

[15] Epitope Mapping of Allergens for Rapid Localization of Continuous Allergenic Determinants

By BRADLEY J. WALSH and MERLIN E. HOWDEN

Introduction

An antigen acts as an allergen when it stimulates receptors on B lymphocytes, causing them to differentiate and form mast cells, which in turn manufacture and secrete immunoglobulin E (IgE) specific to that allergen. Study of the binding between allergens and IgE is most important, as this interaction is the trigger for all type I (immediate) allergic reactions. Some of the symptoms of such reactions are asthma, urticaria, rhinitis, eczema, and conjunctivitis. Allergens occur in many different sources, such as dusts (e.g., grass pollens, animal danders, and dust mites), where the route of sensitization is by inhalation, and foods (e.g., milk, egg, fish, and wheat), where it is by ingestion. Allergen-specific IgE is usually measured by the radioallergosorbent test (RAST), which reacts patient serum samples with allergen bound to a solid-phase support. Any such IgE is then detected by reaction with ^{125}I -labeled anti-IgE. Results are expressed as a percentage ratio of radioactive label bound to label added.

The term allergenic epitope or allergenic determinant is used to denote a peptide sequence from a protein allergen that binds specific IgE. Such peptide allergenic determinants may be prepared by enzymatic or chemical degradation of the parent protein or by solid-phase peptide synthetic methods.¹ Allergenic determinants may be of two types: continuous or conformational. A continuous determinant refers to a linear amino acid sequence in the protein chain. A conformational allergenic determinant is formed from residues that are in close proximity due to the three-dimensional structure of the protein.² Thus a continuous allergenic determinant will endure after removal of tertiary structure, for example by reduction and carboxymethylation of cysteine residues, but a conformational allergenic determinant will not. Our experience is that continuous determinants appear to be important in binding to IgE. For example, reduction and carboxymethylation of a cow milk allergen (β -lactoglobulin, a dimer with a single disulfide bridge) resulted in IgE binding that was as high for the monomer as for the native protein.³ Reduction and carboxymethylation of an allergen from wheat flour (the 0.28 α -amylase inhibitor,

¹ H. Atassi and M. Z. Atassi, *FEBS Lett.* **188**, 96 (1985).

² M. Z. Atassi and J. A. Smith, *Immunochemistry* **15**, 609 (1978).

³ S. L. Adams, B. J. Walsh, and M. E. H. Howden, unpublished results (1987).

0.28 AI) also resulted in as high binding of specific IgE as that recorded for the native protein.⁴ These results are consistent with the notion of linear determinants binding IgE from serum samples.

Only two allergens have been studied in the detail required to provide information on their IgE-binding sites. These are allergen M from cod and the ragweed pollen allergen Ra3. Allergen M is a glycoprotein of 113 amino acid residues and contains 1 glucose moiety.⁵ The molecule is divided into three homologous domains called AB, CD, and EF. As denaturation does not affect the allergenicity of the native molecule, it is presumed to contain continuous IgE-binding sites. This was confirmed by cleaving the molecule into two fragments, both of which were antigenic⁶ and allergenic.⁷ Further study localized the allergenic determinants to four distinct sequences. The first allergenic determinant was residues 13–32 in the AB loop, which, it was concluded, functions as a divalent allergenic determinant.⁸ The second and third were residues 33–44 and 65–74, which join the AB to CD and CD to EF loops, respectively. These allergenic determinants also bind IgE *in vitro* and have sequence homology in the regions 35–41 and 67–73.⁹ The fourth allergenic determinant was residues 41–64 in the CD loop.¹⁰

Atassi and Atassi¹ identified continuous allergenic determinants on ragweed pollen allergen Ra3 by the use of solid-phase peptide synthesis. Ten overlapping pentadecapeptides spanning the whole sequence were synthesized. These were then tested separately for their ability to bind IgE in a pooled serum from patients allergic to ragweed pollen. The peptides 1–15, 21–35, 31–45, 51–65, and 71–85 bound specific IgE to varying degrees. It was suggested by the authors that the allergenic determinants fell within the regions encompassed by the synthetic peptides and that the allergenic determinants may be only part of the sequence of the synthetic peptide in each case.

