

Comparison of skin-prick test assay and reverse enzyme immunoassay competition (REINA-C) for biological activity of allergens

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Summary

An *in vitro* technique for measuring the relative potency of allergenic extracts has been developed and compared with the dose–response skin-prick test. The *in vitro* technique is a modification of an ELISA based on the method of antigen capture using polystyrene microtitre wells as solid phase (REINA-competition) and is also based on a dose–response. Both methods have been applied to allergen extracts of *Dermatophagoides pteronyssinus*, *Phleum pratense*, *Secale cereale*, *Lolium perenne*, *Plantago lanceolata*, *Artemisia vulgaris*, *Salsola kali* and *Parietaria judaica*. For each allergen, the slope of the regression log-log dose–response lines displayed by skin-prick test and REINA-C does not show a statistically significant difference, being parallel ($P < 0.001$), and indicating that the allergen–ligand binding kinetics are identical in both methods.

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Introduction

Numerous methods have been used to evaluate the potency and composition of allergen extracts. Many of them, like weight/volume or protein nitrogen content, did not correlate with allergenic activity. Since its first description in 1974 [1] the RAST inhibition has been the tool most widely used *in vitro* for allergen standardization, making it possible to compare the allergen extract under study with a reference extract. *In vivo* methods to estimate the relative potency on allergen extracts by skin testing are well documented and the methodological errors have been carefully analysed and accepted [2]. Skin testing represents the only feasible method of obtaining extracts standardized biologically in sensitive individuals [3], which give skin reactions of a comparable size, whatever the allergen species tested. Usually, manufacturers standardize their reference extracts in biological units, and then compare their in-house reference preparations with freshly prepared extracts by means of allergen-related *in vitro* assays.

The *in vitro* method REINA (reverse enzyme immunoassay), which is based on the method of antigen capture using polystyrene microtitre wells as solid phase [4–6] and

which has proved to show a good correlation with RAST [7] in the measurement of specific IgE antibodies to common allergens, has been modified to assess the biopotency of allergen extracts. We report here the comparative study between *in vivo* testing for relative potency and *in vitro* inhibition of allergen-specific IgE antibody binding by an enzyme-linked immunosorbent assay of competition, REINA-competition (REINA-C).

Materials and methods

Allergens

Freeze-dried total non-dialysable allergenic components from the house dust mite *Dermatophagoides pteronyssinus* and ether-defatted pollen of *Phleum pratense*, *Secale cereale*, *Lolium perenne*, *Plantago lanceolata*, *Artemisia vulgaris*, *Salsola kali* and *Parietaria judaica* were obtained from Laboratorios CBF-LETI (Madrid, Spain). For the REINA-C procedure, these allergens were conjugated with horseradish peroxidase (HRPO). The method of Nakane and Kawaoi [8] was used with some slight modifications [7]. Briefly, 5 mg of HRPO (Boehringer Mannheim) in 1 ml of 10 mM phosphate-buffered saline (PBS) was treated with 200 μ l acetone solution of 1% fluorodinitrobenzene (Eastman) for 30 min at room temperature. Sodium metaperiodate, 200 μ l 0.3 M, was

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then added and, after 30 min, the mixture was sieved through a column of Sephadex G-25 equilibrated with PBS; the tan-coloured enzyme fraction was collected. Five milligrams of the freeze-dried allergen was added to the activated HRPO and then, after overnight incubation in the cold, 5 mg of sodium borohydride, followed by extensive dialysis. The HRPO-labelled allergen was stored in the freeze-dried state.

For the prick test, serial dilutions at 10, 1, 0.1, 0.01 and 0.001 mg of freeze-dried extract/ml were prepared in 50% glycerol.

