

Use of Monoclonal Antibodies in the Standardization of *Parietaria judaica* Allergenic Extracts

C. Afferni, C. Pini*, R. Tinghino, S. Palumbo, M. M. Biocca,¹ G. Bruno,¹ A. Mari and G. Di Felice
Department of Immunology, Istituto Superiore di Sanita', and ¹I Clinica Medica,
Universita' degli Studi 'La Sapienza', Roma, Italy

Abstract. Two monoclonal antibodies (MoAb) specific for *Parietaria judaica* allergenic components were selected on the basis of their capability to recognize either the *Parietaria judaica* major allergen (MoAb 1A6/1D1) or several other allergenic components (MoAb 1A4/2F8) except the major allergen. These two antibodies, either individually or combined, were used to develop an ELISA-inhibition system using a reference *Parietaria judaica* extract (in-house Reference preparation, IHR). The assays performed with these reagents were firstly standardized by testing the IHR several times. A good reproducibility, evaluated both at the level of 50% inhibition values, and in terms of analysis of the variance of the slopes of the regression curves, was obtained. Subsequently, the potency of several *Parietaria judaica* extracts, either obtained by manufacturing companies or produced in other laboratories, was evaluated by these tests. Data obtained by interpolation with the IHR values and expressed in terms of arbitrary units (AU) were compared with those obtained by classical human IgE inhibition, performed with sera from allergic patients.

Results indicate that the monoclonal antibodies produced in our laboratory can be successfully employed, either individually or combined, in the standardization of allergenic preparations in addition to, and possibly replacing, the classical IgE-based standardization procedures which require human specimens often available in limited amounts only.

Introduction

The lack of carefully standardized allergenic extracts represents one of the most critical points in the diagnosis and therapy of allergic diseases.^{1–4} Several attempts have been made in the past to standardize such preparations with the aim of having extracts showing reproducible behaviour at the diagnostic as well as therapeutic level.⁵ Besides the *in vivo* standardization approach, which requires the physical presence of a panel of allergic patients, the most common alternative *in vitro* approach for allergen standardization was set up by Yman *et al.*, in 1975,⁶ who developed an IgE-inhibition technique which has been subsequently modified essentially introducing new solid phases and properly developed statistical approaches. However, despite the great and obvious advantages of this approach, as compared with *in vivo* methods, the need for large amounts of patients' sera was often a limitation. The scarcity of patients

allergic to a particular allergen only rendered any standardization approach difficult, if not impossible particularly for less frequent allergens. More recently, after the monoclonal antibody (MoAb) technology became available, many attempts were made to apply this technique successfully to the standardization of allergenic extracts⁷ by measuring the major allergen content.⁸ Several approaches have been followed according to the availability of MoAbs and a number of allergenic components have also been purified and characterized.^{9–19} In some instances, methods employing labelled purified molecules as well as sandwich methods with MoAbs specific for two different epitopes have been used.^{20–29}

As regards *Parietaria judaica* pollen allergens (one of the most relevant allergenic sources in the Mediterranean area) several attempts have been made to purify, characterize and quantitate the most important allergenic components.

The major allergen was firstly purified by conventional bio-chemical methods,^{12,30–32} and further characterized by using MoAbs.^{10,33} Moreover, the major allergen *Par j I* has also been quantitated by a MoAbs

*Author to whom correspondence should be addressed: Dr Carlo Pini, Dept. Immunology, Istituto Superiore di Sanita', Viale Regina Elena 299, 00161 Roma, Italy.

assay, and the evaluation of the major allergen content has been used to standardize the *Parietaria judaica* pollen extract.²³ However, it should be pointed out that the standardization of allergenic extracts based upon a MoAb specific for a single component might lead to confusing results due to the fact that only a single epitope in the extract is recognized and monitored. This is particularly true when the stability of such extracts is evaluated, since the overall stability is, in the end, the stability of a single epitope present on a single allergenic component.

