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Immunoaffinity analysis of cross-reacting allergens by protein blotting

IgE antibodies from sera having reactivity against ryegrass pollen protein allergens, wheat endosperm protein allergens and also several other cereal protein allergens were adsorbed with either ryegrass pollen or the wheat/globulin fraction immobilised on solid phases and subsequently eluted with low pH buffer. The eluted antibodies were reacted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) blots of the different allergens. Antibodies adsorbed and subsequently eluted from the two allergen sources recognised different spectra of proteins in the ryegrass pollen and cereal allergen sources and indicated the degree of immunological cross-reactivity. Intra-species cross-reactivity of IgE antibodies was demonstrated employing similar methods to those used for the pollen and cereal allergens by using a recombinant allergen from the venom of the ant *Myrmecia pilosula* as the immunoadsorbant protein on the solid phase.

1 Introduction

Several reports in the literature have described allergic subjects who are hypersensitive to pollens and who also exhibit reactivity to nonrelated food substances. Groups of subjects allergic to grass pollens, on the basis of skin-prick testing, for example, have been shown to be also reactive to cereal grains [1]. Pollinosis to several different pollens has been shown to be also associated with oral allergy to a number of nonrelated nuts, fruits and vegetables [2–7].

Broad serological IgE reactivity by 78 allergic subjects hypersensitive to ryegrass pollen and to a range of cereal grain endosperm proteins was observed in approximately a quarter of these allergic subjects [8]. In some of these subjects the IgE antibodies identifying ryegrass pollen polypeptides could be completely adsorbed by wheat endosperm protein extracts immobilised onto nitrocellulose membrane. This suggested that the IgE antibodies from some of the subjects were cross-reacting with related structural elements from both the pollen extract and wheat proteins.

This paper describes simple immunoaffinity techniques in which whole native or denatured allergen extracts or recombinant allergens can be immobilised onto solid supports and used to immunoadsorb IgE antibodies from the sera of allergic subjects. These adsorbed antibodies can then be eluted from the solid supports and used to probe blotted proteins from other allergens to investigate suspected serological IgE antibody cross-reactivity.

2 Materials and methods

2.1 Allergens

Pure, dry ryegrass pollen (*Lolium perenne*) was obtained from Greer Laboratories Inc. (Lenoir, NC, USA). Wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and rye (*Secale cereale*) flours and oatmeal (*Avena sativum*) were obtained from commercial milling companies. Extracts of the allergens were prepared as previously described [8, 9]. *Myrmecia* ants (*Myrmecia pilosula*) were collected live from several locations in southeastern Australia. Ant venom extracts were prepared as described previously [10]. Ant venom cDNA clones were isolated from a lambda gt11 Sfi-Not cDNA library prepared from poly (A) + mRNA from *Myrmecia pilosula* by immunoscreening with a pool of sera from venom-allergic individuals, as previously described [11].

2.2 Sera and IgE-radioimmunoassay

Subjects hypersensitive to ryegrass pollen and/or cereal endosperm proteins had histories of inhalant allergy to ryegrass pollen and/or ingestion allergy to cereal endosperm products and all exhibited a positive skin prick test result to extracts of these allergens. Subjects allergic to ant stings had a history of severe reactions to the stings, including anaphylaxis in some instances. Sera from hypersensitive individuals were tested for the presence of allergen-specific IgE antibodies in an IgE antibody-specific radioimmunoassay (IgE-RIA). Allergen-coated nitrocellulose or CNBr-activated Whatman 54 paper discs (5 mm dia) [12] prepared as described below were reacted with undiluted serum samples for 3 h, washed, and then reacted for 18 h with ¹²⁵I-labelled anti-human IgE (20 000 cpm/assay). The discs were then washed and counted in an LKB 1275 Mini-Gamma counter [10, 11]. The IgE-RIA results are expressed as percentage uptakes by the discs of the total radioactivity used in the assays.