Patients

Adult patients clinically sensitive to *D. pteronyssinus* ($n=20$), *P. pratense* ($n=19$), *S. cereale* ($n=19$), *L. perenne* ($n=23$), *P. lanceolata* ($n=22$), *A. vulgaris* ($n=21$), *S. kali* ($n=19$) and *P. judaica* ($n=24$) were prick-tested according to the rules of the Nordic Council on Medicines [9] with slight modifications. Briefly, the test was done, in duplicate, on the volar side of the forearm using the following concentrations of the same batch of the allergen: 10, 1, 0.1, 0.01 and 0.001 mg of freeze-dried extract/ml. Histamine diphosphate, 10 mg/ml, was used as a positive control and the glycerinated saline solution was used as a negative control.

All the reactions were recorded 15 min after the application and weal areas were outlined with a fine tip marker and transferred to paper with transparent tape. The area of each weal was calculated as a product of the two diameters, the longest one (D) and the midpoint orthogonal (d). For statistical calculations the geometric mean of the areas provoked by each of the concentrations of the allergen was employed, and any weal area having a value of 0 was replaced by a value of 1. A serum sample was collected from each patient and a pool of sera from the patients sensitive to and tested for each allergen was prepared and stored in 0.5 ml aliquots at -70°C until use.

Procedure for REINA-C

The commercially available reagent kits and test procedure (REINA®, Laboratorios CBF-LETI, Madrid, Spain) were used throughout this work. REINA is a 'reverse' system based on the principle of antigen capture [4-7] in which the wells are first coated with murine monoclonal anti-human IgE, which subsequently binds IgE from human serum samples; the level of allergen-specific antibody is then assessed by incubation with enzyme-labelled allergen, thus avoiding the possible interference of specific IgG antibodies. In the REINA-C test, binding of the allergen-enzyme conjugate by the immobilized antibody is inhibited by the addition of serial

Dilution of free unlabelled allergen	OD1	OD2
Blank	0.011	0.095
Blank	0.036	0.074
2	0.508	0.504
4	0.701	0.681
8	0.889	0.904
16	1.115	1.069
32	1.292	1.241

Fig. 1. Example of the raw data of a typical competitive allergen (*Dermatophagoides pteronyssinus*) inhibition assay.

amounts of free allergen to obtain a standard curve. Since a restricted number of antibodies are available, the enzyme activity is lowered.

For specific anti-allergen IgE inhibition, the following schedule was used: as a first step 50 μl of positive pool serum sample was mixed with an equal volume of REINA diluent in duplicate to the corresponding well and allowed to stand for 30 min at room temperature. During this incubation, five serial dilutions (in a factor of 2) of free unlabelled allergen were prepared with REINA diluent. Once it was ready, a constant amount of labelled allergen was dispensed to all the tubes.

As a second step, and after this 30 min incubation and four subsequent washing steps with PBS-T (phosphate-buffered saline supplemented with 0.1% Triton X-405), 100 μl of HRPO-labelled allergen with the dilution of unlabelled allergen was added to the corresponding wells and the mixture left for 30 min at room temperature. After four washing steps with PBS-T, the wells were incubated at room temperature for 30 min with 100 μl of development solution (3,3'-4,4'-tetramethylbenzidine- H_2O_2 mixture in 0.1 M citrate/phosphate buffer). The

Table 1. Parameters (slope, standard error of the slope and correlation coefficient) of the log-log regression line for prick-test and result of the F test with the corresponding *P* value for linearity

Allergen	Slope	s.e.	<i>r</i>	F test	<i>P</i> value
<i>Dermatophagoides pteronyssinus</i>	0.308	0.02	0.994	241.266	0.0006
<i>Plantago lanceolata</i>	0.363	0.013	0.998	727.681	0.0001
<i>Phleum pratense</i>	0.289	0.025	0.989	135.034	0.0014
<i>Salsola kali</i>	0.237	0.022	0.988	118.132	0.0017
<i>Secale cereale</i>	0.337	0.017	0.996	414.742	0.0003
<i>Parietaria judaica</i>	0.354	0.052	0.969	43.743	0.0064
<i>Lolium perenne</i>	0.372	0.021	0.995	317.56	0.0004
<i>Artemisia vulgaris</i>	0.4	0.023	0.995	296.971	0.0004

