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A method for the detection of IgE binding sequences of allergens based on a modification of epitope mapping

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An ELISA method for the rapid determination of IgE binding sites (allergenic determinants) of proteins is reported. The method utilizes the epitope mapping kit (Geysen et al., 1984) to synthesize hexapeptides of an allergen of interest, followed by a biotin-avidin system to detect peptide-bound IgE. The technique allows rapid localisation of determinants from allergens of known sequence without the need to purify large amounts of allergen nor to generate peptides by cleavage of it. Using the results of the epitope mapping experiments a putative allergenic peptide containing 18 amino acid residues from the sequence of a wheat allergen was identified and synthesised on polyamide resin. Testing of this peptide by radioallergosorbent test (RAST) inhibition showed that it bound specific IgE in the sera of patients allergic to wheat.

Key words: Allergen-specific IgE; Epitope mapping; Avidin-biotin system

Introduction

Only two allergens have been studied in the detail required to elucidate their allergenic determinants or to identify peptides containing their IgE-binding sites. These are allergen M from cod and ragweed pollen allergen Ra3. Allergen M is a glycoprotein from codfish muscle of *M_r* 12 328 (Elsayed and Bennich, 1975). The molecule is divided into three homologous domains called AB, CD and EF (Elsayed and Apold, 1983). Study over a number of years localized the allergenic

determinants to four distinct sequences. The most interesting region containing allergenic determinants consisted of residues 41–64 in the CD loop (Elsayed et al., 1982). The region contained three homologous IgE-binding tetrapeptides separated by hexapeptide spacers.

Atassi and Atassi (1985) have greatly decreased the time taken to identify sequential allergenic determinants of an allergen by the use of solid-phase peptide synthesis. For the Ra3 molecule, ten overlapping pentadecapeptides were synthesised, thus spanning the whole sequence. These were then tested separately for their ability to bind a pooled serum containing human IgE against ragweed pollen. Four of these peptides were found to bind specific IgE. It was suggested by the authors that the allergenic determinant may be only part of the whole synthetic peptide in each case. For both allergen M and Ra3 the allergenic determinants, or peptides containing them, were

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; Fmoc, fluorenylmethoxycarbonyl; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; RAST, radioallergosorbent test.

also antigenic determinants. That is, they also bound specific IgG.

In the studies on both allergen M and Ra3 RAST and RAST inhibition were done only with pooled sera. This reduced the reliance on scarce individual sera. However, the studies by Sutton et al. (1984), Tovey and Baldo (1984) and Ford et al. (1985) indicated that there were considerable differences in the patterns of individual reactivities to various allergens. If this was so for allergen M and Ra3, then other allergenic determinants may exist which were not detected, since the serum pool either did not contain IgE binding to them, or, binding to such allergenic determinants was masked by one strong reactor whose serum contained little or no IgE directed towards those other allergenic determinants. This has major implications when considering an allergen or allergenic determinant for standardization and immunotherapy (Tovey and Baldo, 1984).

A second difficulty in the work on localization of allergenic determinants has been the time it takes to purify enough allergen to generate the necessary quantities of peptides for localisation, and the problems in purifying such peptides. Synthetic peptides allow much more rapid determination of IgE binding sequences. However, even the 'blanket' approach of Atassi and Atassi (1985) can still require lengthy work. Epitope mapping (Geyssen et al., 1984) when modified for IgE offered a suitable alternative for probing the binding of the IgE from the sera of allergic individuals to peptides from allergens of known amino acid sequence. Epitope mapping was developed for IgG binding. In contrast to the above methods, only a short time is required to synthesize peptides and probe them with several antisera. The peptides produced by us on the epitope mapping kit pins made up the sequence of an allergen of interest, known as the 0.28 wheat α -amylase inhibitor (Kashlan and Richardson, 1981). The protein had M_r 15 500, contained 124 amino acid residues and was shown by us to be an allergen of wheat flour (Walsh and Howden, unpublished results). To probe for the allergenic determinant(s), overlapping hexapeptides were produced. The synthesis began with the amino terminal hexapeptide and then moved along two amino acids at a time to produce the next overlapping hexapeptide, and so on. This set of

peptides, bound to pins, was probed with the sera of allergic patients thus mapping the allergenic determinants of the whole molecule.

Materials and methods

Epitope mapping kit

An epitope mapping kit was purchased from CRB (Cambridge, England) and used according to the manufacturer's instructions. Briefly, Fmoc amino acid-activated esters were covalently bound to the polypropylene pins supplied with the kit in order to produce overlapping hexapeptides from the sequence of the 0.28 α -amylase inhibitor (Fig. 1). The first hexapeptide was made up of residues 1–6 and the second was made up of residues 3–8. This overlap of two amino acids per adjacent peptide was maintained through the whole molecule to form the set of test peptides.