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Abbreviations: BSA, bovine serum albumin; RIA, radioimmunoassay; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffer saline

2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using either 9–27% gradient gels as previously described [13, 14] or the Tricine system [15] using a 16.5% total, 6% cross-linker running gel. Solutions of the freeze-dried allergen extracts for electrophoresis were prepared as described previously [8, 11]. Electrophoretic transfer of the separated polypeptides to nitrocellulose membrane (0.1 μ pore size, Schleicher and Schuell) after SDS-PAGE was carried out as previously described [13, 14]. Blots were blocked by incubation for 1 h in TBS (0.05 M Tris, pH 8.0, 0.15 M NaCl) containing 0.1% Tween 20 [16] and dried in air at room temperature. The dried blots were stored dry at 4° until use.

2.4 Immunoblotting

Electroblots were cut into 4 mm strips and incubated by slow rocking in a Bio-Rad (Richmond, CA, USA) small incubation tray with sera from allergic individuals diluted tenfold with 0.005% v/v Tween 20, 0.5% w/v bovine serum albumin (BSA, Fraction V, Sigma, St. Louis, MO, USA), 0.1% w/v sodium azide in TBS or with antibody solutions prepared as described below for 18 h. The solutions were aspirated and the strips vigor-

ously washed three times with 5 mL 0.05% Tween 20 in TBS and then incubated for 18 h with ^{125} I-labelled anti-human IgE (80 000 counts per strip) diluted with 0.005% Tween 20, 0.5% BSA in TBS. After washing, as described above, the strips were dried in air at room temperature, mounted on cardboard and exposed to X-ray film (Fuji Rx-safety) at -70°C.

2.5 Preparation of allergen-coated supports: adsorption and elution of IgE antibodies

2.5.1 Native allergens

Ryegrass pollen extract was prepared as described previously [9] except that the extract was extensively dialysed against extraction buffer prior to freeze-drying. The freeze-dried extract was determined to be 49% w/w protein by the BCA protein determination method (Pierce, Rockford, IL, USA). Wheat total proteins were prepared as described previously [8] and determined to be 49% w/w protein. Wheat endosperm albumins and globulins were extracted from wheat flour by overnight mixing at room temperature with 20 volumes of 10% w/v NaCl. The material was centrifuged (17 000 g, 20 min) and the supernatant extensively dialysed against water. The precipitated globulins were dispersed to yield a uniform suspension (45.9% w/w protein) and freeze-dried.

Allergens (wheat endosperm total proteins and the wheat endosperm albumin and globulin fraction) were immobilized on nitrocellulose membrane discs (50–85 mm, 0.1 μ pore size). Nitrocellulose discs were coated with allergen (Fig. 1A) by incubation for 18 h at room temperature with allergen extracts. Wheat total protein extract was dissolved in SDS-PAGE transfer buffer (2 mg/mL protein in 25 mM HCl, pH 8.3, 192 mM glycine, 0.1% w/v SDS, 20% v/v methanol). Wheat albumins and globulins were dissolved in TBS and made to 0.1% sodium azide. The protein solutions, after incubation with the discs, were aspirated and the discs washed four times with 5 mL of TBS. The discs were then treated for 15 min with a 0.25% v/v solution of glutaraldehyde, washed four times with 10 mL of TBS and then reacted with 0.1 M ethanolamine, pH 9.0, for 3 h to eliminate free glutaraldehyde groups. The filters were then blocked by vigorously washing 4 times with 10 mL of 0.1% Tween 20 in TBS (20 min each wash) and stored in TBS containing 0.1% sodium azide until required.

Coupling of ryegrass pollen extract to CNBr-activated paper discs (Fig. 1B) was carried out by incubating freshly activated discs overnight at 4°C with allergen solution (2 mg/mL in 0.1 M NaHCO₃). The allergen solution was aspirated and the discs washed three times with 10 mL of 0.1 M NaHCO₃ and, to eliminate unreacted groups, was reacted with 0.1 M ethanolamine, pH 9.0, for 3 h at room temperature with occasional mixing. The solution was aspirated and the disc then washed three times with 10 mL of 0.1 M sodium acetate, pH 4.0, followed by three washes with TBS. The discs were stored in TBS, pH 8.0, containing 0.1% w/v sodium azide.

