

Using the methods described, we could easily produce rabbit antisera which reacted strongly with chicken SC. It is more difficult to produce an antiserum specific for chicken SC by absorbing unwanted activity towards the chicken α chain because chicken IgA devoid of SC is difficult to prepare [22]. However, we anticipate that this problem should be overcome by the use of anti-chicken IgA monoclonal antibodies.

Received June 17, 1985; in revised form September 2, 1985.

5 References

- 1 Heremans, J., in Sela, M. (Ed.), *The Antigens*, vol. 2, Academic Press, New York 1974, p. 365.
- 2 Mostov, K. E., Friedlander, M. and Blobel, G., *Nature* 1984. 308: 37.
- 3 Mostov, K. E., Kraehenbuhl, J. P. and Blobel, G., *Proc. Natl. Acad. Sci. USA* 1980. 77: 7257.
- 4 Mullock, B. M., Hinton, R. H., Dobrota, M., Peppard, J. and Orlans, E., *Biochim. Biophys. Acta* 1979. 587: 381.
- 5 Kuhn, L. C. and Kraehenbuhl, J. P., *Trends Biochem. Sci.* 1982. 7: 299.
- 6 Lebacqz-Verhayden, A., Vaerman, J. P. and Heremans, J. F., *Immunology* 1972. 22: 165.
- 7 Peppard, J., Rose, M. E. and Hesketh, P., *Eur. J. Immunol.* 1983. 13: 566.
- 8 Rose, M. E., Orlans, E., Payne, A. W. R. and Hesketh, P., *Eur. J. Immunol.* 1981. 11: 561.
- 9 Orlans, E., Peppard, J. V., Payne, A. W. R., Fitzharris, B. M., Mullock, B. M., Hinton, R. H. and Hall, J. G., *Ann. N. Y. Acad. Sci.* 1983. 409: 411.
- 10 Peppard, J., Orlans, E., Payne, A. W. R. and Andrew, E., *Immunology* 1981. 42: 83.
- 11 Peppard, J. V., Jackson, L. E., Hall, J. G. and Robertson, D., *Immunology* 1984. 53: 385.
- 12 Schneider, C., Newman, R. A., Sutherland, D. R., Asser, V. and Greaves, M. F., *J. Biol. Chem.* 1982. 257: 10766.
- 13 Laemmli, U. K., *Nature* 1970. 227: 680.
- 14 Anderson, N. G. and Anderson, N. L., *Anal. Biochem.* 1978. 85: 331.
- 15 Anderson, N. L. and Anderson, N. G., *Anal. Biochem.* 1978. 85: 341.
- 16 Morrissey, J. H., *Anal. Biochem.* 1981. 117: 307.
- 17 Towbin, H., Staehelin, T. and Gordon, J., *Proc. Natl. Acad. Sci. USA* 1979. 76: 4350.
- 18 Brandtzaeg, P., *Scand. J. Immunol.* 1974. 3: 707.
- 19 Mostov, K. E. and Blobel, G., *Proc. Natl. Acad. Sci. USA* 1982. 257: 11816.
- 20 Altamirano, G. A., Barranco-Acosta, C., Van Roost, E. and Vaerman, J.-P., *Mol. Immunol.* 1980. 17: 1525.
- 21 Kuhn, L. C. and Kraehenbuhl, J.-P., *Proc. Natl. Acad. Sci. USA* 1979. 254: 11066.
- 22 Kobayashi, K. and Hirai, H., *J. Immunol.* 1980. 124: 1695.

Hammad Atassi and
M. Zouhair Atassi

Marrs McLean Department of
Biochemistry, Baylor College of
Medicine, Houston

Antibody recognition of ragweed allergen Ra3: localization of the full profile of the continuous antigenic sites by synthetic overlapping peptides representing the entire protein chain*

A comprehensive synthetic approach, for the localization of the full profile of the continuous antigenic sites on proteins, previously introduced by this laboratory, was applied here to localize the continuous antigenic sites of ragweed allergen, Ra3. The following 10 consecutive peptides, each comprising 15 residues (except for peptide 91–101) and overlapping each of its neighbors by 5 residues, were synthesized and purified: 1–15, 11–25, 21–35, 31–45, 41–55, 51–65, 61–75, 71–85, 81–95 and 91–101. Quantitative radiometric titrations of protein and peptide adsorbents were performed with ^{125}I -labeled anti-Ra3 IgG antibodies from rabbit, outbred mouse and human antisera. The specificity of antibody binding to peptide adsorbents was confirmed by inhibition experiments. These studies established the full profile of antigenic (IgG-binding) sites of Ra3 and permitted comparison with the allergenic (IgE-binding) sites recently localized. It was found that the recognition by IgG antibodies was independent of the host species in which the antibodies were raised. Furthermore, the regions recognized by human IgE antibodies coincided with those recognized by IgG antibodies in three different species. Thus, Ra3 was found to have 4 continuous antigenic sites which occupy the same locations as the allergenic sites.