The major drawbacks of the work on both allergen M and Ra3 were that tedious trial-and-error methods were used to localize the determinants

⁴ B. J. Walsh, Ph. D. Thesis, "The Molecular Basis of Allergenicity and Cross-Allergenicity in Certain Wheat Proteins." Macquarie University, Sydney, 1988.

⁵ S. Elsayed and H. Bennich, *Scand. J. Immunol.* **4**, 203 (1975).

⁶ J. Apold, S. Elsayed, L. Aukrust, and H. Bennich, *Int. Arch. Allergy Appl. Immunol.* **58**, 337 (1979).

⁷ S. Elsayed, J. Apold, K. Aas, and H. Bennich, *Int. Arch. Allergy Appl. Immunol.* **52**, 59 (1976); S. Elsayed and J. Apold, *Int. Arch. Allergy Appl. Immunol.* **54**, 171 (1977).

⁸ S. Elsayed, U. Ragnarsson, and B. Nettland, *Scand. J. Immunol.* **17**, 291 (1983).

⁹ S. Elsayed and J. Apold, *Allergy* **38**, 449 (1983).

¹⁰ S. Elsayed, S. Sornes, J. Apold, K. Vik, and E. Floorvag, *Scand. J. Immunol.* **16**, 77 (1982).

and that the RAST and RAST inhibition studies were done only with pooled sera. This reduced the reliance on scarce individual sera. However, studies by Sutton *et al.*,¹¹ Tovey and Baldo,¹² and Ford *et al.*¹³ indicated that there were considerable differences in the patterns of individual reactivities, in the various allergen sources examined by them. If this was so for allergen M and Ra3, then other allergenic determinants may exist that were not detected, since the serum pool may not contain IgE binding to them. This has major implications when considering an allergen or allergenic determinant, for use in standardization and immunotherapy.¹²

Epitope Mapping: Application to Localization of Allergenic Determinants

The need thus arose for a method that would allow the continuous allergenic determinants to be rapidly identified for allergic patients whose serum IgE bound to various purified allergens. A method was reported that allowed rapid localization of *antigenic* determinants (IgG binding) by using solid-phase peptide synthesis on an array of polypropylene pins.¹⁴ The peptides synthesized are those corresponding to overlapping sequences covering the complete antigen being studied. The procedure is similar to that used for fluorenylmethoxycarbonyl (Fmoc) peptide synthesis with minor modifications. By using a direct enzyme-linked immunosorbent assay (ELISA) with enzyme-linked antisera the continuous antigenic determinants of the protein can be mapped.

Because of the small amount of IgE (range, 0–437 ng/ml) relative to IgG (range, 7–15 mg/ml) present in serum, an enzyme-linked anti-IgE was not suitable for direct probing of the pins, as the resulting signal would not be strong enough to distinguish it from background. We therefore developed a method using epitope mapping and employing an avidin–biotin system to increase the signal and thereby detect binding of allergen-specific IgE to the pins. Thus localization of continuous allergenic determinants only requires the sequence of the allergen to be known and samples of serum to be available from patients hypersensitive to that allergen. At least two sera can be probed per week to give a full map of IgE-binding peptides

¹¹ R. Sutton, J. H. Skerritt, B. A. Baldo, and C. W. Wrigley, *Clin. Allergy* **14**, 93 (1984).

¹² E. R. Tovey and B. A. Baldo, *Int. Arch. Allergy Appl. Immunol.* **75**, 322 (1984).

¹³ S. A. Ford, E. R. Tovey, and B. A. Baldo, *Int. Arch. Allergy Appl. Immunol.* **78**, 15 (1985).