Probe reagents and allergic sera

Each reagent in the test was optimised for the strongest binding of antibodies to peptide and the lowest non-specific binding. The results for optimisation are not shown. These preliminary tests were carried out on unfractionated wheat albumins (Baldo and Wrigley, 1978) bound to microtitre trays. Dilutions were similar to those used by Subba-Rao et al. (1983). Allergic sera were selected which were positive in the RAST to the 0.28 α -amylase inhibitor. The sera from two allergic subjects (Arm and Bro) were exposed to the peptides on the pins. These sera had percent radioactive uptakes in the RAST of 16 and 44% respectively to the 0.28 α -amylase inhibitor. To

	10	20	30
S G P W S W C D P A T G Y K V S A L T G C R A M V K L Q C V			
	40	50	60
G S Q V P E A V L R D C C Q Q L A D I N N E W C R C G D L S S			
	70	80	90
M L R S V Y Q E L G V R E G K E V L P G C R K E V M K L T A			
	100	110	120
A S V P E V C K V P I P N P S G D R A G V C Y G D W A A Y P			
D V			

Fig. 1. The complete amino acid sequence of the 0.28 α -amylase inhibitor (Walsh and Howden, unpublished).

TABLE I

ANTIBODY DILUTIONS FOR ALL COMPONENTS USED IN THE IgE EPI TOPE MAPPING ELISA SYSTEM

Reagent	Dilution (v/v)	Incubation time (h)	Temperature °C
(1) Human IgE	1/7	22	4
(2) Goat anti-human IgE	1/2000	20	4
(3) Biotinylated rabbit anti-goat IgG	1/10000	2	25
(4) Avidin peroxidase	1/400	2	25
(5) <i>o</i> -phenylenediamine and hydrogen peroxide	—	5 min	25

test for non-specific binding of any of the reagents a sample of 100 IU/ml of IgE not directed against the 0.28 α -amylase inhibitor was also included. This sample was referred to as the 'high IgE control'.

The dilution buffer used consisted of 2% w/v bovine serum albumin, 0.05% w/v Tween 20 in phosphate-buffered saline (PBS). PBS was 0.1 M phosphate and 0.15 M NaCl at pH 7.4. Lyophilized affinity-purified goat anti-human IgE (Biorad, Richmond, CA, U.S.A.) was made up in dilution buffer at a concentration of 1 mg/ml. This antibody was tested by the manufacturer and shown not to cross-react with other human immunoglobulin subclasses. Biotinylated rabbit anti-goat IgG, avidin peroxidase and *o*-phenylenediamine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The rabbit anti-goat IgG was supplied at a concentration of 0.6 mg/ml. The avidin-peroxidase was made up by dissolving it in dilution buffer to a concentration of 1 mg/ml. All antibody dilutions were made according to Table I.

Detection of IgE binding to pins

Microtitre plates were used (low binding Immulon, Nunc, Denmark) to incubate the reagents with the peptides on the pins. The pins and plates were first blocked in dilution buffer for 1 h at room temperature and washed 3×10 min with 0.05% Tween 20 in PBS. The serum and then the reagents necessary to detect IgE bound to the peptides on the pins were then added in the order given in Table I. For the incubations, 0.175 ml of

each reagent was placed into the wells of a plate and the pins were lowered into it. The pins and plate were then sealed in a polypropylene box for the duration of each incubation. A fresh plate was used with each solution. Between each addition of reagent the pins were washed four times with 300 ml 0.05% Tween 20 in PBS for 10 min per wash. Following binding of the avidin-peroxidase the substrate was made up (just prior to use) as 0.05% w/v phenylenediamine, 0.01% H_2O_2 in 0.1 M sodium citrate, 0.1 M NaPO_4 at pH 5.0. The mixture was agitated and the reaction was allowed to proceed in the dark for 5 min. It was then stopped by the addition of 0.05 ml 4 M H_2SO_4 . The absorbances of the solutions in the wells were read at 492 nm in a Titertek Multiskan MCC plate reader (Flow Laboratories, U.S.A.). Since any non-specific IgE binding to peptides on the pins was a possible source of error, these were also separately probed with a sample of high IgE control. The results were corrected for non-specific binding by subtracting the values obtained for the high IgE control from the results obtained with sera from subjects Arm or Bro. In order to probe the pins with the serum of another subject, bound antibodies from a previous test were removed from the pins according to the procedure of Geyssen et al. (1984). This sequence of tests could be repeated a number of times.