Table 2. Parameters (slope, standard error of the slope and correlation coefficient) of the log-log regression line for REINA-C and result of the F test with the corresponding *P* value for linearity

Allergen	Slope	s.e.	<i>r</i>	F test	<i>P</i> value
<i>Dermatophagoides pteronyssinus</i>	0.364	0.04	0.981	75.153	0.0032
<i>Plantago lanceolata</i>	0.338	0.02	0.995	281.422	0.0005
<i>Phleum pratense</i>	0.314	0.015	0.997	462.848	0.0002
<i>Salsola kali</i>	0.236	0.057	0.922	17.001	0.0259
<i>Secale cereale</i>	0.368	0.027	0.992	191.824	0.0008
<i>Parietaria judaica</i>	0.319	0.046	0.97	47.196	0.0063
<i>Lolium perenne</i>	0.382	0.008	0.999	255.638	0.0001
<i>Artemisia vulgaris</i>	0.275	0.012	0.997	503.612	0.0002

Table 3. Upper and lower 95% confidence limits expressed as percentage of the unlabelled allergen that produces the 50% inhibition in REINA-C

Allergen	95% confidence limits	
	Upper (%)	Lower (%)
<i>Dermatophagoides pteronyssinus</i>	152	66
<i>Plantago lanceolata</i>	116	86
<i>Phleum pratense</i>	123	81
<i>Salsola kali</i>	187	54
<i>Secale cereale</i>	138	72
<i>Parietaria judaica</i>	137	73
<i>Lolium perenne</i>	122	82
<i>Artemisia vulgaris</i>	134	75

enzymatic reaction was terminated by the addition of 100 μ l of 2 N sulphuric acid. The absorbency was read at 450 nm against blanks in an automatic plate reader (Multiskan® MCC 340, Flow, UK). The results were expressed in optical densities (OD) recorded under the conditions of

assay. All determinations were performed in duplicate. An example of the values obtained with an allergen (*D. pteronyssinus*) is shown in Fig. 1.

Statistical analysis

For REINA, using the method of least squares, the dose-response relationship is estimated by regression line analysis $\log(Y) = a + b \times \log(X)$, in which *Y* is the arithmetic mean of the two ODs obtained and *X* is the dilution of free allergen added for inhibition. Upper and lower confidence limits (95%) for the percentage of unlabelled allergen that produces 50% of inhibition were calculated.

For the prick-test, also using the method of least squares, the bioassay of dose-response relationship of the allergen and the skin reaction is estimated by regression line analysis $\log(A) = a + b \times \log(C)$, in which *A* is the geometric mean of all the weal areas provoked by a known allergen concentration and *C* is this allergen concentration.

Regressions were carried out with the program Stat-View on an Apple SE/30. Linearity was tested by the F

Table 4. Results of the *t* test with the corresponding *P* value when comparing the slopes of the prick-test and REINA-C

Allergen	Slope		<i>t</i> -test	<i>P</i> value
	Prick-test	REINA-C		
<i>Dermatophagoides pteronyssinus</i>	0.308	0.364	1.2521981	<0.001
<i>Plantago lanceolata</i>	0.363	0.338	1.0480545	<0.001
<i>Phleum pratense</i>	0.289	0.314	0.8574929	<0.001
<i>Salsola kali</i>	0.237	0.236	0.0163671	<0.001
<i>Secale cereale</i>	0.337	0.368	0.9716007	<0.001
<i>Parietaria judaica</i>	0.354	0.319	0.5041323	<0.001
<i>Lolium perenne</i>	0.372	0.382	0.4449942	<0.001
<i>Artemisia vulgaris</i>	0.400	0.275	4.8183959	<0.001

test (analysis of variance) comparing the variance due to deviations of *Y* (in REINA-C) or *C* (in prick-test) from the fitted line (deviations from regression) with the variation due only to *Y* or *C* (the pooled error from the *Y* or *C* replicates, the within mean square).

For testing the regression lines for parallelism (i.e. equality of slopes) the *t* test was used [10].