¹⁴ H. M. Geysen, R. H. Meloan, and S. J. Barteling, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3998 (1984).

10	20	30
<u>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</u>		
40	50	60
<u>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</u>		
70	80	90
SM <u>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</u>		
100	110	120
A A S V P E <u>V C K V P I P N P S G D R A G V C Y G D W A A Y</u>		

P D V

FIG. 1. Sequence of the wheat 0.28 AI.¹⁶ Allergenic determinants selected by the epitope mapping method are shown in boldface and underlined.

for each individual. The peptide determinants selected by this method can then be synthesized on a larger scale and tested for their ability to inhibit IgE binding to the whole protein in the RAST.¹⁵ The technique is cost effective, rapid, selective, and sensitive. A major advantage is that it is not necessary to purify large amounts of the allergen of interest, apart from that initially required for sequencing. If the DNA or protein sequence is known, no purification procedures are required at all, thus removing the need for an often long and arduous task.

The peptides produced on the pins in this work were from the sequence of an allergen of interest (the 0.28 AI).¹⁶ To probe for the allergenic determinant(s), overlapping hexapeptides were produced. Synthesis began at 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 all of their allergenic determinants (see Fig. 1).

Enzyme-Linked Immunosorbent Assay for Epitope Mapping

The principle of detecting IgE binding to the pins with biotin and avidin peroxidase is as follows¹⁶: IgE in serum is reacted with the peptides

¹⁵ G. J. Gleich, J. B. Larsen, R. T. Jones, and H. Baer, *J. Allergy Clin. Immunol.* **53**, 158 (1974).

¹⁶ B. J. Walsh and M. E. H. Howden, *J. Immunol. Methods* **121**, 275 (1989).

on the pins. The peptide-IgE complex is reacted with goat anti-human IgE. A third antibody, rabbit anti-goat IgG, is reacted with the peptide-IgE-anti-IgE complex. The rabbit anti-goat IgG is labeled with biotin. The final reagent that is bound to the complex is a peroxidase enzyme labeled with avidin. Avidin is a protein with four high-affinity binding sites for biotin. This high-affinity quarternary reagent binds tightly to each biotin molecule, resulting in a greatly increased sensitivity of the assay. The complex can then be detected spectrophotometrically following incubation with substrate, which gives a colored product on reaction with the enzyme.

Stock Solutions

Phosphate-buffered saline (PBS): 0.1 *M* phosphate and 0.15 *M* NaCl at pH 7.4

Wash buffer: 0.05% Tween 20 in PBS (w/v)

Dilution buffer: Used to dilute all reagents, consists of 2% bovine serum albumin and 0.05% Tween 20 (w/v in PBS)

Lyophilized goat anti-human IgE (Bio-Rad, Richmond, CA): Made up in dilution buffer at a concentration of 1 mg/ml

Biotinylated rabbit anti-goat IgG, avidin peroxidase, and *o*-phenylenediamine: From Sigma Chemical Co. (St. Louis, MO); the rabbit anti-goat IgG is supplied at a concentration of 0.6 mg/ml and the avidin peroxidase is made up by dissolving in dilution buffer at a concentration of 1 mg/ml

Note: All antibodies are aliquoted and stored at -20° . Avidin peroxidase is stored at 4° .

The substrate reagent is made up just prior to use as 0.05% *o*-phenylenediamine (w/v), 0.01% H_2O_2 (v/v) in 0.1 *M* sodium citrate, 0.1 *M* Na_2HPO_4 (pH 5.0). The mixture is agitated and the reaction is allowed to proceed in the dark for 2 to 5 min. It is then stopped by removal of the pins and the addition of 0.05 ml 4 MH_2SO_4 .

Microtiter plates are used (low-binding Immulon; Nunc, Denmark) to incubate the reagents with the pins. The plates and pins are blocked separately in dilution buffer for 1 hr at room temperature (0.2 ml/well). Following blocking, the plates are washed three times, 10 min each, with wash buffer. The dilution, time, and temperature of incubation for each antibody are given in Table I.