Synthesis and sequencing of peptide 9–26

The sequence from residues 9–26 (Fig. 1) of the 0.28 α -amylase inhibitor was selected as it corresponded to a region of strong binding in the epitope maps. The corresponding peptide was synthesised on polyamide resin using the Fmoc method (Sheppard, 1986). Following HPLC purification on a C18 reversed phase column the product was sequenced on an ABI gas phase sequencer essentially according to the method of Hunkapiller et al. (1983).

RAST inhibition

RAST inhibition was performed by the method of Gleich et al. (1974) using the sera from four baker's asthma patients. Serum (0.05 ml) was incubated for 3 h at room temperature with 0.05 ml of inhibitor solution (1 mg/ml) or phosphate-buffered saline to give a result for non-inhibition.

Positive and negative control sera were also incubated with inhibitor to check for any non-specific reactions. The mixture was then added to nitrocellulose discs coupled to the 0.28 α -amylase inhibitor. The percentage of ^{125} I-labelled anti-IgE which remained bound to the disc was calculated as counts per minute in the sample divided by counts per minute in 50 μ l aliquot of anti-IgE for both inhibited (*i*) and non-inhibited (*n*) samples. Percent RAST inhibition was then calculated as:

$$100 \times \frac{(1-i)}{n}$$

Results and discussion

The allergenic determinant maps for IgE binding are shown in Fig. 2. The IgE binding peptides were mapped for the sera of two subjects with baker's asthma (Arm and Bro) and the high IgE control. The results for the control serum are shown on each allergenic determinant map as dotted lines.

Figs. 2A and 2B show the peaks for IgE binding to the 0.28 α -amylase inhibitor peptides for Arm and Bro. Table II shows the ten highest peaks for each subject and gives the percentage increase of the result from the test sera over the result for the control high IgE serum. Five peaks of IgE binding were obtained with the sera of both subjects. The other five highest peaks did not correspond to those obtained with the serum of the other individual. This result is consistent with a varying sensitivity of patients to allergenic determinants.

A second wheat allergen was tested (results not shown) and comparable results were obtained. The sequence spanning residues 9–26 (Fig. 1) corresponded to high IgE binding in the epitope map (Fig. 2) as well as in the maps for a second wheat allergen with over 60% sequence homology (results not shown). After subtraction of the control serum values from those of both test sera the region 9–26 still showed significant binding for both wheat allergens and was thus selected for further study. It was synthesised by the Fmoc polyamide method and found to bind IgE in a

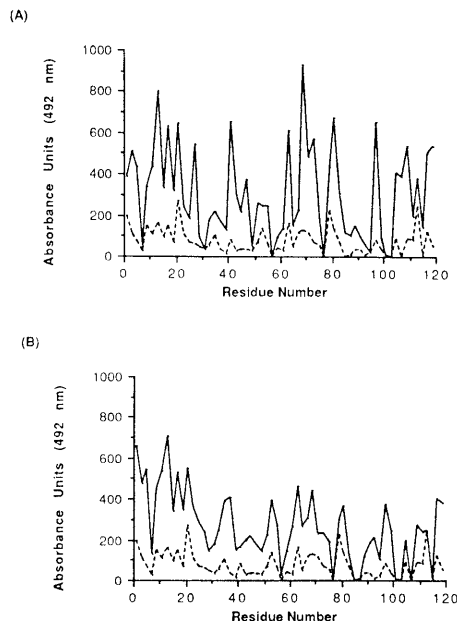


Fig. 2. Epitope maps for the 0.28 α -amylase inhibitor resulting from probing with IgE in the sera of two patients (A) Arm and (B) Bro with baker's asthma. The dashed lines correspond to values for a non-wheat protein specific IgE control. The results are expressed as absorbance of the product from the ELISA measured at 492 nm. Residue number refers to the number of the first residue in each hexapeptide with respect to the sequence of the allergen numbered from the N to C terminus.

modified RAST test for the sera of 11 subjects with clinically diagnosed baker's asthma (Table III). This provided confirmation of the effectiveness of the modified epitope mapping technique. The purified peptide was sequenced on the Model 470A protein sequencer to check that the desired peptide had been synthesised and purified. The sequence was found to be correct and there were no peptide impurities detected.