The choice of the most appropriate coupling method for the coating of the solid support for adsorption and elution of antibodies was determined by preliminary experiments. Allergen-coated discs (50–85 mm diameter) were reacted for 18 h at room temperature with serum from allergic subjects diluted tenfold in TBS, pH 8.0, containing 0.005% v/v Tween 20, 0.5% w/v BSA, and 0.1%

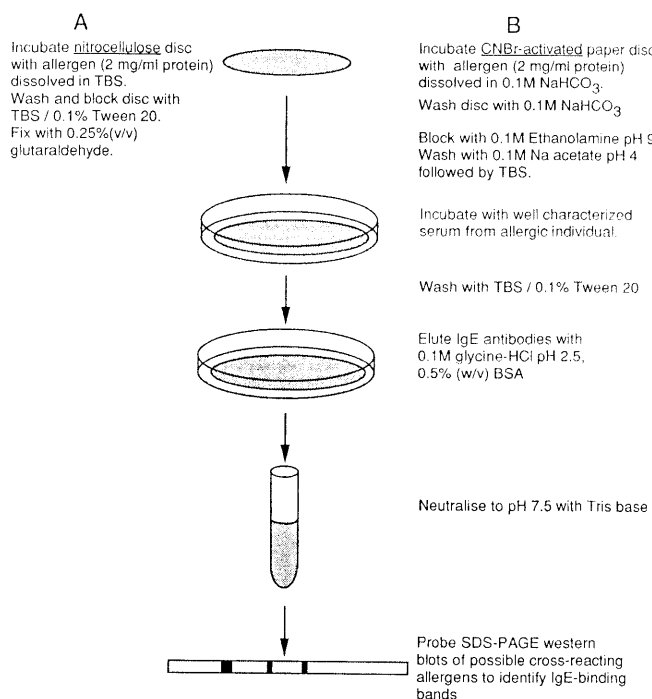


Figure 1. Diagrammatic representation of the procedure followed for the adsorption and elution of IgE antibodies from allergens bound to solid phase and subsequent probing of separated allergens with eluted antibodies. (A) Nitrocellulose disc. (B) CNBr-activated paper disc.

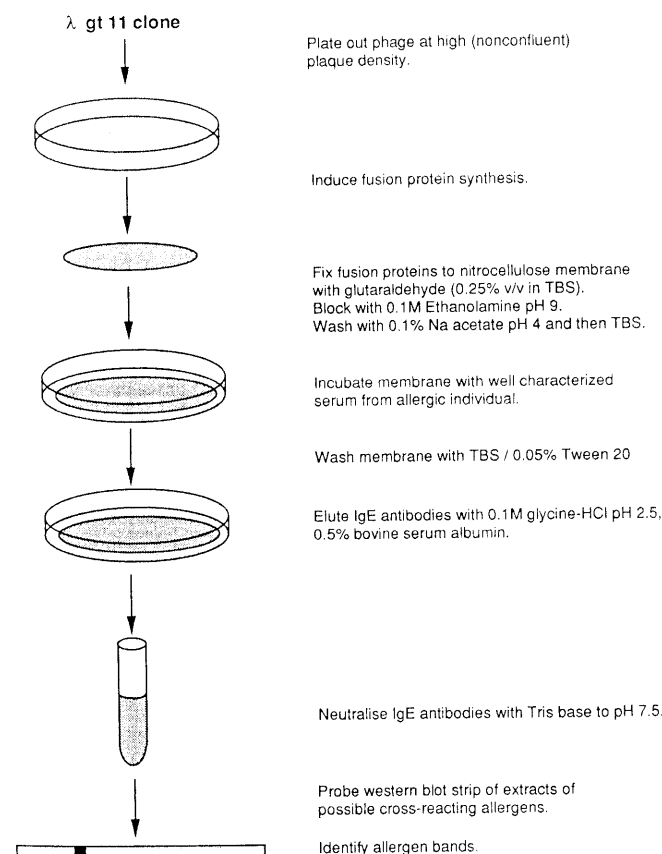


Figure 2. Diagrammatic representation of the procedure followed for the adsorption and elution of IgE antibodies from cloned allergens bound to solid phase and subsequent probing of separated allergens with eluted antibodies.

