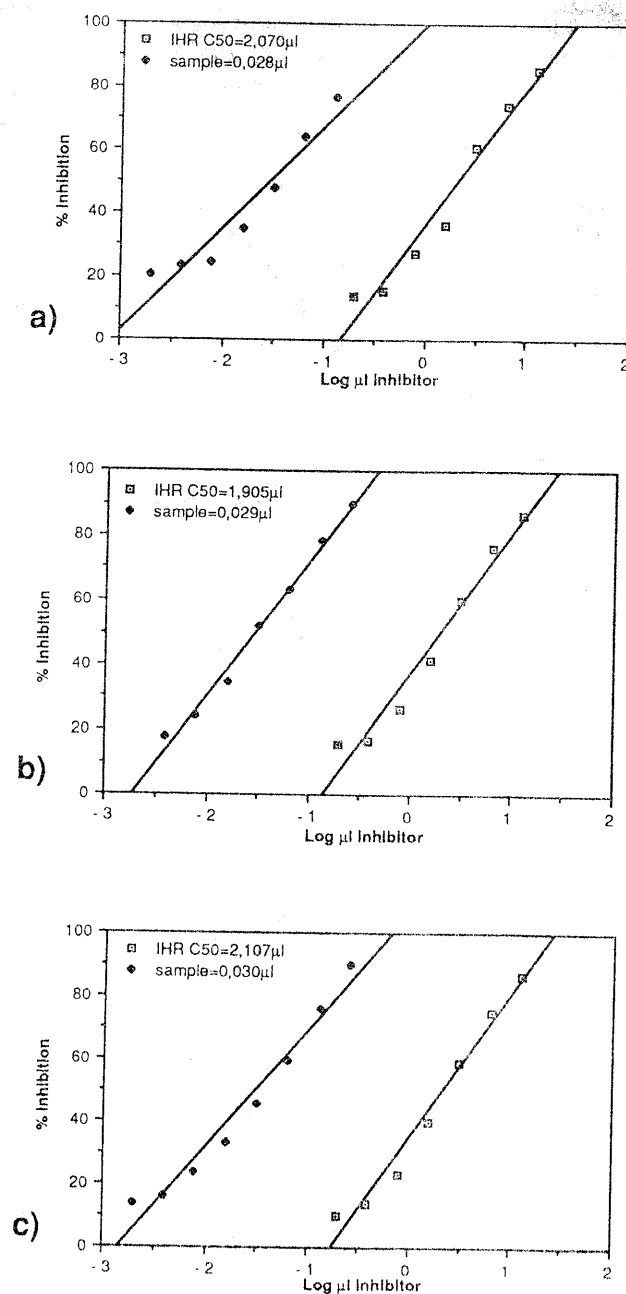


Fig. 3. REAST inhibition. Three separate (a, b, c) analyses of PAR sample in comparison to PAR IHR reference preparation. Regression lines and C_{50} values, corresponding to 50% inhibiting doses, are reported. OD of uninhibited serum was 2.650.

ardization purposes is unquestionable. At present some procedures for the quantitative determination of major allergens in certain extracts are

available, e.g., *Der p I* in *Dermatophagoides pteronyssinus* extracts (Luczynska et al., 1989), but satisfactory procedures for the determination of