All antibody dilutions are made in dilution buffer. Following each incubation the plates are washed four times with wash buffer for 10 min/wash. For the incubations, 0.175 ml of each reagent is placed into the wells of a plate and the pins are lowered into it. The pins and plate are then

TABLE 1
ANTIBODY DILUTIONS FOR COMPONENTS USED IN IGE EPIOTOPE-MAPPING ELISA SYSTEM

Reagent	Dilution (v/v)	Incubation time	Temperature (°C)
Human IgE	1:7	22 hr	4
Goat anti-human IgE	1:2,000	20 hr	25
Biotinylated rabbit anti-goat IgG	1:10,000	2 hr	25
Avidin peroxidase	1:400	2 hr	25
<i>o</i> -Phenylenediamine and hydrogen peroxide	—	5 min	25

sealed in a polypropylene box for the duration of each incubation. A fresh plate is used with each solution. Between each addition of reagent the pins are washed four times with 300 ml of wash buffer for 10 min/wash. Substrate for the peroxidase enzyme is *o*-phenylenediamine (150 μ l/well). The results are read at 492 nm in a Titertek Multiskan MCC plate reader (Flow Laboratories, McLean, VA).

Since any IgE binding nonspecifically to peptide on the pins is a possible source of error, the pins are also probed with a sample of serum

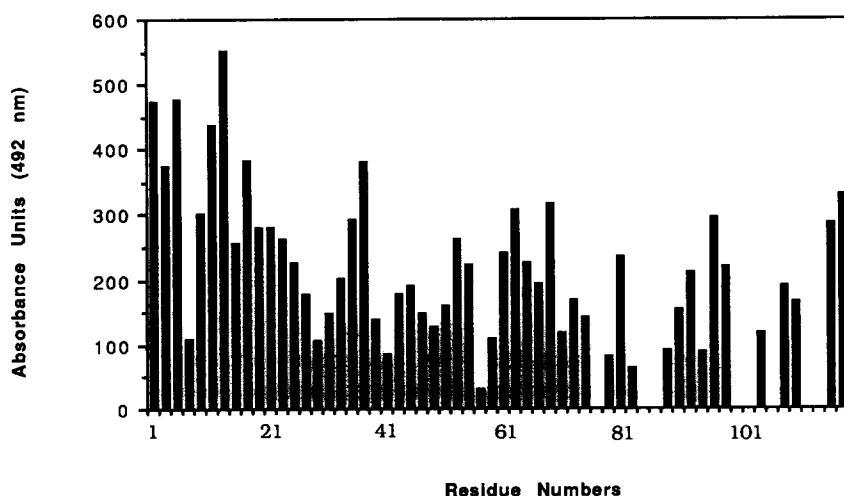


FIG. 2. Epitope maps for the 0.28 AI resulting from probing with IgE in the serum of patient Bro allergic to wheat. The results are expressed as absorbance of the product ($\times 1000$) from the ELISA measured at 492 nm. Residue number refers to the number of the first residue in each hexapeptide with respect to the sequence of the allergen numbered from the N to C terminus.

containing 100 kilounits (kU)/liter of IgE nonspecific for wheat proteins. Results are corrected for nonspecific binding by subtracting the values obtained for this high-IgE serum test from the results obtained for sera from the patient. An example of the resultant epitope map for the 0.28 AI is given in Fig. 2. The map has been corrected for nonspecific binding. Table II gives the 10 highest peaks for two serum samples probed expressed as a percentage increase in absorbance over the background absorbance for each pin.

Procedure for Removal of Bound Antibodies from Pins

The blocks of pins are placed in polypropylene baths containing 300 ml 1% (w/v) sodium dodecyl sulfate (SDS), 0.1% (v/v) 2-mercaptoethanol, 0.1 M NaH₂PO₄ (pH 7.2) prewarmed to 60°. They are then sonicated for 30 min, removed, and fully immersed in water at 60°, three times. After the water washes the blocks are finally immersed in a bath of gently boiling methanol for 2 min before being air dried. After removal of bound antibodies by the disruption procedure, pins are probed with further sera in the same manner.