w/v sodium azide (10 mL/85 mm disc; 4 mL/50 mm disc). After incubation the serum was aspirated and the discs were washed vigorously four times with 10 mL of 0.005% v/v Tween 20 in TBS for 15 min each wash. The adsorbed IgE antibodies were eluted by vigorous agitation (10 min each elution) in four aliquots of 5 mL each of 0.1 M glycine-HCl, pH 2.5, containing 0.5% w/v BSA. The eluted antibodies were neutralized with 3 M Tris, pH 8.8. The eluted antibodies were then reacted with blot strips of native allergens separated by SDS-PAGE as described above. A schematic presentation of the method is given in Fig. 1.

2.5.2 Recombinant lambda gt11 allergens

A modification of the method described for the rapid immunological analysis of recombinant phage clones positive to sera raised against *Plasmodium falciparum* antigens [17] was used to select IgE antibodies. Lambda gt11 cDNA clones were plated on 90 mm plates at high plaque density (10^4 pfu/plate). After plaque formation at 42°C (3 h) expression was induced at 37°C by overlaying the plates with nitrocellulose filters that had been impregnated with a 10 mM solution of isopropyl- β -D-thiogalactopyranoside. After 2 h the filters were quickly placed reverse-side-down for another 2 h so that both sides of the filter were coated with recombinant protein. The filters were then treated in the same manner as the nitrocellulose filters impregnated with native allergens. A schematic presentation of the method is given in Fig. 2.

3 Results

3.1 Immunoaffinity analysis using native allergen extracts

The three subjects' (A, B and C) sera used in this study to illustrate the method all exhibited high IgE-RIA results (Table 1) with ryegrass pollen and several cereal endosperm extracts. The binding profiles of the serum IgE to ryegrass pollen extract and the different cereal endosperm extracts by allergic individuals and the IgE antibody eluates, after these sera had been adsorbed by wheat albumin/globulin, are shown in Fig. 3.

3.1.1 Subject A

The IgE antibodies from the serum that recognized wheat albumin/globulin were adsorbed from the serum by albumin/globulin immobilized on solid support. The

eluted antibodies (Fig. 3A (b)) faithfully reproduced the IgE-binding profiles observed on blots of untreated serum (Fig. 3A (a)). The results also showed IgE antibody recognition to barley (3A (c, d)), and rye (3A (e, f)) was shared by IgE antibody eluates from wheat albumin/globulin. This was not true however, for oatmeal (3A (g, h)) and ryegrass pollen (3A (i, j)). Oatmeal extract failed to bind antibodies eluted from wheat albumin/globulin after adsorption of this serum since the antibody eluate (Fig. 3A (h)) gave only weak binding to the polypeptides having molecular masses greater than the major IgE-binding band of untreated serum (Fig. 3A (g)). This latter profile can also be considered to be a good experimental control and it indicated that the adsorption-elution technique was not due to non-specific association of IgE antibodies with protein bound to the solid phase. The pattern of binding of the eluted antibodies from wheat albumin/globulin reacting with ryegrass pollen (3A (i, j)) showed that it was principally the ryegrass pollen proteins between 50–67 kDa that shared some common IgE-binding elements with wheat albumin/globulin.

3.1.2 Subject B

The IgE-binding profiles showed a weak quantitative interaction of the antibodies eluted from wheat albumin/globulin to barley (3B (a, b)) and, in the case of rye, a specific interaction of the eluted antibodies with a 35 kDa rye polypeptide (3B (c, d)).

3.1.3 Subject C

The binding to ryegrass pollen polypeptides by IgE antibodies eluted from total wheat protein (3C (b)) and from wheat albumin/globulin (3C (c)) were qualitatively different. Wheat protein is comprised of approximately 20% albumins and globulins, the remainder being the sparingly water-soluble gliadins and the insoluble glutenins [18]. These elution profiles reflect the quantitative differences between the total protein and the albumin/globulin fraction. The ryegrass pollen polypeptides identified by the IgE antibodies eluted from the albumin/globulin fraction (3C (c)) can also be identified in the ryegrass pollen IgE-binding profile of antibodies eluted from the wheat total protein extract (3B (b)). The wheat total protein extract, however, also adsorbed IgE antibodies which recognized a group of higher molecular weight ryegrass pollen polypeptides. These antibodies therefore were also recognised by components of the gliadin/glutenin fraction of wheat in the adsorption of antibodies from the serum.