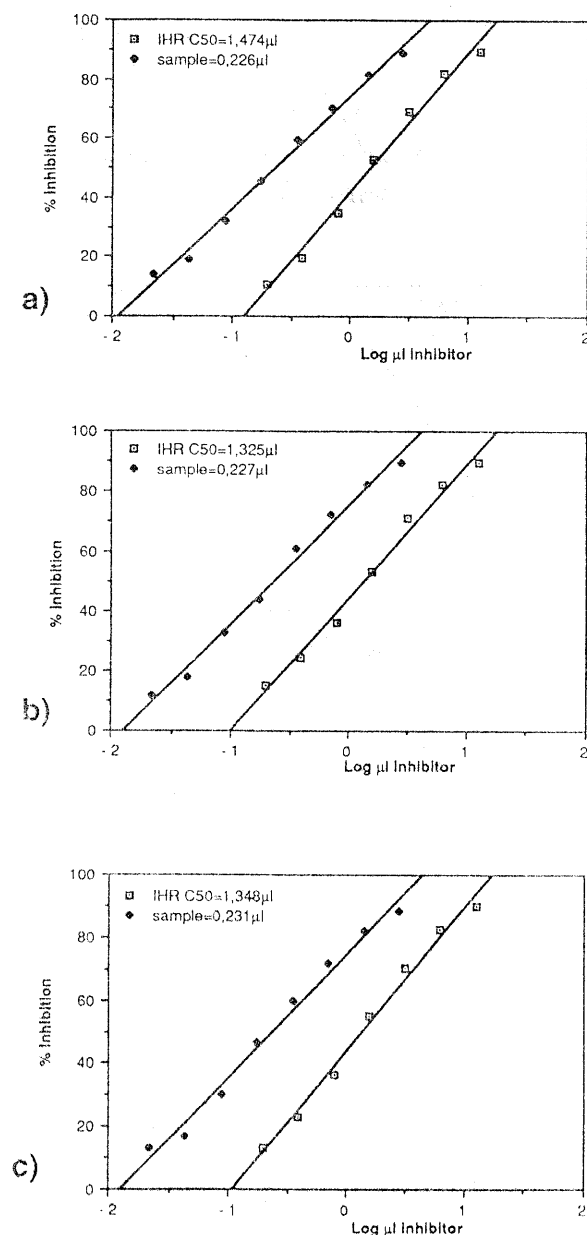


Fig. 4. RAST inhibition. Three separate (a, b, c) analyses of DER sample in comparison to DER IHR reference preparation. Regression lines and C_{50} values, corresponding to 50% inhibiting doses, are reported. OD of uninhibited serum was 2.560.

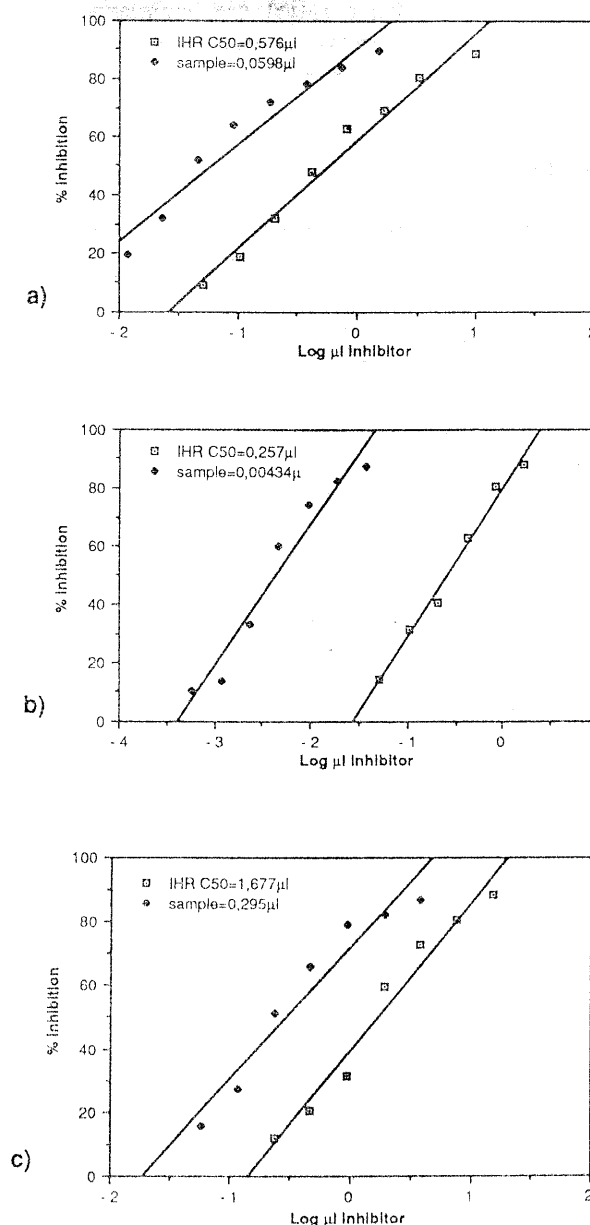


Fig. 5. RAST inhibition. Analysis of GRASS (a), PAR (b) and DER (c) samples and corresponding IHR reference preparations. Regression lines and C_{50} values, corresponding to 50% inhibiting doses, are reported.

total allergenic potency are not yet established. At present two procedures are used to this purpose: skin test titration on volunteers and RAST inhibition. The disadvantages of skin test titrations include the great variability of the assay and

Table 1
Comparison of REAST inhibition and RAST inhibition: C_{50} values, expressed as μ l of inhibitor extract, are reported. The tests were performed simultaneously