In our laboratory, MoAbs specific for several allergenic components present in the *Parietaria judaica* pollen extract were produced in BALB/c mice and characterized by ELISA and immunoblotting.³³ Two clones, named 1A4/2F8 and 1A6/1D1 were chosen on the basis of their specificity. The former was able to recognize several cross-reacting allergenic components different from the major allergen, while the latter reacted with the major allergen only. These antibodies, either individually or combined, were used to develop an ELISA-inhibition system employing an in house reference (IHR) allergenic preparation.

The aim of the present paper is to investigate whether these reagents, as well as the method developed, can be successfully applied to the standardization of *Parietaria judaica* allergenic extracts in addition to, or possibly replacing, the classical IgE-based methods requiring large volumes of highly specific human sera which may present handling problems and are often available only in very limited amounts.

Materials and methods

Antisera

Human allergic sera. Human sera were obtained from patients sensitive to *Parietaria judaica* only, as proven by skin test and radio-allergo-sorbent test (RAST). According to standardized criteria,³⁴ highly positive sera (RAST class 4) were pooled, aliquoted and stored at -30°C until used. The sera considered for the pool were all reactive with *Par j* I and with many other allergenic components, as evaluated by immunoblotting.

Monoclonal antibody. MoAbs were prepared and characterized as previously described.³³ Briefly, three mice were injected three times intraperitoneally with *Parietaria judaica* pollen extract (100 μg of protein for each injection) emulsified in Complete Freund's Adjuvant. Three days before the fusion, one mouse,

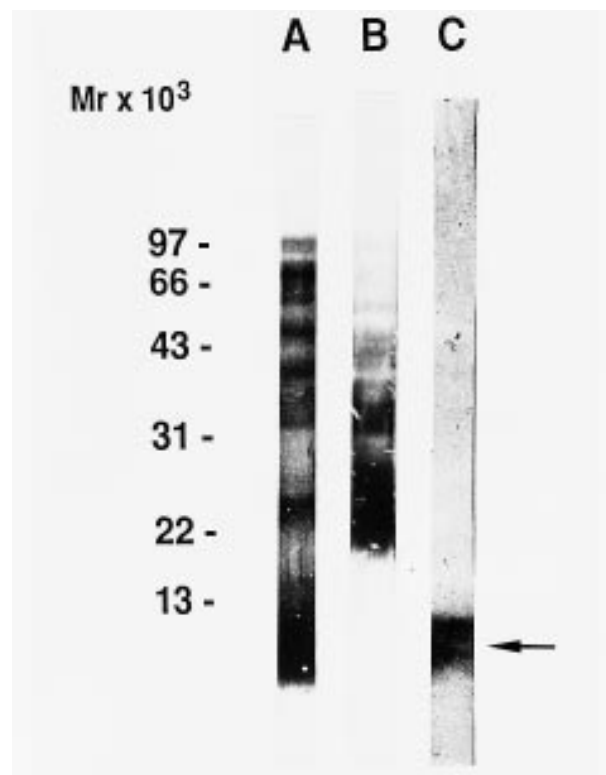


Figure 1. Immunoblotting pattern of *Parietaria judaica* pollen extract. Lane A: immunoblotting performed with pooled human IgE; lane B: immunoblotting performed with mouse monoclonal antibody 1A4/2F8; lane C: immunoblotting performed with mouse monoclonal antibody 1A6/1D1. Arrow indicates the *Par j* I major allergen.

chosen on the basis of the intensity of the antibody response evaluated by ELISA, was boosted intravenously with the same amount of antigen in saline. After the fusion, cells were seeded in selecting medium and growing hybrids were tested by ELISA and immunoblotting. Hybrids of interest were cloned twice by limiting dilution and expanded *in vitro*. Supernatants were concentrated ten times by precipitation at 50% ammonium sulphate saturation. Supernatants from clone 1A4/2F8, specific for several allergenic components of the extract except the major allergen, and from clone 1A6/1D1, specific for the major allergen only, were used throughout the experiments. The pattern of reactivity of these antibodies is reported in Fig. 1.