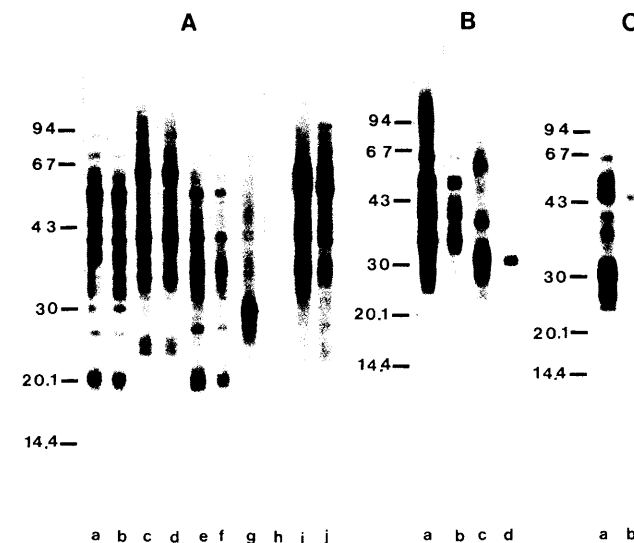


Figure 3. IgE-binding profiles of allergic subjects' sera (see Table 1) and IgE antibodies eluted from wheat albumin/globulin (a/g) or wheat total protein immobilised on solid supports to blots of cereal and ryegrass pollen allergen extracts after separation by SDS-PAGE. Subject (A): (a) wheat a/g, (b) wheat a/g eluate binding to wheat a/g, (c) barley, (d) wheat a/g eluate binding to barley, (e) rye, (f) wheat a/g eluate binding to rye, (g) oats, (h) wheat a/g eluate binding to oats, (i) ryegrass pollen, (j) wheat a/g eluate binding to ryegrass. Subject (B): (a) barley, (b) wheat a/g eluate binding to barley, (c) rye, (d) wheat a/g eluate binding to rye. Subject (C): (a) ryegrass pollen, (b) wheat total protein eluate binding to ryegrass pollen, (c) wheat a/g binding to ryegrass pollen.

3.2 Immunoaffinity analysis using recombinant allergens

The rapid method of immunological analysis of recombinant phage clones described in Section 2.5.2 (see Fig. 1) has been used by us to immunocharacterize cDNA clones encoding *Myrmecia pilosula* ant venom allergenic polypeptides [11]. Figure 4 shows how the polypeptides encoded by a cDNA clone can be used in conjunction with sera from allergic subjects to identify the allergen encoded by the cDNA clone. Lane 4 (a) shows the recognition profile by a serum, which recognised at least five IgE-binding polypeptides ranging in size from 2 kDa to 8.5 kDa. Four of these bands (8.5, 7.5, 5.5 and 2 kDa) were considered both on the intensity of binding and on the frequency of recognition by allergic subjects to be major allergenic components. Lane 4 (b) shows the IgE-binding profile of a subject whose IgE recognition was restricted to the 7.5 and 5.5 kDa bands. The IgE antibody binding profile of the eluted antibodies (Lane 4c) to the blot, after adsorption of this serum to the expressed cloned allergen (see Fig. 2), showed that both of these bands were recognised. This occurred even though the IgE antibodies were eluted after adsorption to a single recombinant protein. Non-recombinant lambda gt11 Sfi-Not was used in place of the IgE-binding clone as the control. The absence of IgE-binding using the eluate from the induced control (lane 4d) indicated that the adsorption and elution of the IgE by the recombinant IgE-binding clone was clone-specific and not due to non-specific binding of IgE to *E. coli* proteins. The recognition of two bands, the 7.5 and 5.5 kDa bands, by the antibody eluate from the recombinant IgE-binding clone, representing a single polypeptide species, indicated that these bands shared common or highly structurally related antibody combining sites.

