

JIM 06111

Measurement of IgE specific to a major allergen of house dust mite: Der p I

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(Received 6 February 1991, revised received 20 May 1991, accepted 24 July 1991)

A monoclonal antibody assay has been developed to measure Der p I specific IgE in sera of *D. pteronyssinus* sensitive patients. In this assay a specific monoclonal antibody was bound to the solid-phase and this complex was used for insolubilization of the allergen. Two procedures using two different solid-phases, CNBr activated paper discs and microtitre plate wells, were compared. In the paper disc assay about 90% of specific IgE was bound to the solid-phase. A study of 80 sera from mite sensitive children confirmed the importance of Der p I; indeed all the sera contained Der p I specific IgE and IgE anti Der p I contributed from 5% to 100% (mean = 39%) of the mite specific IgE response. In the microtitre plate assay only 45% of specific IgE was immobilized and it was necessary to express the results in arbitrary units. The correlation with the paper disc assay was significantly positive ($r = 0.89$) but five samples were found to be negative. However, this assay appears to be of interest for studying the affinity of specific IgE in different samples. The use of specific monoclonal antibodies as allergosorbents is a useful approach to a better standardization of the in vitro diagnostic reagents.

Key words: Allergen; Specific IgE; Monoclonal antibody; Immunoassay

Introduction

Immediate hypersensitivity to house dust mites of the genus *Dermatophagoides* is a major component of allergic disease. The allergens involved in these reactions have been fully studied (Chap-

man et al., 1980; Lind et al., 1985; Heymann et al., 1986, 1989) and reviewed by Platts-Mills and Chapman (1987). Two main groups have been defined: (i) group I includes Der p I allergen from *Dermatophagoides pteronyssinus* and Der f I allergen from *Dermatophagoides farinae* and (ii) group II includes Der p II allergen (*D. pteronyssinus*) and Der f II allergen (*D. farinae*). These two groups appear to be structurally and antigenically different but there is extensive cross reactivity for IgE binding and structural homology between Der p I and Der f I on the one hand and between Der p II and Der f II on the other hand.

The examination of allergic patients includes several aspects: (i) clinical history and examination performed by the allergist, (ii) skin and

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Abbreviations: RAST, radio allergosorbent test; IU, international unit; SAS, saturated ammonium sulphate; PBS, phosphate buffer solution; EIA, enzymeimmuno assay; BSA, bovine serum albumin; OPD, orthophenylenediamine; WHO/IUIS, World Health Organisation/International Union of Immunological Societies; NIBSC, National Institute for Biological Standards and Control; U, arbitrary unit.

provocation tests, and (iii) biological assays made in the laboratory. At the present time the most useful biological tests are the serum specific IgE measurements since these antibodies are especially involved in the mechanisms of immediate hypersensitivity. Several different possibilities are open to the clinician: quantitation of total IgE (low specificity), and specific IgE measurement using mixed (screening tests) or individual allergens. Each individual allergenic extract is in fact a mixture of several different allergenic molecules.

There have been great advances in the standardization of allergenic extracts during the last few years but differences in the allergenic components of extracts from commercial suppliers remain. For in vitro tests such as the RAST assay, the composition of the immobilized allergens is still unknown. Monoclonal antibodies directed against allergens are now available (Chapman, 1988) and their use to bind allergens on a solid-phase has been proposed for a better standardization of the reagents used for specific IgE measurement. However, there have been relatively few publications on this subject (Kemeny et al., 1986; Van der Zee et al., 1988; Heymann et al., 1989). Kemeny first described the possibility of measuring specific IgE using mono- or polyclonal antibodies to bind allergens in an ELISA assay. Van der Zee et al. (1988) have measured the Der p I and Der p II specific IgE in sera from mite allergic patients. However the procedure used (Sepharose as solid-phase and radiolabelled anti IgE) limits the extent of this method. Heymann et al. (1989) described a monoclonal antibody modified RAST to detect group II specific IgE in human serum. This present study describes the investigation of this approach, taking Der p I allergen as an example.