Parietaria judaica pollen extracts

Extract preparations. *Parietaria judaica* pollen was kindly supplied by Lofarma (Milan, Italy). The in-house extract was prepared according to Giuliani *et al.*³²

One extract was kindly supplied by Dr A. Ruffilli (International Institute of Genetics and Biophysics, Naples, Italy). Commercially available extracts were kindly supplied by the producers Lofarma (Milan, Italy), S.A.R.M. (Rome, Italy) and Pharmacia (Uppsala, Sweden).

Establishment of an 'In House-Reference (IHR)' preparation. In order to standardize the inhibition assays, the *Parietaria judaica* pollen extract, prepared in our laboratory (see above) was fully characterized by biochemical and immunochemical techniques as previously reported,³³ and was assigned 10 000 arbitrary units (AU) of activity. This extract was used as an IHR preparation. For each ELISA plate, the IHR was run together with the samples, and the potency as well as the parallelism of the latter was evaluated by using a parallel line program, kindly provided by Prof. G. Doria (ENEA, Casaccia, Italy).³⁵

Enzyme-linked immunosorbent assay (ELISA) inhibition

ELISA inhibitions were performed according to the principle developed by Yman *et al.*⁶ Microelisa plates (Dynatec M 129A, Plochingen, Germany for MoAbs assays or Falcon 3912, Becton Dickinson, Oxnard, CA, U.S.A. for human IgE assays) were coated with the antigen for 3 h at 37°C, and then overnight at 4°C. For each well, 0.2 ml of 20 µg/ml of *Parietaria judaica* pollen extract in 0.05 M carbonate buffer; pH 9.6, 0.02% NaN₃, were added. Two different ELISA inhibitions, and therefore two slightly different sets of conditions were used, in relation to the antibody under investigation.

Monoclonal antibody inhibition. The 10-times concentrated MoAbs supernatants were pre-diluted 1:160 and incubated for 1 h at room temperature (r.t.) with twofold serial dilutions of inhibitors. The mixtures were then transferred to the plates and further incubated for 1 h at r.t. After washings, the plates were incubated for 1 h at 37°C with peroxidase-labelled rabbit anti-mouse IgG (Bio-Rad, Richmond, CA, U.S.A.) (1:3000). The plates were washed again and developed for 30 min by adding 0.2 ml of orthophenyldiamine solution (Sigma, Milan, Italy) (0.5 mg/ml in 0.1 M citrate/0.2 M phosphate buffer, pH 7.2, containing 0.01% hydrogen peroxide). The reaction was stopped by adding 50 µl 0.5 N sulphuric acid and the plates read at 495 nm in a Titertek Multiskan ELISA plate reader (Flow Laboratories, Milan, Italy).

The two monoclonals combined in an optimal ratio, found on the basis of preliminary results to be equal to 1:1, was prediluted 1:1200 and incubated for 1 h at r.t. with twofold serial dilutions of inhibitors: the assay was continued as above.

Human IgE-inhibition. Inhibition of specific IgE was carried out by incubating the human pool, diluted 1:5, for 3 h with two-fold serial dilutions of inhibitors. After this period, the mixture was transferred to the plates, previously blocked for 1 h at r.t. with 3% gelatin, and further incubated for 3 h at r.t., followed, after washings, by an overnight incubation with a peroxidase-labelled rabbit IgG anti-human IgE (KPL, Gaithersburg, MD, U.S.A.) (1:2000). The plates were developed as above.

Statistical evaluation

The reproducibility of the various inhibition assays was evaluated by calculating the coefficient of variation of the results, expressed in µg protein/ml of inhibitor producing 50% inhibition, obtained by testing the IHR several times. The correlation of the results (AU of potency) obtained with the different ELISA inhibition systems by using all the available extracts was evaluated by regression analysis³⁶ performed after logarithmic transformation of the data.

