

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.

Received February 2, 1993

5 References

- [1] Bar Sela, S., Ben-Zvi, A. and Rubinfeld, A., *J. Clin. Immunol.* 1980, 77, 196.
- [2] Dreborg, S., *Ann. Allergy* 1988, 61, 41-46.
- [3] Eriksson, N. E., *Allergy* 1978, 33, 189-196.
- [4] Eriksson, N. E., Formgren, H. and Svenonius, E., *Allergy* 1982, 37, 437-443.
- [5] Pauli, G., Bessot, J. C. and Dietemann-Molard, A., *Clin. Allergy* 1985, 15, 372-379.
- [6] Anderson, L. B. Jr., Frefuss, E. M. and Logan, J., *Allergy* 1970, 45, 310-319.
- [7] Ortolani, C., Ispano, M., Pastorello, E., Bigi, A. and Ansaloni, R., *Ann. Allergy* 1988, 61, 47-52.
- [8] Donovan, G. R. and Baldo, B. A., *Clin. Exp. Allergy* 1990, 20, 501-509.
- [9] Ford, S. A. and Baldo, B. A., *Int. Arch. Allergy and Appl. Immunol.* 1986, 81, 193-203.
- [10] Ford, S. A., Baldo, B. A., Weiner, J. and Sutherland, S., *Clin. Exp. Allergy* 1991, 21, 167-171.
- [11] Donovan, G. R., Baldo, B. A. and Sutherland, S., *Biochim. Biophys. Acta* 1993, 1171, 272-280.
- [12] Ceska, M., Eriksson, R. and Varga, J. M., *J. Allergy Clin. Immunol.* 1972, 49, 1-9.
- [13] Tovey, E. R. and Baldo, B. A., *Int. Arch. Allergy Appl. Immunol.* 1985, 78, 82-85.
- [14] Ford, S. A., Tovey, E. R. and Baldo, B. A., *Int. Arch. Allergy Appl. Immunol.* 1985, 78, 15-21.
- [15] Schagger, H. and Von Jagow, G., *Anal. Biochem.* 1987, 166, 368-379.
- [16] Bateiger, B., Newhall, W. and Jones, R., *J. Immunol. Methods* 1982, 55, 297-307.
- [17] Ozaki, L. S., Mattei, D., Jendoubi, M., Druille, P., Blisnick, T., Guillotte, M., Pujalon, O. and Da Silva, L. P., *J. Immunol. Methods* 1986, 89, 213-219.
- [18] Donovan, G. R., Lee, J. W. and Hill, R. D., *Cereal Chem.* 1975, 54, 638-645.
- [19] Baldo, B. A. and Harle, D. G., in: Baldo, B. A. (Ed.), *Monographs in Allergy* 1990, 28, 11-51.
- [20] Ford, S. A., Baldo, B. A., Geraci, D. and Bass, D., *Int. Arch. Allergy Appl. Immunol.* 1986, 79, 120-126.
- [21] Ford, S. A. and Baldo, B. A., *J. Allergy Clin. Immunol.* 1987, 79, 711-720.
- [22] Baldo, B. A., Panzani, R. C., Bass, D. and Zerboni, R., *Molecular Immunol.* 1992, 29, 1209-1218.
- [23] Montelaro, R., *Electrophoresis* 1987, 8, 432-438.

Geraldine M. O'Neill¹
Brian A. Baldo^{1,2}

¹Kolling Institute of Medical Research, Royal North Shore Hospital, St. Leonards

²Department of Medicine, University of Sydney

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 probe 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% Na₂S₂O₈, followed by ¹²⁵I-labelled anti-human anti-IgE (Bioclone, 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.

Correspondence: Dr. B. A. Baldo, Kolling Institute of Medical Research, Royal North Shore Hospital, St. Leonards, N. S. W. 2065, Australia

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

scribed above were resuspended in 0.1 M NaHCO₃ to a final protein concentration of 40 µg/mL, as determined using the Pierce BCA protein determination kit (Pierce, Rockford, IL, USA), enhanced protocol. Fifty mm CNBr activated paper discs were cut in half and 1.0 mL of the protein solution was added to one half of a CNBr-activated paper disc and then incubated on a platform rocker at room temperature overnight. After incubation with the protein solution, discs were washed three times with 0.1 M NaHCO₃ and unreacted groups were then blocked by incubation for 5 h with 0.1 M ethanolamine, pH 9.0. Discs were then washed three times with 0.018 M sodium acetate buffer, pH 4.0 followed by a further three washes with PBS containing 0.05% Tween 20. Polypeptide coupled paper discs were incubated overnight with sera from allergic patients, diluted 1 in 10 in PBS, 0.05%