Materials and methods

Reagents

Monoclonal antibodies with specificity for the Der p I allergen (5H8 and biotinylated 4C1) were provided by Dr. M. Chapman (University of Virginia, Charlottesville, VA, U.S.A.). The monoclonal 5H8 is Der p I specific but the monoclonal 4C1 is directed against an epitope present on the

Der p I and Der f I allergens (Luczinska et al., 1989).

An allergenic extract of *D. pteronyssinus* was prepared from a mite culture (Allerbio, Varennes, France) by extraction over 18 h at 4 °C into 0.1 M NaHCO₃, pH 9.5. The extract was then centrifuged and filtered through a 0.45- μ M membrane (Millex-Millipore, St. Quentin Yvelines, France). The extract was aliquoted and kept frozen at -20 °C. This extract contained 380 μ g/ml Der p I. A human serum pool was prepared by mixing 15 sera from mite sensitive patients. The level of IgE anti *D. pteronyssinus* in this pool was determined by Stallerzym (bioMérieux, Marcy l'Etoile, France) to be 21 IU/ml.

Mite allergic population

Sera were obtained from 28 patients, 6–15 years old, with asthma and who had tested positive in *D. pteronyssinus* and *D. farinae* Stallerzym assays (> 0.70 IU/ml) and given positive skin tests to mite extracts (wheal > 3 mm).

Der p I quantitation

The procedure described in detail by Luczinska et al. (1989) was used with certain modifications: the saturation solution was 0.1 M phosphate buffer pH 7.4, containing 23.4 g/l NaCl and 1 g/l BSA. The samples were incubated for 3 h. The revealing reagent was 100 μ l of 1/5000 streptavidin-biotin-peroxidase complex (Amersham, Les Ulis, France) in 0.1 M phosphate buffer, pH 7.4. The plates were then washed five times and incubated with 100 μ l of 1/1000 biotinylated anti Der p I 4C1 in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature. After a further five washes 100 μ l of a 1/5000 streptavidin-biotin-peroxidase complex (Amersham, Les Ulis, France) in 0.1 M phosphate buffer, pH 7.4, were added for 30 min at room temperature. The plates were washed five times and developed with 100 μ l of OPD in phosphate citrate buffer containing H₂O₂ (bioMérieux). The color reaction was stopped after 15 min with 200 μ l of H₂SO₄ (1.8 N) and the plates read at 492 nm using a Titertek Multiscan plate reader (Flow laboratories, Les Ulis, France). The standard curve was established with dilutions of the WHO/IUIS ref-

erence preparation of *D. pteronyssinus* (NIBSC code 82/518) containing 12.5 µg/vial Der p I (Ford et al., 1985). The concentration range was 5–250 ng/ml Der p I.

D. pteronyssinus specific IgE measurement

CNBr activated paper discs were coated with *D. pteronyssinus* extract according to the method of Brighton et al. (1974). The discs were incubated with 50 µl of serum for 18 h at room temperature. After three washes over 10 min with 2 ml of washing buffer (see above), the discs were incubated with 100 µl of monoclonal anti IgE antibody horseradish peroxidase conjugate (0.1 µg/ml) (Stallerzym, Dye, bioMérieux) for 1 h at room temperature. The discs were washed three times and developed with 300 µl of OPD in phosphate citrate buffer containing H₂O₂ (bioMérieux). The color reaction was stopped after 30 min with 1 ml of H₂SO₄ (1.8 N) and, after shaking, the color intensity of the disc supernatant was quantitated at 492 nm using an AXIA 2 spectrophotometer (bioMérieux). The standard curve was established with anti IgE coated discs and dilutions of IgE solutions standardized against the second international reference preparation for IgE (75/502). The monoclonal anti IgE antibody coated on the paper disc (LE 27) and the anti IgE conjugated with peroxidase recognize two different epitopes of human IgE. Thus they may be used in a sandwich immunoassay to measure human IgE (Knutti-Müller et al., 1986). The concentration range was 0.35–60 IU/ml IgE.