Protein assay

The protein assay was performed according to Bradford.³⁷

Results

In order to find out the optimal conditions for the ELISA-inhibition systems, the two MoAbs, individually and combined, were titrated by a direct ELISA. Thus, hybridoma supernatants concentrated 10 times were individually tested at different serial dilutions. Results, reported in Fig. 2, show that different curves were obtained according to the specificity of the MoAb used. In fact, as expected, the MoAb 1A6/1D1, specific for *Parietaria judaica* major allergen only gives a low optical density (OD) value, as the epitope recognized by the antibody is only partially represented on the whole extract absorbed on the solid phase. In contrast, the MoAb 1A4/2F8, specific for the epitope shared by several allergenic components, gives a much higher O.D. value, in agreement with the fact that a greater number of molecules bearing the specific epitope are available on the solid phase.

On the basis of the experimental curves obtained,

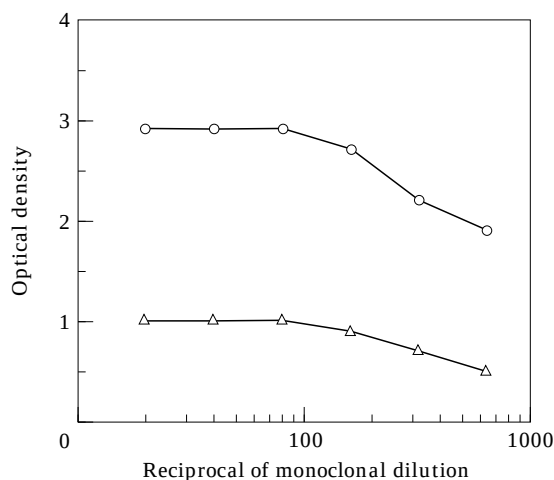


Figure 2. Titration of monoclonal antibody 1A4/2F8 (○) and 1A6/1D1 (△). Optical density was evaluated at 495 nm.

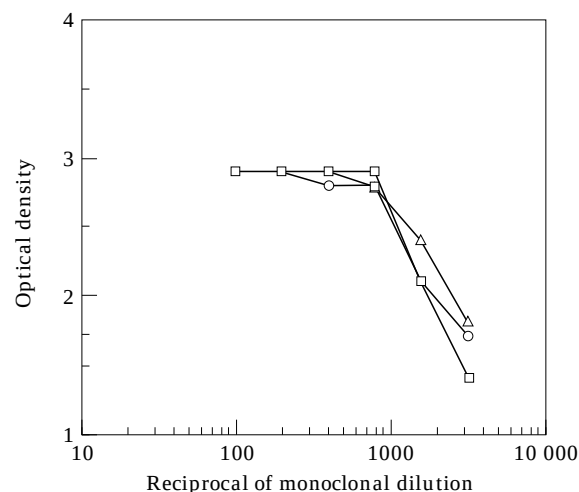


Figure 3. Titration of the mixture of the two monoclonal antibody 1A4/2F8 and 1A6/1D1 tested at the three different ratios 1:1 (△), 1:2 (○) and 1:4 (□). Optical density was evaluated at 495 nm.

the dilution 1:160 for both 1A6/1D1 and 1A4/2F8 was chosen in order to carry out the inhibition studies at approximately 80–90% of the maximum binding value in each system.

As regards the mixture of the two MoAbs, several ratios have been tested in order to find out the optimal conditions. Thus, one volume, two volumes and four volumes of 1A6/1D1 (diluted 1:160), were added to a constant amount of 1A4/2F8 (diluted 1:160). The resulting mixtures were titrated by ELISA as reported above (Fig. 3). It can be seen that no notable differences between the three conditions could be detected, as for all the three conditions a value of 1:1200 was the optimal dilution giving about 80–90% of the maximum O.D. value. These similarities were also confirmed by inhibition studies (Fig. 4). Therefore, the 1:1 ratio was chosen for all subsequent experiments.