RAST inhibition

A further experiment was performed to test further the IgE binding of the peptide. If the peptide contained a determinant it should inhibit binding of IgE to the parent protein from which the peptide sequence was taken. Thus RAST in-

TABLE II

TEN PEAKS WITH THE HIGHEST ABSORBANCE AT 492 nm FOR THE RESULTS OF EPI TOPE MAPPING USING THE SERA FROM TWO SUBJECTS

They are listed in order of highest to lowest absorbance for each of the two subjects (Arm and Bro). The first residue number refers to the position in the sequence (Fig. 1) of the N-terminal amino acid of each hexapeptide. The percentage increase over background absorbance (i.e., the absorbance obtained with the control serum) for the results from each allergenic determinant map are also given.

Sequence	First residue	% increase for sera from subject Arm	Sequence	First residue	% increase for sera from subject Bro
ELGVRE	69	626	YKVSAL	13	335
YKVSAL	13	393	SWCDPA	5	654
VCKVPI	97	705	SGPWSW	1	240
DCCQQL	41	675	TGKYVS	11	395
GCRKEV	81	400	ALTGCR	17	247
REGKEV	73	741	AVLRDC	37	1103
AAYPDV	119	880	PWSWCD	3	341
LQCVGS	27	759	AAYPDV	119	593
ALTGCR	17	312	ELGVRE	69	244
LRSVYQ	63	280	LRSVYQ	63	186

hibition was performed and a serum having high, non-wheat specific IgE was used as a control. No binding of IgE to the synthetic peptide or the 0.28 α -amylase inhibitor was observed with this control serum. For each serum the synthetic peptide inhibited binding of IgE to the 0.28 α -amylase inhibitor (Table III).

The method of epitope mapping of allergens should prove to be widely applicable in the study of IgE binding sites of proteins. The technique will certainly assist in rapid localisation of continuous allergenic determinants when the sequence of the protein of interest is known.

TABLE III

RAST INHIBITIONS SHOWN AS PERCENT DECREASE IN RADIOACTIVE UPTAKE IN THE RAST OF THE 0.28 α -AMYLASE INHIBITOR AFTER INCUBATION OF SERA WITH THE SYNTHETIC PEPTIDE

The quantity of inhibitor was 50 μ g in each case.

α -amylase inhibitor inhibited with	Degree of RAST inhibition (%) with serum code indicated				
	Bro	Spa	Pas	Wil	High
Peptide 5–25	44	27	13	17	0

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A fluorescence sandwich ELISA for detecting soluble and cell-associated human interleukin-2

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A fluorescence sandwich ELISA was developed for the quantitation of human interleukin-2 (IL-2) using a combination of two anti-human IL-2 antibodies, L61 and biotinylated anti-IL-2 rabbit IgG. The minimum level of IL-2 detectable by this ELISA was 0.3 pg/ml, indicating that this method is about 100 times more sensitive than the usual colorimetric human IL-2 ELISA. The ELISA specifically detected human IL-2 in cell culture supernatants and serum, and also in extracts from mitogen-stimulated peripheral blood mononuclear cells. All of the IL-2 species detected in the supernatants, sera and cell lysates were shown to have a molecular weight of 15 000, as estimated by size exclusion high performance liquid chromatography.

Key words: ELISA, fluorescence sandwich; Interleukin-2, soluble and cell-associated human

Introduction

Interleukin-2 (IL-2) promotes the proliferation of activated T cells by interaction with a specific high-affinity membrane receptor (Smith, 1984). In addition, it directly or indirectly plays an important role in the proliferation of other cell types during the immune response (Henney et al., 1981; Dempsy et al., 1982; Farrar et al., 1982). Those effects depend quantitatively on a source of IL-2

and the expression of receptors for the cytokine (Robb et al., 1981). For the quantitation of IL-2, a bioassay has most frequently been employed, using murine IL-2-dependent T cell lines, CTLL-2 cells (Gillis et al., 1978b) or HT-2 cells (Watson, 1979). However, some risk of false negatives and positives is commonly involved in the measurements, since the proliferation and viability of target cells can be affected by many reagents (i.e., buffer salts, pH, drugs, etc.), suppressive factors (Hardt et al., 1981; Honda et al., 1985; Maki et al., 1986; Martin et al., 1987) and enhancing cytokines (Ohara and Paul, 1985). An IL-2 ELISA, using polyclonal and monoclonal antibodies (Robb, 1985; Moriya et al., 1987), has the advantage that it is able to discriminate IL-2 from contaminants which might affect the response of the target cells in the bioassay. Because of variation in the sensitivity of this assay, a highly sensitive ELISA test specific for

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Abbreviations: IL-2, interleukin-2; FS-ELISA, fluorescence sandwich enzyme-linked immunosorbent assay; r, recombinant; b, biotinylated; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; n, natural.