Der p I specific IgE measurement: paper disc assay

CNBr activated paper discs were coated with monoclonal anti Der p I antibody 5H8 (5 µg/disc). These discs were incubated for 90 min with shaking with 100 µl of *D. pteronyssinus* extract diluted 1/190. This dilution contained 2 µg/ml Der p I. After three washes (10 min each) with 2 ml of washing solution, the procedure used was exactly the same as that for the *D. pteronyssinus* specific IgE measurement and the same calibration curve was used. Two specificity controls were performed. (a) Every serum was tested with anti Der p I coated disc without further incubation with *D. pteronyssinus* extract in order to verify the absence in the samples of anti mouse

IgE. (b) Every serum (non-diluted) was tested with a disc coated with monoclonal anti rabbit IgG and then further incubated with *D. pteronyssinus* extract in order to show that the Der p I was only bound to the disc by allergen specific antibody. All of these control IgE levels were lower than 0.35 IU/ml.

Der p I specific IgE measurement: microtitre plate assay

The plates used were the same as those used for Der p I quantitation. All further incubation steps were made at room temperature with shaking. After five washes with 300 µl of washing buffer, 100 µl of *D. pteronyssinus* extract diluted 1/190 containing 2 µg/ml Der p I were added to the wells and incubated for 90 min. The plates were washed five times and then incubated with 100 µl of serum for 3 h. After a further five washes, 100 µl of 1/2 diluted monoclonal anti IgE–peroxidase conjugate (Stallerzym, Dye, bioMérieux) were added for 30 min. After five washes the plates were developed using the same method as for Der p I quantitation. The standard curve was established with dilutions of the pool of sera containing mite specific IgE. The Der p I specific IgE level of this pool was assigned arbitrarily at 100 U/ml. The concentration range was 0.4–100 U/ml.

Quantitative aspects of specific IgE measurement

D. pteronyssinus or Der p I paper disc assay. The *D. pteronyssinus* or Der p I specific IgE were measured in five individual sera and in the positive serum pool using the paper disc assay. After the 18-h sample incubation the supernatant was removed and re-incubated on a new allergen disc for a further 18-h period. The two assays were concluded as described above. The two IgE levels obtained (1° step and 2° step in Table I) were added and the percentage bound in the first step (the ratio of specific IgE after the first step/specific IgE after both steps) was calculated (third column in Table I). The first step was performed in quadruplicate so that four supernatants were obtained and mixed in order to give sufficient volume to perform the second step in duplicate.

Der p I microtitre plate assay. The same samples were incubated in the microtitre plate as for

the specific IgE measurement, but after the 3-h incubation period the supernatant was removed and re-incubated on a Der p I paper disc for 18 h. After this incubation the supernatant was removed and re-incubated on a new Der p I coated disc for 18 h. The two paper disc assays were finished as described above. The first column in Table I lists the specific IgE levels obtained after overnight incubation (on a disc) of the supernatant from the serum incubation over 3 h in a microtitre plate well (1° step). The second column indicates the specific IgE concentrations obtained after overnight incubation of the supernatant from the previous step on a paper disc (2° step). The third column shows the difference between the sum of specific IgE levels in the two steps of the paper disc assay and the same sum for the microtitre plate assay. This difference (IgE bound on the plate) was divided by the sum of specific IgE levels in the two steps of the paper disc assay and then expressed as a percentage (third column in Table I).

Inhibition experiments

The positive serum pool was diluted 1/3 in serial dilutions of *D. pteronyssinus* extract and incubated for 1 h at room temperature with shaking. The Der p I specific IgE was also measured in this mixed solution by either the paper disc or the microtitre plate assay.

Results

Der p I specific IgE assay development

The principle of the measurement is based on the use of a Der p I coated solid-phase. Instead of using purified Der p I, which is difficult to obtain, a monoclonal anti Der p I antibody is immobilized and a non-purified extract of *D. pteronyssinus* is used as a source of Der p I.