As regards the human IgE system, all conditions were already defined in our laboratory on the basis of previous inhibition studies carried out with a standard human pool, currently employed at a dilution of 1:5.³³

In order to evaluate the reproducibility of the various ELISAs developed, the IHR was tested several times applying the established conditions. The curves were obtained by plotting the per cent of inhibition obtained in each experiment vs the amount of inhibitor and analyzed for their parallelism and for the reproducibility of the amount of inhibitor giving 50% of inhibition. Figure 5 reports on the results obtained with the MoAbs 1A4/2F8 and 1A6/1D1, with the 1:1 mixture and with the IgE-positive pooled sera.

When data were analyzed for parallelism, it was possible to demonstrate that in all instances a good parallelism between the various runs was present ($P < 0.01$). In order to assess the variability of the ELISAs in detail, the mean amount of inhibitor requested to produce 50% inhibition and its coefficient of variation (Table 1) were calculated. The variability of the ELISA inhibition performed with MoAbs 1A4/2F8, 1A6/1D1, individually and combined and with human IgE, expressed as a coefficient of variation (CV), ranged between 12.5% and 14.3% with the mono-

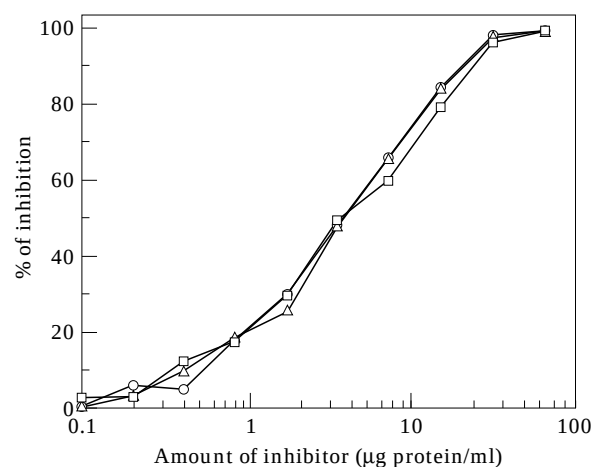


Figure 4. Inhibition by IHR of the ELISA performed with an optimal dilution of the mixture of the two monoclonal antibodies 1A4/2F8 and 1A6/1D1 tested at three different ratios 1:1 (△), 1:2 (○) and 1:4 (□).