Preparation and Testing of Allergenic Determinant Peptides

Residues 9–25 from the 0.28 AI, which bound IgE in the epitope mapping experiments, are synthesized on a larger scale. These amino acid residues were selected because they are in the set of 10 highest peaks and they bind IgE in the sera of both of the individuals tested (Table II). Solid-phase peptide synthesis is performed essentially according to Atherton *et al.*,¹⁷ either by using a simple, semiautomated instrument or a 9050 PepSynthesizer (MilliGen Division, Millipore, Burlington, MA). The sequences of the synthetic peptides are confirmed to be correct by gas-phase sequencing via Edman degradation and by amino acid analysis.

Coupling of Proteins to Disks

The 0.28 AI is coupled to nitrocellulose disks by the method of Walsh *et al.*¹⁶ RAST buffer is made as follows: to PBS is added 3% bovine serum albumin (BSA) and 1% Tween 20 (w/v). Coupled and blocked nitrocellulose disks are stored dry at 4°.

¹⁷ E. J. Atherton, C. J. Logan, and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1* **38**, 538 (1981).

TABLE II
PERCENTAGE INCREASE OVER CONTROL FOR 10
HIGHEST PEAKS FROM 0.28 AI EPI TOPE MAP^a

Peptide sequence	Increase (%) over control for sera of patients ^b	
	Arm	Bro
SGPWSW	—	240
PWSWCD	—	341
SWCDPA	—	654
TGKYVS	—	395
YKVSAL	393	335
ALTGCR	312	247
LQCVGS	759	—
AVLRDC	—	1103
DCCQQL	675	—
LRSVYQ	280	186
ELGVRE	626	244
REGKEV	741	—
GCRKEV	400	—
VCKVPI	705	—
AAYPDV	880	—

^a Above are given the 10 peaks with the highest absorbance at 492 nm for the results of epitope mapping using the sera from two subjects. They are listed in order of highest to lowest absorbance for each subject. The sequence for each hexapeptide is also given from N to C terminus. The percentage increase over background absorbance (i.e., the absorbance obtained with the control serum) for peptide is given for each serum.

^b Arm and Bro are patient codes.

RAST

The RAST is employed to identify the presence of IgE binding to allergenic proteins. The ¹²⁵I-labeled anti-IgE is raised in rabbit and the IgG fraction is affinity purified. The antibody does not cross-react with other immunoglobulin classes and gives low levels of nonspecific binding. The antibody may be purchased from either Pharmacia Diagnostics (Uppsala, Sweden) or Kallestad Laboratories, Inc. (Austin, TX), and should be used

within 1 month of receipt. The RAST method is performed according to the instructions of the manufacturer (Pharmacia Diagnostics, Uppsala, Sweden). Each disk is incubated with 0.05 ml of either fetal cord or test serum (diluted with PBS if necessary) in a 6-mm diameter tube for 3 hr at room temperature. The disks are washed three times in wash buffer (2.5 ml each, 10 min/wash). Then 0.05 ml ^{125}I -labeled anti-IgE is placed in each tube and allowed to react overnight. Unbound anti-IgE is removed with three more washes. The percentage of ^{125}I -labeled anti-IgE that remains bound to the disk is calculated as percentage radioactive uptake:

$$\text{Percentage uptake} = \left(\frac{\text{Total counts bound to disk}}{\text{Total counts in 0.05-ml aliquot anti-IgE}} \right) 100$$

Specific IgE binding is calculated by deducting the percentage radioactive uptake for cord serum (minus the value for its blank disk) from the value for the test serum (minus its blank).