Paper disc assay. This assay was developed in order to obtain maximum binding of specific IgE on to the solid-phase. In order to satisfy this condition it is necessary to immobilize the highest quantity of Der p I and accordingly of monoclonal anti Der p I on the disc. Our experience suggested that a level of 5 µg/disc was sufficient to saturate a CNBr activated paper disc with a monoclonal antibody (Grassi et al., 1986). Serial dilutions of *D. pteronyssinus* allergenic extract were tested in order to optimise the Der p I concentration and to saturate all the anti Der p I sites (data not shown). A dilution (1/190) of the *D. pteronyssinus* allergenic extract containing 2 µg/ml of Der p I was chosen to perform all the further Der p I specific IgE measurements in patients. The Der p I was quantitated in this dilution after incubation of 100 µl with an anti Der p I coated paper disc for 90 min with shaking. A level of 1.4 µg/ml was found, suggesting

TABLE I

QUANTITATIVE ASPECTS OF *D. pteronyssinus* AND Der p I SPECIFIC IgE MEASUREMENT BY PAPER DISC AND MICROTITRE PLATE ASSAYS

For explanations see materials and methods section.

Serum	<i>D. pteronyssinus</i> disc assay			Der p I disc assay			Der p I microtitre plate assay		
	1° step	2° step	% bound in the 1° step	1° step	2° step	% bound in the 1° step	1° step	2° step	% bound on the well
	(IU/ml)	(IU/ml)		(IU/ml)	(IU/ml)		(IU/ml)	(IU/ml)	
No. 1	25.5	9.3	73	18	2.4	88	10.7	1.3	41
No. 2	9.3	2.9	76	5.3	0.5	91	3.4	0.3	36
No. 3	35.4	6.0	86	9.3	0.9	91	4.1	1.3	56
No. 4	26	11	70	6.1	0.7	90	3.2	0.3	49
No. 5	5.6	1.1	84	3.4	0.4	89	1.7	< 0.3	50
Pool	19	5.3	78	8.1	1	89	5	0.6	38

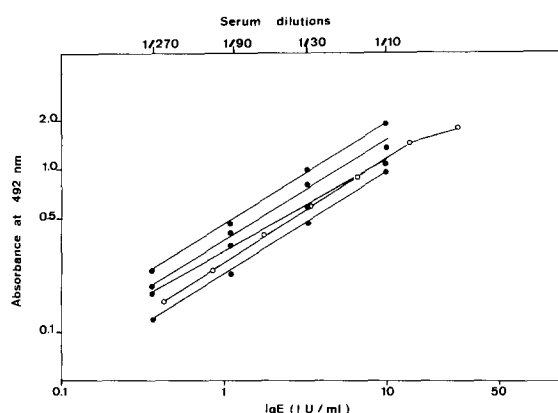


Fig. 1. Parallelism between the total IgE standard curve and the Der p I specific IgE dilution curves using the paper disc assay. ○ — ○ standard curve; ● — ● serum dilutions.

that one anti Der p I coated disc was able to bind about 60 ng of Der p I.

In order to obtain the most quantitative assay possible a long incubation time (18 h) was chosen for incubation of the serum with the Der p I coated solid-phase. We have already shown (Grassi et al., 1986) the importance of incubation time if all the specific IgE in the sample is to be immobilized. As the same monoclonal anti IgE-peroxidase conjugate was used to reveal the IgE bound to the anti IgE discs (used for total IgE quantitation) or to the Der p I discs, the results could be expressed in IU/ml of IgE (Grassi et al., 1986). In order to check this assumption, dilution curves of different samples were compared to a total IgE standard curve (Fig. 1). The parallelism observed between standard curve and several dilution curves permitted the expression of the results of Der p I specific IgE measurements in IU/ml of IgE.

The specificity of the assay was shown by inhibition with a *D. pteronyssinus* extract. The results (Fig. 2) showed a slight decrease of the signal. Non-diluted extract did not completely inhibit the IgE contained in the serum pool and the difference from the negative control remained significant. A dilution weaker than 1/27 was necessary in order to obtain a significant decrease of the signal compared to the positive control.