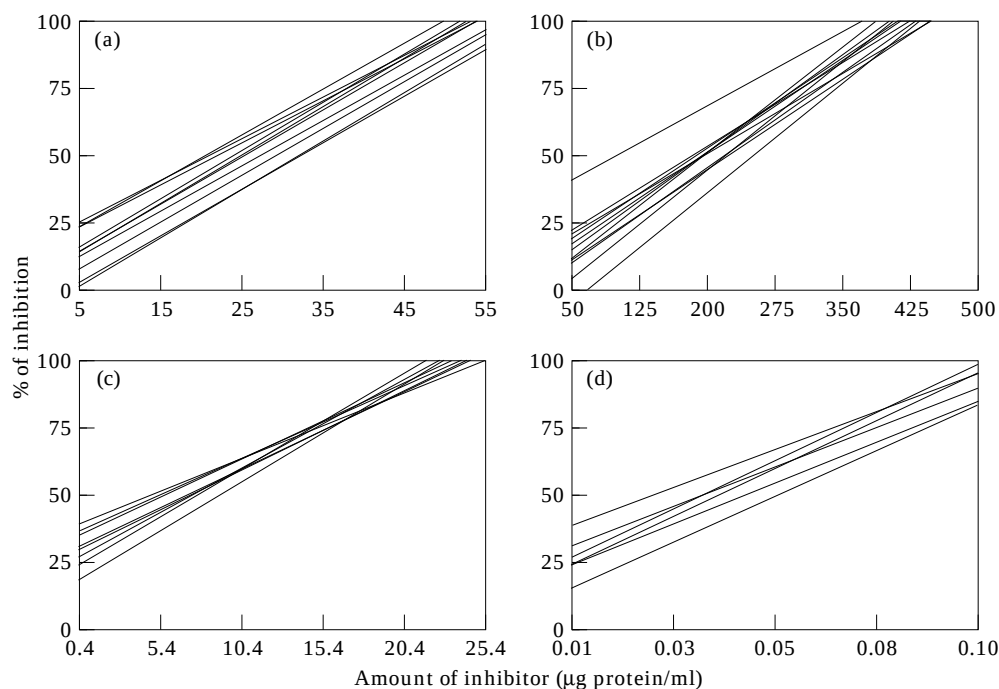


Figure 5. Inhibition curves obtained by testing the IHR several times in the four ELISA inhibition systems: MoAb 1A4/2F8 (a), MoAb 1A6/1D1 (b), their 1:1 mixture (c) and the human IgE (d).

clonal antibody systems, and was equal to 25% with human IgE. Taken together, these results support an overall reproducibility of the tests.

Once the ELISAs performed with the various reagents were properly validated, they were used to test the potency, expressed in AU, of 12 *Parietaria judaica* extracts as measured against the IHR, in which 10 000 AU were assigned. The results obtained are reported in Fig. 6. It is evident that a great heterogeneity can be observed in all the different systems used, as all the samples were distributed in the range from 1000 up to 25 000 AU. Data obtained are detailed in Table 2.

Table 1. Variability of the ELISA inhibition performed with the in house reference (IHR) extract

Antibody employed	Number of experiments	Mean $\pm$ SD ($\mu\text{g/ml}$)*	Coefficient of variation (%)
1A4/2F8	11	25.9 $\pm$ 3.7	14.3
1A6/1D1	11	203 $\pm$ 27.5	13.5
Mixture	11	4 $\pm$ 0.5	12.5
IgE	6	0.04 $\pm$ 0.01	25

* Values represent the mean concentrations of inhibitor (IHR) giving 50% of inhibition.

In order to assess whether any correlation could be demonstrated between the potency measured with the MoAb-based systems and the human IgE system, potency values obtained with 1A4/2F8, 1A6/1D1, individually and combined, were compared with

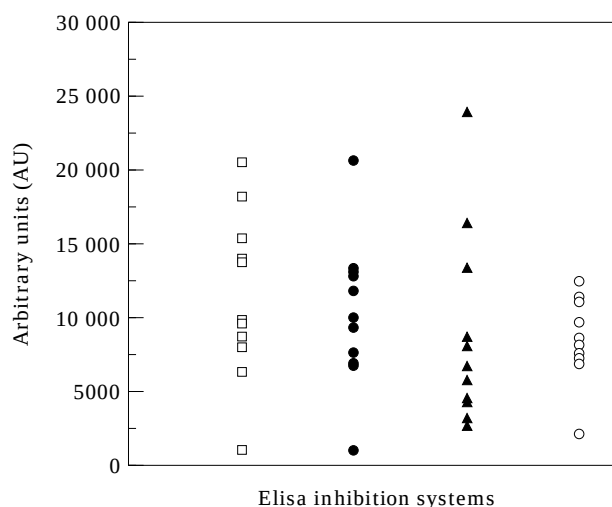


Figure 6. Distribution of the potency (AU) of 12 different *Parietaria judaica* pollen extracts as titrated by the four ELISA systems by using the IHR, in which 10 000 AU were given. 1A4/2F8 ($\square$), 1A6/1D1 ($\bullet$), their 1:1 mixture ($\blacktriangle$) and the human IgE ($\circ$).

Table 2. Potency data (arbitrary units) of 12 *Parietaria judaica* pollen extracts obtained from different sources and tested by the four ELISA inhibition systems

Antibody employed	Potency (Arbitrary Units)+			
	Range	Mean $\pm$ SD	Median	CV (%)*
1A4/2F8	1000–20 642	11 217 $\pm$ 5454	9721	48.6
1A6/1D1	1000–20 810	10 219 $\pm$ 4861	9789	47.6
Mixture	2807–24 138	8831 $\pm$ 6304	6841	71.4
IgE	2314–12 773	8880 $\pm$ 2826	8607	31.8

+ Potency was calculated by interpolation on the standard curve obtained by testing the IHR on the same ELISA plate.