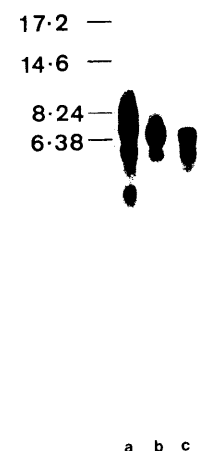


Figure 4. IgE-binding profiles of the serum of subjects allergic to the venom of *Myrmecia pilosula*. (a) Subject allergic to four major venom allergens, (b) subject allergic to two major allergens, (c) cloned allergen IgE antibody eluate, (d) nonrecombinant lambda gt11 IgE antibody eluate.

Table 1. IgE-RIA of subjects allergic to cereals and ryegrass pollen

Subject	Ryegrass pollen	Wheat albumin/globulin	Wheat total protein	Barley	Oat	Rye
A	57.2 ^a	49.2	30.8	22.3	22.3	14.6
B	28.6 ^a	35.1	45.7	29.2	47.6	18.9
C	56.8 ^a	37.7	26.7	15.0	6.8	16.6

a) Percentage of radioactive uptakes of total ^{125} I-labelled anti-human IgE in IgE-RIA by allergen-coated nitrocellulose or CNBr-activated paper discs.

4 Discussion

Serological reactivity of IgE antibodies to allergens from nonrelated sources would, in most instances, be expected to be due to different antibody populations arising as a result of separate sensitizations. However, where a consistent pattern of allergen recognition is observed between allergens from different sources, either as a result of skin-prick testing, or as a result of serological reactivity, IgE antibody cross-reaction must be considered as a possible reason for this relationship. There are, for example, non-proteinaceous small molecules such as anaesthetic drugs and muscle relaxants to which subjects have had powerful IgE-mediated allergic reactions although these agents had never before been administered [19]. Such adverse reactions can only be explained by IgE antibody cross-reactivity arising as a result of sensitization to other substances, presumably sharing structurally related allergenic determinants. Where protein allergens might be expected to be closely structurally related because of taxonomic considerations (i.e. the grass pollens), cross-reactivity might be expected to be the probable cause of serological IgE reactivity to these different allergens. However, in the absence of experimental methods to test the hypothesis, it cannot be unequivocally established that the multiple reactivity is due to cross-reaction.

Immunoabsorption and elution of antibodies provides a simple and reasonable method of establishing if serological activity to a wide range of allergens is due to antibody cross-reaction. The technique also demonstrated the ability of IgE antibodies adsorbed by nondenatured native allergens on solid phase to react with allergens denatured by SDS, heat, and reduction and subsequently separated by SDS-PAGE and electroblotted onto nitrocellulose membrane. This strongly suggests that the allergenic determinants recognised in the undenatured allergen were also recognised in the denatured allergens and were thus more likely to be sequential rather than the nonsequential, conformational type. There is, however, the possibility that some renaturation of proteins occurs during the transfer process such that the recognition might involve native conformational determinants, but there is insufficient evidence for these possible renaturations or how they might be quantified.

Pollen-allergic subjects often recognise several allergenic components in blotted allergen extracts and many subjects often share a common multiple band recognition profile [20-22]. The extent to which these multiple bands share common allergenic determinants, however, is not known. Methodology employing immunoabsorption and elution of antibodies could also be suitably applied with polypeptides desorbed from SDS-PAGE gel nitrocellulose blots with aqueous acetonitrile solutions [23]. These polypeptides could be used to coat solid supports using the same protocols as described for whole

allergen extracts to determine the extent of immunological cross-reactivity between individual IgE-binding polypeptides from the same allergen source.

Recombinant proteins have the advantage of representing a single allergenic polypeptide and, although possibly not in conformations identical to those of the native allergens, they are, however, certainly capable of recognising sequential determinants which might form component structures of conformational determinants. In the experiment described in this manuscript it was possible, using a recombinant allergen, to deduce that two major allergen bands recognised by sera from allergic subjects in fact shared common IgE-binding determinants.