Microtitre plate assay. This assay was developed in order to provide a simple less time consuming assay. The binding capacity of a mi-

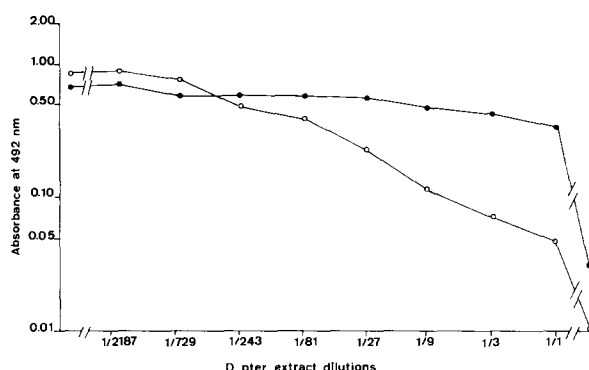


Fig. 2. Inhibition of the Der p I specific IgE measurement with *D. pteronyssinus* extract dilutions. The values on the left were obtained without inclusion of allergenic extract (positive control). The values on the right were obtained without inclusion of the positive serum pool (negative control). ○ — ○ microtitre plate assay; ● — ● paper disc assay.

crotitre plate well is known to be weaker than that of a CNBr activated paper disc (Hamilton et al., 1981; Kauffman et al., 1985). The same dilution of *D. pteronyssinus* extract (containing 2 µg/ml Der p I) was used to coat the wells as was used for disc coupling. It was also established that a weaker dilution did not permit the binding of more specific IgE (data not shown). Quantitation of Der p I after incubation of 100 µl of this dilution in a microtitre plate well led to an estimation of 30 ng of Der p I bound to the well. A typical calibration curve is shown in Fig. 3. In the case of the paper disc assay, the specificity of the test was shown by inhibition with *D. pteronyssinus*

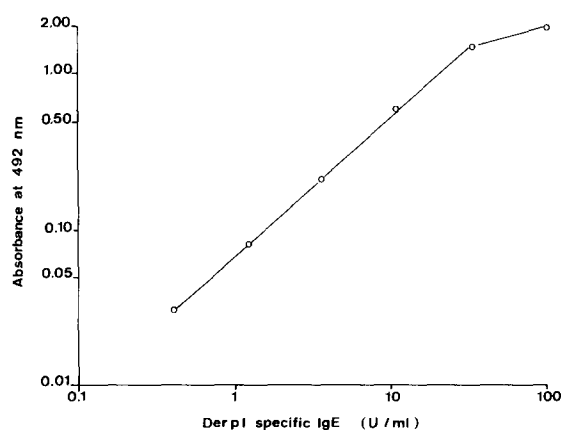


Fig. 3. Typical standard curve of Der p I specific IgE measurement by microtitre plate assay.

extract. In comparison with the disc assay the results (Fig. 2) showed a more significant signal decrease. Furthermore this decrease commenced with the first extract dilutions used (1/729–1/243), unlike the paper disc assay where no decrease was shown before the 1/27 dilution. Non-diluted extract was able to inhibit almost all IgE antibodies of the serum pool. Indeed there was only a slight difference between this and the negative control.

Correlation between the two assays

A comparison was made between the paper disc assay and the microtitre plate assay by measuring Der p I specific IgE in 80 sera of mite allergic patients. The results (Fig. 4) reveal a significant correlation ($r = 0.89$) between these two methods. However the correlation was less good ($r = 0.75$) for the 22 sera having a Der p I specific IgE level of less than 5 IU/ml by paper disc assay.

Correlation between the *D. pteronyssinus* and the Der p I disc assays

D. pteronyssinus and Der p I specific IgE levels were measured in 80 sera of mite allergic patients by the paper disc assays. All of these sera contained Der p I specific IgE. The correlation between the two measurements was significantly positive ($r = 0.85$) (Fig. 5). The contribution of IgE anti Der p I to the total IgE response against

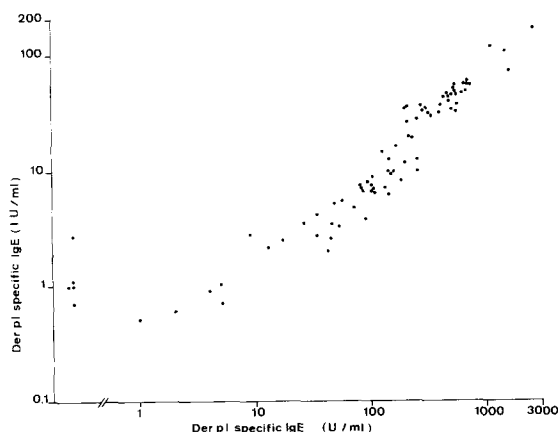


Fig. 4. Correlation between the paper disc (ordinate) and the microtitre plate (abscissa) assays for Der p I specific IgE measurement in 80 sera of mite sensitive patients.