Results

Table 1 shows the results of the regression analysis (slope and correlation coefficient) and the test for linearity of the prick test. The value of the slopes ranged from 0.289 (*Phleum*) to 0.400 (*Artemisia*), with a correlation coefficient > 0.96 in all cases. The F test of analysis of variance had a *P* value ranging from 0.0064 in *P. judaica* to 0.0001 in *P. lanceolata*.

The results for REINA-C are summarized in Table 2. The value of the slopes ranged from 0.236 (*Salsola*) to 0.368 (*Secale*), with a correlation coefficient > 0.92 in all cases. The F test of analysis of variance had a *P* value ranging from 0.0259 in *S. kali* to 0.0001 in *L. perenne*. Table 3 shows the upper and lower 95% confidence limits for 50% inhibition, ranging from the lower limit of 54% for *S. kali* to 86% for *P. lanceolata*, and the upper limit of 187% for *S. kali* to 116% for *P. lanceolata*. For all the allergens these 95% confidence intervals are fitted into the limit values of 50 and 200%.

In all cases, the correlation was quite good ($r > 0.92$) and the test for linearity was also very good (F test had a *P* value < 0.05 in all cases).

The test of parallelism, using the *t* test for comparing the two slopes, showed that in all the comparisons the result of *t* had a *P* value < 0.001 (Table 4). Figure 2 illustrates the observed parallelism for all the allergens tested.

Discussion

When comparing a new method of dose-response with a previous existing one, with the aim that the results of measuring the relative potency of the same compound could be comparable, the assumption that the two lines satisfy particularity, linearity and parallelism must be met.

Although REINA-C, as a radioligand assay, is perhaps not strictly a bioassay, since it does not depend upon responses measured in living organisms or tissues, it is so similar in structure that it needs to be considered from the viewpoint of bioassay [11]. The method of the skin-prick test used in this study is a real bioassay, in which the measurable skin response is related to the allergen concentration. In consequence, the two methods, since they are based on a bioassay of measured dose-response for assessing the relative potency of allergenic extracts, could be compared.

For both methods, the F test proves that there is no evidence for lack of linearity, the log-log regression of dose-response being practically linear. As a consequence, in the REINA-C method, the upper and lower 95% confidence limits for the 50% inhibition are practically into the range of 150% and 70%, which is a narrow range for a log-log regression. Moreover, these 95% confidence limits are tighter than the 200% and the 50% of the stated potency accepted [12] for assays of total *in vitro* allergenic activity.

The *t*-test proves the identity in the slopes of the log-log regression dose-response, the lines being, as a consequence, parallel. Since the same extract is used for both methods, and proving that the slope parameter assessed on IgE binding inhibition in REINA-C is identical to that obtained with skin-prick test titration, the assumption of similarity in the kinetics of the allergen-ligand interaction must be made. If a non-parallelism was obtained, it could