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Intra-species cross-reactivity of house dust mite allergens separated by protein blotting and detected by selective elution of mite components and IgE antibodies

A complex and varied antibody response to allergens, in particular *Dermatophagoides pteronyssinus*, is observed in the serum of allergic patients. To examine the role of cross-reacting antigenic determinants in allergy, a strategy has been devised for isolating antibody populations that bind to individual polypeptides of an allergen source separated by sodium dodecyl sulfate-polyacrylamide electrophoresis and electroblotted to nitrocellulose. Polypeptides are identified on the blots, desorbed and coupled to CNBr-activated paper discs. The coupled discs are then used as immunoabsorbent surfaces for the binding of antibodies from allergic sera. Antibodies eluted from the polypeptide immunoabsorbents are then used to reprobe protein blots of the allergen. Results are presented which demonstrate strong cross-reaction between two major mite allergens, *Der p* I and *Der p* III, and some weaker intra-species cross-reactivities.

The separation of individual components of an allergen source by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), protein blotting and subsequent probing with allergic patients' sera has become a commonly used technique for locating allergens in a variety of biological extracts [1]. Comparison of different patients' antibody binding patterns often reveals a complex and varied response to the same allergen source. It is frequently unclear whether individual proteins from an allergen source elicit a unique antibody response or whether allergenic cross-reaction occurs between proteins from the same source. Inter-species allergenic cross-reaction is well known to exist for grass pollens [2, 3], insects [4], fungi [5] and a number of other allergen sources, but intra-species cross-reactivity of allergens is a neglected area of study.

Separation of the individual proteins of an allergen source by SDS-PAGE, selective probing with sera from allergic patients together with blotting studies using IgE antibodies eluted from individual protein bands offers a means of identifying and studying allergenic cross-reactivities within a single source. Previous studies [6] have described a method for preparing antibodies directed against individual polypeptides. However, the authors of this paper have found it is often not possible to prepare sufficient antibody using this method, possibly due to low antibody affinities for different polypeptides or low titres of particular antibody populations directed against different polypeptides. A strategy for the concentration of individual polypeptides separated by SDS-PAGE was therefore devised. Experiments with the house dust mite (*Dermatophagoides pteronyssinus*) are described in which selected polypeptides are concentrated, covalently bound

to a paper disc and used as immunoabsorbents. The results clearly demonstrate binding of several different mite proteins to the same population of IgE antibodies. The strategy outlined offers a general approach for studying intra-species cross-reactions in a variety of other allergen sources.

Half a gram of *D. pteronyssinus* bodies, from Commonwealth Serum Laboratories (CSL, Parkville, Victoria, Australia) were extracted as previously described [7]. Extracts were concentrated using a 2000 Da cut-off filter in an Amicon ultrafiltration unit. Mite proteins were separated on gradient (9-27%) SDS-polyacrylamide gels and electroblotted to nitrocellulose membranes (0.1 µ pore size) as previously described [7]. Protein molecular weight markers (Pharmacia LKB Biotechnology, Uppsala, Sweden) were run in wells on either side of the preparative well. The molecular weight markers were excised from the blot and stained with Amido Black. Strips were excised from either side and the centre of the blot and probed with mite allergic serum, diluted 1/10 in phosphate-buffered saline (PBS), 0.05% Tween 20, 0.5% bovine serum albumin (BSA) and 0.1% Na₂S₂O₅, followed by ¹²⁵I-labelled anti-human anti-IgE (Biolclone, Australia) and prepared for autoradiography as previously described [7]. These strips together with the molecular weight markers were used to determine the position of the major IgE-binding bands on the remaining unstained portion of the blot (Fig. 1). Transverse sections of the unstained blots corresponding to the identified IgE-binding bands were excised. A control region at the bottom of each blot was excised and subsequently treated in the same manner as the strips with the proteins being studied. The proteins bound to the nitrocellulose sections were desorbed by incubation with 20% acetonitrile in 0.1 M ammonium acetate, pH 8.9, as described by Montelaro [8] (Fig. 1) and lyophilized.

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Abbreviations: BSA, bovine serum albumin; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

CNBr activation of 50-mm-diameter Whatman 54 paper discs was carried out as previously described [9] and protein coupling was carried out with the following modifications: desorbed, lyophilised proteins prepared as de-