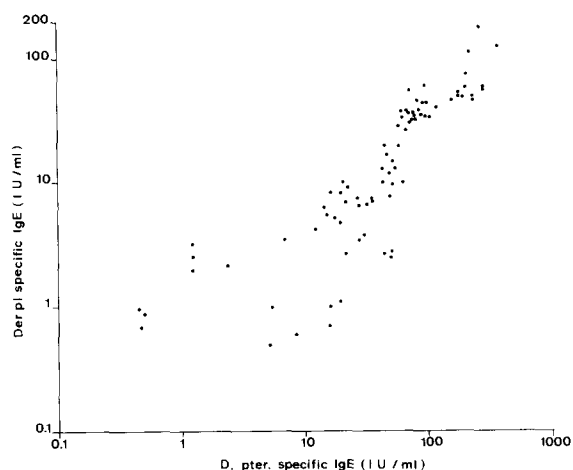


Fig. 5. Correlation between *D. pteronyssinus* and Der p I specific IgE levels in 80 sera of mite sensitive patients using paper disc assays.

D. pteronyssinus ranged from 5% up to 100% (mean = 39%).

Quantitative aspects of specific IgE measurements

To obtain exact quantitative measurements of specific IgE the sum of an infinite series of incubations should be performed. We have approximated this level from the sum of the two first steps. This leads to a maximum overestimation of 10% for the percentage of IgE bound in the first step. The results (Table I) show that with the *D. pteronyssinus* paper disc assay, 70–86% (mean = 78%) of the specific IgE was bound to the solid-phase. A slightly higher percentage was obtained with the Der p I disc assay (88–91%, mean = 90%). On the other hand with the Der p I microtitre plate assay the percentage of bound IgE was much lower (36–56%, mean = 45%).

Discussion

This work demonstrates the quantitation of specific IgE to Der p I allergen, using specific monoclonal antibody immobilized on a solid-phase. This antibody facilitates the isolation of the Der p I allergen from a *D. pteronyssinus* extract and its binding on the solid-phase, which is later used to measure the Der p I specific IgE of the samples. The advantage of this method

compared with the RAST assay is the use of well defined immobilized reagents (a monoclonal antibody and an allergen). Furthermore this method does not depend on the previous purification of the allergen. The measurement is possible because the Der p I specific antibody (5H8) does not inhibit human IgE binding to Der p I allergen (Chapman et al., 1984, 1987). Indeed the monoclonal antibodies raised against Der p I allergen recognize four species-specific epitopes which are non-overlapping antigenic sites on Der p I and one single epitope which is common to Der p I and Der f I (Heymann et al., 1986). Immunoabsorption experiments suggest that 80–95% of human IgE is directed against cross-reacting sites on Der p I and Der f I (Heymann et al., 1986). Consequently the antibody 5H8 which is directed against a species-specific epitope showed no consistent inhibition of human IgE binding (Chapman et al., 1987). However, Lind et al. (1988), using another method to study the specificity of mouse IgG and human IgE, found no essential differences between these two types of antibody. These authors concluded that further studies of the fine specificity of human IgE were needed in order to define the epitopes on Der p I for IgE.

The human IgE response is polyclonal and probably differs greatly from one person to another. Thus it is of interest to check the IgE binding of many individual patients to well defined allergens such as Der p I. Relatively little data are available on this point because the experiments were performed using radiolabelled purified Der p I which is difficult to obtain (Chapman et al., 1978, 1980) or using a rather complex method (Van der Zee et al., 1988).

To perform a measurement of Der p I specific IgE that is as quantitative as possible, a maximal amount of this allergen must be immobilized on the solid-phase. The comparison between the CNBr activated paper disc and the microtitre plate well showed that the disc bound twice as much Der p I as a well. Therefore the use of paper discs is more appropriate for quantitative determinations. Secondly, it is important to incubate the samples for a prolonged time period in order to immobilize almost all the IgE molecules, even those with low affinity. Thus the standard curve can be made with anti-IgE paper discs and

IgE standard solutions and the results can be expressed in IU/ml of IgE (Grassi et al., 1986). With such a procedure about 90% of Der p I specific IgE can be bound to the solid-phase.