* CV = coefficient of variation.

potency data obtained with the IgE system. The correlation was evaluated by linear regression analysis performed, after logarithmic transformation of the AU, in order to obtain the best fitting of the experimental points: the results, reported in Fig. 7, show the best-fit lines obtained for the three systems. All the MoAb-based systems showed a significant correlation with the IgE system ($P < 0.001$ for 1A4/2F8, $P < 0.001$ for 1A6/1D1 and $P < 0.01$ for the combined MoAbs).

Discussion

To date, one of the most important problems in the diagnosis and therapy of allergic diseases has been the availability of well defined, characterized and standardized preparations whose biological potency has been established in a reproducible manner. The most common system developed so far for the evaluation of the allergenic activity of an extract is the quantitative skin prick test (SPT), an *in vivo* approach which has been developed for diagnostic purposes, and which has been also used by companies for the calibration of an internal reference allergenic extract (IHR). However, due to the difficulties in patient recruitment required for the *in vivo* tests, this approach cannot be used for testing each batch of product in which potency has to be evaluated. Therefore, alternative *in vitro* methods have been developed, and are now largely used to compare subsequent batches with the IHR *in vivo* standardized extract. Thus, in order to overcome the problem of patients' availability linked to the SPT, a method based on specific IgE-inhibition was developed in 1975.⁶ This method, based on the inhibition of the IgE-binding with a reference extract, had both the

advantage of being an IgE-dependent, but patient-independent, test. However, in some circumstances the availability of high IgE-level serum samples is limited by the low number of untreated patients available and by the limited amount of serum sample which can be taken from each individual patient.

Thus, several methods based on animal polyclonal antibodies raised by injecting the whole extracts have also been developed,⁷ although the low availability, and the heterogeneity, of these reagents was regarded as a limit to the large diffusion of this approach.

Therefore, the availability of MoAbs has greatly improved the standardization approach, as the reagents are of defined and constant specificity, easily handled and available in unlimited amounts.

Several MoAbs have been prepared against a panel of common allergens and a variety of immunoassays have been developed in order to quantitate relevant allergens in the whole extract. In the case of *Parietaria judaica*, Corbi *et al.*²³ have published an interesting method which is able to quantitate the amount of the *Parietaria judaica* major allergen (*Par j* I) by a sandwich method based on MoAbs. However, although these authors have obtained a good correlation of the MoAb-based assay with IgE inhibition values (amount of major allergen vs arbitrary units of IgE-binding activity), the fact that only one out of the several allergenic components of *Parietaria judaica* extract is measured may remain a critical point, as compared with the polyclonal IgE system which, when carefully carried out with properly selected sera, is able to give a balanced measure of the overall allergenic potency.

The present paper describes an ELISA inhibition developed with two previously selected MoAbs³³