Tween 20, 0.5% BSA and 0.1% NaN₃. Discs were then washed three times for 15 min each wash with PBS, 0.05% Tween 20 and then washed for 10 min with 1.0 mL of elution buffer (0.1 M glycine-HCl, 1.0% BSA, pH 2.5). The eluted antibodies were neutralized with 3M Tris, pH 8.8, made to 0.1% NaN₃ and stored at 4°C. The eluted antibodies were incubated with strips of a mite Western blot. Bound IgE antibodies were detected using ¹²⁵I-labelled anti-human anti-IgE (Bioclone, Australia) as described above. Strips were exposed to X-ray film and autoradiographed. The sections on a mite protein blot corresponding to (i) 30–32 kDa polypeptides (ii) 25 kDa polypeptide and (iii) a control strip from the bottom of the blot, were excised. Regions (i) and (ii) were chosen as they corresponded to *Der p* III [10] and *Der p* I [11], two mite allergens frequently recognised by mite-allergic

sera. Polypeptides were desorbed and coupled to CNBr discs as outlined above.

Two strongly reactive mite allergic sera (serum A and serum B) were used to probe the polypeptide-coupled paper discs. After incubation with the diluted sera the discs were washed and antibodies eluted with elution buffer. The eluted antibodies were used to probe strips of mite Western blots (Fig. 2). Serum A and serum B antibodies eluted from the 30–32 kDa polypeptide disc bound to both the 30–32 kDa polypeptides and the 25 kDa polypeptide on the blot. Serum A and serum B antibodies eluted from the 25 kDa polypeptide disc bound to both the 25 kDa polypeptide and the 30–32 kDa polypeptides on the blot (Fig. 2). In addition, serum A antibodies eluted from the 25 kDa disc bound to a protein of 24 kDa and serum B antibodies eluted from the 30–32 kDa disc bound to a 15 kDa protein. Antibodies eluted from the control disc in both cases displayed no binding to the blots.

In each case the antibodies rebound to the corresponding region of the blot from which they had been eluted and, additionally, they bound to other proteins on the blot. This suggested that there were cross-reacting

determinants present on the different allergens recognised by the antibodies. There appears to be a strong cross-reaction occurring between the 30–32 kDa and the 25 kDa polypeptides of the mite and a weak cross-reaction occurring between the 25 kDa and the 24 kDa polypeptide of the mite as well as the 30–32 kDa and 15 kDa polypeptides. A previous study [6] also noted that IgE antibodies eluted from the 30–32 kDa component bound to the 30–32 kDa component and to a lesser extent to the 25 kDa component.

The observation of apparently cross-reacting IgE antibody determinants is not unexpected in light of the well-known heterogeneity of the antibody response [12]. Each polypeptide has a number of different antigenic determinants, some of which may be cross-reacting and others unique. Populations of antibodies are produced in response to both cross-reacting and unique determinants, hence some antibodies will bind to a number of different polypeptides while others will bind to only a single polypeptide. This paper gives examples of serum antibodies binding to cross-reacting determinants; however, antibodies from other sera (results not shown) only bound to the polypeptides from which they had been eluted.

The technique of desorbing polypeptides separated by SDS-PAGE and coupling the desorbed polypeptides to paper discs to be subsequently used as immunoabsorbents provides a useful method for studying cross-reaction between polypeptides from the one allergen source. The strategy provides a means for desorbing a particular polypeptide from several protein blots and concentrating the desorbed polypeptides by coupling them to a single CNBr-activated disc. The strategy may also be used to study cross-reaction between individual polypeptides of inter-species cross-reacting allergen extracts.