	REAST inhibition	RAST inhibition
DER		
Extract	0.228	0.295
IHR	1.382	1.677
IHR/extract ratio	6.1	5.7
PAR		
Extract	0.029	0.0043
IHR	2.027	0.257
IHR/extract ratio	69.9	59.8
GRASS		
Extract	0.054	0.060
IHR	0.677	0.576
IHR/extract ratio	12.5	9.6

difficulties in recruiting volunteers. The disadvantages of RAST inhibition include the scarcity of analytical data about the allergens bound to the solid phase, and, as a consequence, poor standardization and inter-assay reproducibility. Indeed, in the case of RAST inhibition there is a lack of information about the quantity and quality of allergenic fractions bound to the solid phase, which renders the results uncertain of analysis and hinders the possibility of comparisons of analytical data between different laboratories. This

fact is of primary importance, because only the allergens having their counterpart on the solid phase can be detected in RAST inhibition. In contrast, in REAST inhibition the inhibitor (i.e., the extract under assay) is in the same liquid form as the biotinylated allergen. Therefore it is possible to quantify exactly the inhibitory capacity of the extract under study by calculation of the concentration (expressed as protein) of the biotinylated allergen corresponding to 50% inhibition. A further advantage of REAST inhibition compared to RAST inhibition is the possibility of a more precise characterization of the allergenic preparation used in the test. This can be achieved, for example, by SDS-PAGE and blotting and parallel development with IgE from allergic sera (to verify the presence of the main allergens) or with streptavidin-peroxidase (to verify their biotinylation)(unpublished data).

Comparison of REAST and RAST inhibition results showed that for DER and GRASS 50% inhibiting doses (C_{50}) were quite similar: 0.228 and 0.295 respectively for DER and 0.054 and 0.060 for GRASS (Table 1). In contrast, for PAR a consistent difference was observed: 0.029 and 0.043 for REAST inhibition and RAST inhibition respectively. This means that a larger amount of PAR allergen is required to inhibit REAST compared to RAST. On the basis of these data one might speculate that REAST inhibition is less

Table 2
Inter-assay variations of three REAST inhibition analyses performed on different days

	First assay	Second assay	Third assay	Mean (SD)
DER				
Extract	0.147	0.239	0.228	0.205 (24.5%)
IHR	1.153	1.680	1.382	1.405 (18.8%)
IHR/extract ratio	7.8	7.0	6.1	6.85 (12.2%)
PAR				
Extract	0.029	0.037	0.057	0.041 (35.2%)
IHR	1.399	1.211	2.082	1.564 (29.3%)
IHR/extract ratio	48.2	32.7	36.5	38.1 (20.6%)
GRASS				
Extract	0.070	0.076	0.054	0.066 (17.0%)
IHR	0.872	0.780	0.677	0.776 (12.6%)
IHR/extract ratio	12.4	10.3	12.5	11.76 (10.6%)

DER, PAR, GRASS extracts and corresponding IHRs were tested. The results are expressed as C_{50} values (μ l of inhibitor extract), means and standard deviations.

sensitive than RAST inhibition, at least for PAR allergen. In fact, in preliminary experiments the inhibitory activity of a given extract could be shown to vary over a wide range by altering the concentration of biotinylated allergen, in that a higher amount of inhibitor was necessary to inhibit a higher concentration of biotinylated allergen.

Similar observations have been made in RAST inhibition by changing the allergen concentration on the solid phase (unpublished data), but in contrast to REAST, there is no precise data about the quantity of allergen employed in the RAST assay.

In conclusion, REAST inhibition is a valuable new technique for the determination of allergenic potency of extracts.

The main advantage, compared to RAST inhibition, lies in the improved knowledge of the reagents to be inhibited.

Further relevant advantages consist of:

- the possibility of complete automation of the test, which for RAST inhibition is impossible since the allergens are bound to the solid phase;
- REAST inhibition is performed much more rapidly than RAST inhibition (195 min versus 20 h);
- the presence of allergen-specific antibodies belonging to IgG class (very frequent in the sera of food-allergic patients and in the sera of patients submitted to specific immunotherapy) do not interfere with REAST inhibition because they are removed after the first step and before the addition of the biotinylated allergen, whereas in RAST inhibition such antibodies exhibit interference due to competition with IgE antibodies for the same epitopes (Lynch et al., 1975; Zimmerman et al., 1980).

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