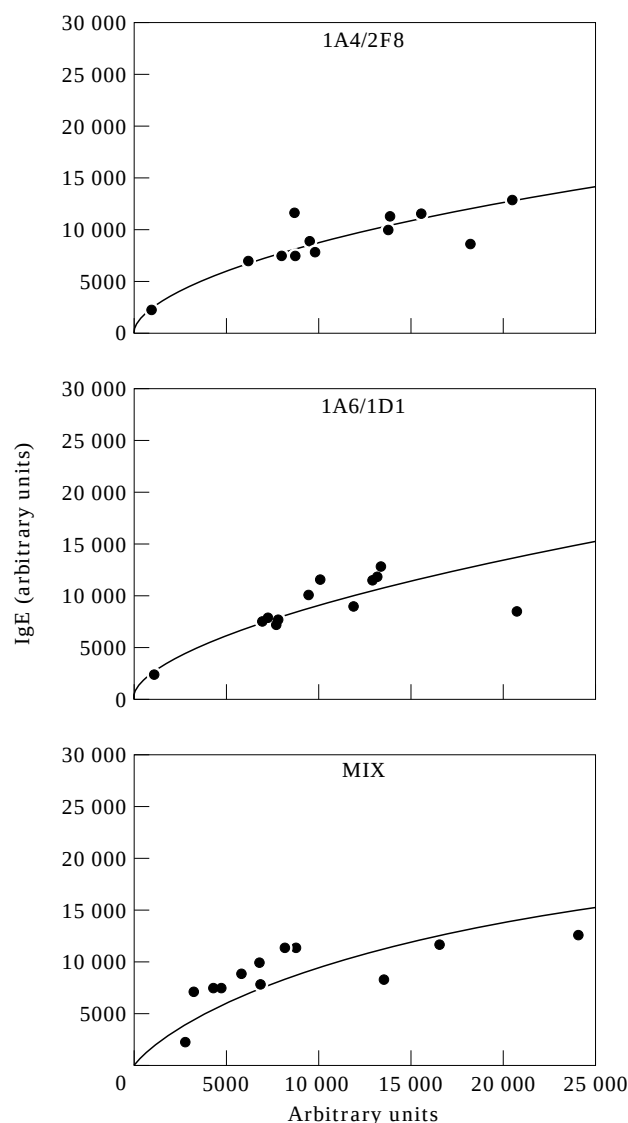


Figure 7. Correlation between the potency (AU) of the 12 different *Parietaria judaica* pollen extracts, as calculated by IgE-inhibition and the potency (AU) obtained when each extract was tested by the MoAb 1A4/2F8, 1A6/1D1 individually and combined 1:1.

Lines represent the best computer fitting obtained after logarithmic transformation of the data.

specific for the *Parietaria judaica* major allergen *Par j* I, and for a public epitope shared by several allergenic components except the major allergen itself, respectively. The idea was to test these two MoAbs either individually or as a defined combination to detect other components besides the major allergen, and to compare data obtained with the results of a classical IgE inhibition assay.

Thus, in order to validate the methods employed,

we evaluated the reproducibility of the various systems. The coefficient of variation (always less than 15% for the systems employing MoAbs and around 25% for the IgE-system) was satisfactory. Moreover, the slopes of the lines obtained in the various experiments were not statistically different from each other (for all the systems $P < 0.01$), thus confirming the reproducibility of the assays.

When several *Parietaria judaica* available extracts, obtained from both other research laboratories and allergen manufacturers, were tested, a great degree of heterogeneity of their relative potency could be observed, thus confirming that different methods of preparation and different source materials may lead to very different finished products. This heterogeneity was demonstrable with all the antibodies used, and all the systems were able to detect in a similar way differences between extracts, although the classical IgE-based system seems to be less able to detect such heterogeneity, as the values obtained are spread over a limited range of values. Moreover, a good correlation between the anti-*Par j* I MoAb and the IgE-inhibition system could be found, especially after logarithmic transformation of the data which support the theoretical assumption that the curve should approximate the origin of both axes. This finding allows us to confirm, with an independent method, that the quantitation of the *Parietaria judaica* major allergen reflects the overall IgE-binding activity of the extract.²³ This result has also been reported for other allergenic extracts, such as those from the cat,²⁴ dust mites²² and olives.²⁵

On the other hand, a good correlation was also demonstrable between the MoAb reactive with the determinant shared by several components of the extract and the IgE system, leading to the conclusion that the contribution from an important part of the extract may be underestimated when a standardization procedure involving the major allergen only is used. In this context, the use of a combination of two or even more MoAbs should ensure that several components are simultaneously monitored, and participate in the determination of the extract potency, thus better resembling the classical potency determination by polyclonal IgE inhibition. Our data obtained by using the two MoAbs combined support this idea.

In conclusion, our data confirm the usefulness of the MoAbs-based approach for the standardization of allergenic extracts and suggest that a mixture of MoAbs, carefully chosen in order to cover a large allergenic panel, could give information better related to the IgE-binding potency values than those obtained by using a single MoAb.

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