Received April 6, 1993

References

- [1] Baldo, B. A. and Tovey, E. R., in: Baldo, B. A. and Tovey, E. R. (Eds.), *Protein Blotting: Methodology, Research and Diagnostic Applications*, Karger, Basel, 1989, pp. 111–136.
- [2] Donovan, G. R. and Baldo, B. A., *Clin. Exp. Allergy* 1990, 20, 501–509.
- [3] Baldo, B. A., Panzani, R. C., Bass, D. and Zerboni, R., *Mol. Immunol.* 1992, 29, 1209–1218.
- [4] Baldo, B. A. and Panzani, R. C., *Int. Arch. Allergy Appl. Immunol.* 1988, 85, 278–287.
- [5] Baldo, B. A. and Baker, R. S., *Int. Arch. Allergy Appl. Immunol.* 1988, 86, 201–208.
- [6] Tovey, E. R., Johnson, M. C., Roche, A. L., Cobon, G. S. and Baldo, B. A., *J. Exp. Med.* 1989, 170, 1457–1462.
- [7] Tovey, E. R. and Baldo, B. A., *J. Allergy Clin. Immunol.* 1987, 79, 93–102.
- [8] Montelaro, R. C., *Electrophoresis* 1987, 8, 432–438.
- [9] Ceska, M., Eriksson, R. and Varga, J. M., *J. Allergy Clin. Immunol.* 1972, 49, 1–9.
- [10] Stewart, G. A., Ward, L. D., Simpson, R. J. and Thompson, P. J., *Immunology* 1992, 75, 29–35.
- [11] Chua, K. Y., Stewart, G. A., Thomas, W. R., Simpson, R. J., Dilworth, R. J., Plozza, T. M. and Turner, K. J., *J. Exp. Med.* 1988, 167, 175–182.
- [12] Kabat, E. A., in: Kabat, E. A. (Ed.), *Structural Concepts in Immunology and Immunochemistry*, Holt, Rinehart and Winston, New York 1976, pp. 223–317.

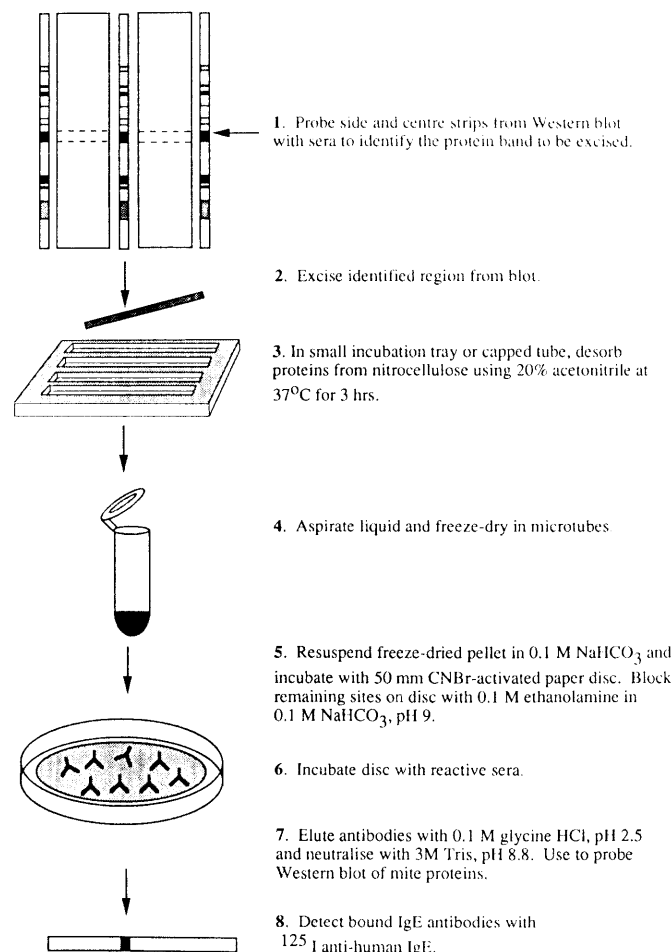


Figure 1. Schematic diagram of the strategy employed in the isolation of antibodies reactive to particular polypeptides.

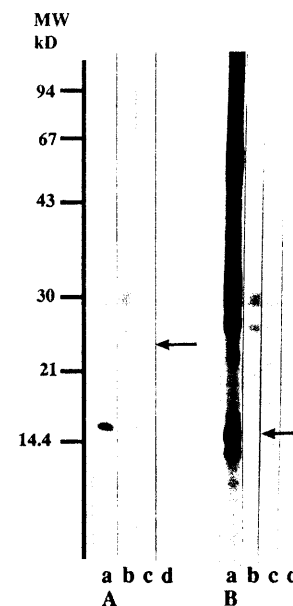


Figure 2. Strips of mite Western blot probed with (a) mite-allergic serum diluted 1/10 in PBS with 0.1% Tween 20 and 0.1% NaN₃, (b) serum antibodies eluted from the 30–32 kDa region coupled to a CNBr activated paper disc, (c) serum antibodies eluted from the 25 kDa region coupled to a CNBr-activated paper disc, and (d) serum antibodies eluted from a control region coupled to a CNBr disc. The two sera used in the experiments were serum A and serum B. Arrows indicate positions of bands displaying weak binding.