Using these conditions the comparison between Der p I specific IgE and *D. pteronyssinus* specific IgE is valuable in spite of the small differences in the efficiencies of the two types of disc. This difference (about 90% for Der p I and about 78% for *D. pteronyssinus* discs) could lead to an overestimation of the Der p I specific IgE contribution to the specific IgE response against mite. On the other hand it cannot be completely ruled out that the monoclonal anti Der p I inhibits the binding of some IgE molecules.

The results show that the Der p I disc assay can detect all of the previously tested *D. pteronyssinus* sensitive patients. The frequency of reactivity is greater than that obtained previously by Van der Zee et al. (1988) (69%), Rawle et al. (1984) (80%), Stewart et al. (1988) (88%), Chapman et al. (1978) (79%) or Krillis et al. (1984) (90%) but similar to that obtained by Stewart et al. (1982) (100%). These differences are probably related to the procedure used: specific monoclonal antibody RAST (Van der Zee et al., 1988), purified allergen RAST (Stewart et al., 1982, 1988), a double antibody assay (Chapman et al., 1978) and CRIE (Krillis et al., 1984). The population studied could also be an important factor and indeed the two studies (this study and that of Stewart et al. (1982)) in which Der p I specific IgE was detected in all patients both focussed on a paediatric population.

For 74 serum samples the Der p I specific IgE level was lower than the *D. pteronyssinus* specific IgE. Such a result is not surprising because the patients have IgE directed against other *D. pteronyssinus* allergens such as Der p II (Van der Zee et al., 1988). More interesting is the fact that the Der p I specific IgE level was greater for the six samples with the lowest *D. pteronyssinus* specific IgE concentrations. Thus it seems that the Der p I specific IgE measurement is more sensitive at low IgE concentrations. One explanation could be that the allergen presentation with the antibody as a spacer improves specific IgE binding. This phenomenon would be more prominent with low IgE levels.

We have also developed a method for Der p I specific IgE measurement that is quick and easy to perform using the wells of a microtitre plate as solid-phase. The choice of a short incubation time (3 h) permits the assay to be made in one day, but suffers from the disadvantage that not all IgE molecules contained in the sample become bound (only about 45%). Thus the standard curve should be performed with dilutions of a reference serum and the results expressed in arbitrary units.

The correlation between these two types of Der p I specific IgE measurements is good, even though some samples with low levels of Der p I specific IgE determined by the paper disc assay are negative in the microtitre plate assay. It appears that it is not only a question of sensitivity because a greater dispersion was observed for the lower concentrations of specific IgE. It could be an indication of differences in the affinities of the IgE antibodies for Der p I. Indeed the wells of microtitre plates have a lower binding capacity than paper discs and one hypothesis is that the plate binds specific IgE with particularly high affinity for Der p I. The different patterns of IgE binding observed with the various samples may reflect differences in the IgE responses of the patients. The differences observed in the inhibition experiments may also be a reflection of the same phenomenon. With the microtitre plate assay the inhibition is early and significant whereas in the case of the paper disc assay it is late and weak. During the first step of the inhibition assay there is binding of specific IgE to soluble Der p I and it is probable that the IgE with the stronger affinity binds first to the allergen, though these IgE molecules cannot bind the Der p I immobilized on the solid-phase during the second step of the inhibition assay. This decrease in IgE level was not seen with the paper disc assay because it is quantitatively not very significant but it is seen in the microtitre plate assay which measures specific IgE with especially high affinity.

In conclusion, our work has confirmed the importance of Der p I allergen in mite sensitization. Der p I specific IgE measurements using a monoclonal antibody seems to be a useful method to detect mite sensitive patients. The use of well defined allergens represents a step forward in the standardization of the reagents for specific IgE

quantitation which may be extended to other allergens. Further clinical studies using such reagents should permit a better understanding of the human IgE response to allergens.

Acknowledgement

We wish to thank Mrs. M. Bertinelli for preparing the manuscript.

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