In order to select sera for the epitope-mapping experiments, specimens collected from wheat allergic patients are tested in the RAST against the 0.28 AI coupled to nitrocellulose disks. Samples giving a high percentage radioactive uptake in the test are used for the epitope-mapping studies. The results for a group of patients allergic to wheat are given in Table III.

TABLE III
RAST RESULTS^a

Serum code	0.28 AI
Arm	16
Bro	40
Pas	16
Spa	14
Wil	8
Cord ^b	0.2
High ^c	0.2

^a RAST (radioallergosorbent test) results are expressed as percentage of total radioactive uptake. A result of 5 times background is considered to be positive.

^b Cord refers to fetal cord serum, which is normally free of IgE.

^c High refers to the serum containing 100 kU/liter IgE nonspecific for wheat proteins.

RAST Inhibition

The ability of the synthetic peptide to act as an allergenic determinant is tested by assessing its ability to inhibit IgE binding to the parent protein attached to nitrocellulose disks in RAST by the method of Gleich *et al.*¹⁵ Serum (0.05 ml) is incubated for 3 hr at room temperature with 0.05 ml of inhibitor solution (1 mg/ml). Positive and negative control sera are also incubated with inhibitor to check for any nonspecific reactions. In our hands, none is found. The mixture is then added to nitrocellulose disks coupled to the purified 0.28 AI. The percentage of ¹²⁵I-labeled anti-IgE that remains bound to the disk is calculated as above for both inhibited (*i*) and uninhibited (*u*) samples. Percentage RAST inhibition is then calculated as

$$\text{Percentage RAST inhibition} = 100(u - i)/u$$

Table IV shows the results for the RAST inhibition studies using the 0.28 AI. In all cases the peptide is able to inhibit IgE binding to the protein from which it was derived. Since the peptide inhibits IgE binding to the parent allergen, it must contain allergenic determinant(s) present in the native conformation of the allergen. The inhibition of binding varies with each serum sample. This is to be expected, since RAST measures all IgEs directed against all of the determinants of the allergen and only one allergenic determinant was used in this RAST inhibition experiment. These results thus demonstrate that the linear peptide can act as an IgE-binding determinant. The control sera do not show any reactivity to the peptides or proteins, thus indicating that the results are not due to nonspecific reactions.

TABLE IV
RAST INHIBITIONS^a

0.28 AI inhibited with	Degree of RAST inhibition (%) with serum code indicated				
	Bro	Pas	Spa	Wil	High ^b
SWCDPATGYKVSALTGCRAMV	44	13	27	17	0

^a Shown as percentage decrease in radioactive uptake in the RAST of the 0.28 AI after incubation of sera with the synthetic peptide. The quantity of inhibitor was 50 µg/50 µl of PBS in each case.

^b High refers to the serum containing 100 kU/liter IgE nonspecific for wheat proteins.

Discussion

This is the first time that the allergenic determinants of a cereal protein have been elucidated and a peptide containing a determinant synthesized. The method given here demonstrates that there are individual reactivities to the allergen. The serum from each patient contained allergen-specific IgE directed against a number of different allergenic determinants. This is consistent with the results for Ra3, for which five allergenic determinants were detected. Four of the determinants bound IgE in the sera of both patients (Table II) tested in the 0.28 AI epitope-mapping experiments.

Comments on Method

The method depends on the absorbance values from the negative control sample being low and those from the test samples being high. Therefore, the anti-IgE used must be affinity purified and also tested in epitope mapping without adding any serum containing IgE. This will indicate if there is any nonspecific binding by the anti-IgE antibody to the peptides on the pins. The problem resulting from such binding can, in principle, be eliminated by preabsorbing the antiserum with the allergen bound to a solid phase.

If serum samples are small, or the protein to be probed is large, a 1-in-10 dilution is possible for allergic sera that give RAST values of greater than 10%, approximately, against the allergen.

The value of this method is its speed and selectivity. When performed in conjunction with large-scale peptide synthesis and RAST inhibition to confirm the results, it is a powerful technique.

Acknowledgments

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