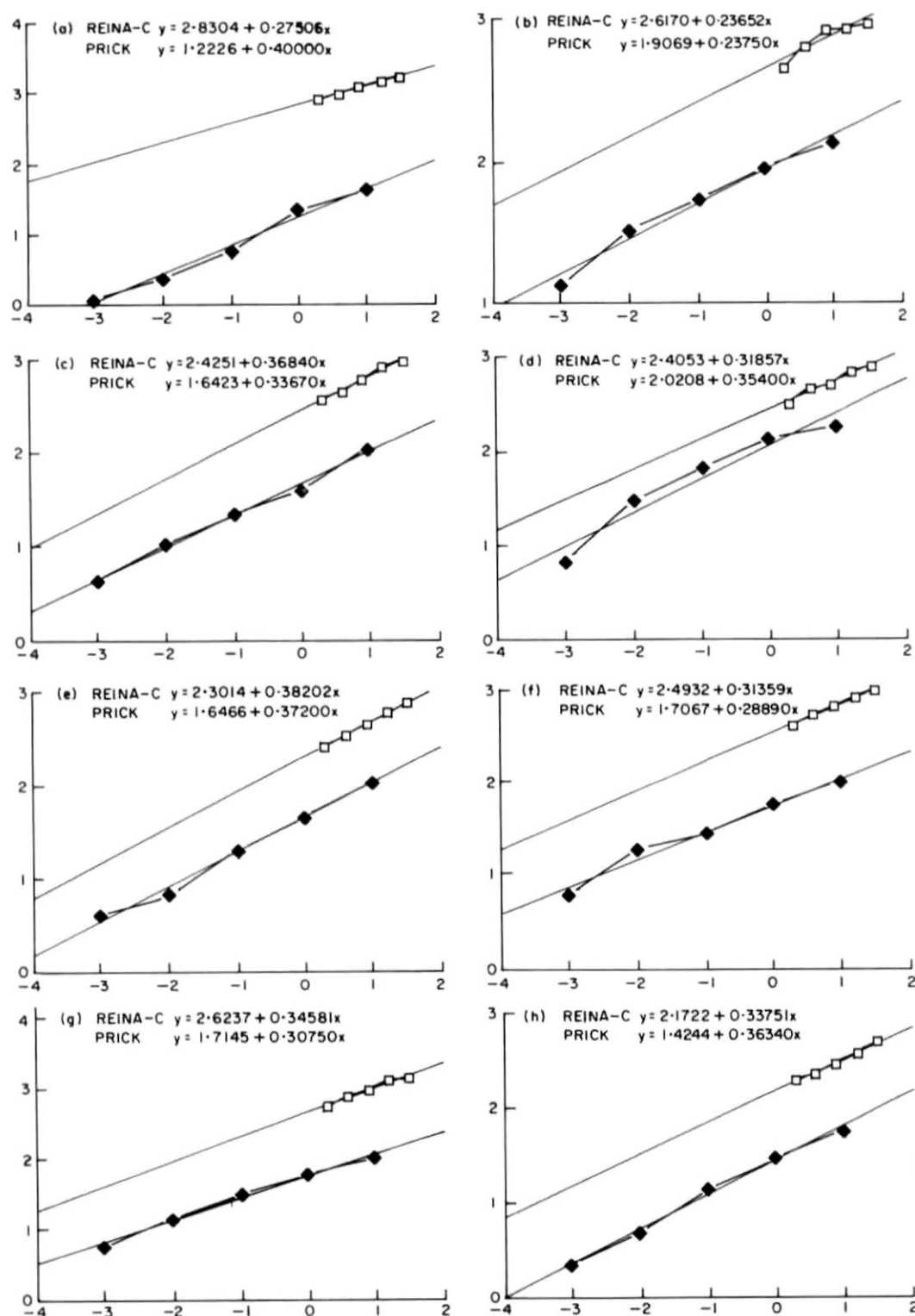


Fig. 2. Scatter diagrams of the log-log regression lines obtained for each allergen with $\blacklozenge$, skin-prick test and $\square$, REINA-C. (a) *Artemisia vulgaris*; (b) *Salsola kali*; (c) *Secale cereale*; (d) *Parietaria judaica*; (e) *Lolium perenne*; (f) *Phleum pratense*; (g) *Dermatophagoides pteronyssinus*; (h) *Plantago lanceolata*.

indicate, to some extent, that the extracts may be compositionally different [13], or that the kinetics of the allergen–ligand interaction are different in both methods.

On the other hand, the REINA-C test is easy to perform, has sufficient sensitivity and processing speed to handle the large numbers of samples generated by production, and has some advantages over the other well-known method of inhibition of specific IgE binding, the RAST-INH method: it is faster and the influence of allergen-specific antibodies of isotypes other than IgE, which could interfere in RAST-INH as happens with normal RAST [14–18], is avoided in REINA-C, which depends on IgE pre-extracted from the serum sample by immobilized monoclonal anti-IgE [4,7,18,19].

The skin test method has been proved to be useful and accepted as the only method feasible for standardization of allergenic extracts [2,3]. In view of the results, with close agreement in the regression dose–response lines, the REINA-C method could also be used as a tool for *in vitro* standardization of allergenic preparations.

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