[I 5120]

* This work was supported in part by the American Heart Association (Grant no. 81-101) and by the Welch Foundation due to the award to MZA of the Robert A. Welch Chair of Chemistry.

Correspondence: M. Zouhair Atassi, Department of Biochemistry, Baylor College of Medicine, Texas Medical Center, Houston, TX 77030, USA

Abbreviation: Ra3: Ragweed allergen Ra3

1 Introduction

Ragweed pollen is one of the most common causative agents of seasonal hay fever. At least 4 different protein allergens (namely antigen E, antigen K, Ra3 and Ra5) have been isolated from ragweed pollen [1–5]. One of these major allergens, Ra3, is a basic glycoprotein having a single polypeptide chain composed of 101 amino acid residues [6]. Clinical testing has shown [7, 8] that Ra3 is highly allergenic in about 30–50% of ragweed-sensitive individuals. The anaphylactic

phenomena which result in the symptoms of organ shock are initiated by reaction of allergen with IgE antibodies elicited in response to the allergen. Therefore, in order to understand the anaphylactic mechanism at the molecular level, the localization of the allergenic (IgE-binding) and antigenic (IgG-binding) sites of allergens is necessary.

Previously, this laboratory had introduced [9] a comprehensive synthetic approach designed to systematically localize the full profile of the continuous antigenic sites on a protein molecule. The approach consists of the synthesis and examination of the immunochemical activities of a series of consecutive overlapping peptides, of uniform size and overlaps, that encompass the entire protein chain. This strategy was first applied to localize the continuous sites recognized by anti-hemoglobin antibodies on the α chain [9, 10] and, more recently, on the β chain [11, 12] of human hemoglobin. It has also been applied for the localization of the sites recognized by T cells on sperm-whale myoglobin [13, 14], hen egg lysozyme [15, 16] and the hemoglobin chains [12]. In the present study we report the application of this strategy to Ra3. The synthesis of 10 overlapping peptides comprising the entire Ra3 molecule is reported. The binding to these peptides of anti-Ra3 IgG antibodies from human and two other host species enabled the localization of the antigenic sites to within fairly small regions on the allergen.

2 Materials and methods

2.1 Isolation of Ra3 from ragweed pollen

All operations were carried out in the cold (0–4°C) unless otherwise specified. Short ragweed pollen was purchased from Hollister-Stier (Spokane, WA). The pollen (200 g) was suspended in 550 ml of 0.01 M sodium phosphate buffer, pH 7.2, 0.15 M NaCl containing phenylmethylsulfonyl fluoride (100 mg) to inhibit proteolysis and p-chloromercuribenzoate (100 mg). The suspension was stirred and, during the first 5 h, the pH was maintained at 7.2 by the addition of 1 M NaOH. After stirring overnight, the first extract was obtained by filtration through a coarse sintered glass funnel. The pollen cake was resuspended (2–3 h) and refiltered three more times with 400 ml each of the above solvent. The four extracts were pooled (1400 ml) and dialyzed against several 5-liter changes of 90% saturated $(\text{NH}_4)_2\text{SO}_4$. The precipitate was recovered by centrifugation ($2000 \times g$, 30 min), resuspended in water (50 ml) and dialyzed against 0.01 M phosphate buffer, pH 7.5 (eight 5-liter changes) using a dialysis tubing with a 3500 molecular exclusion limit (Arthur Thomas, Philadelphia, PA). The suspension (500 ml) was ultracentrifuged at $120\,000 \times g$ to remove lipid. The floating lipid layer was aspirated carefully off the top and discarded along with the sediment at the bottom of the tubes. The resulting solution was dark greenish brown and turbid. Therefore, the afore-mentioned steps of protein precipitation in 90% saturated $(\text{NH}_4)_2\text{SO}_4$, centrifugation, redissolving in water and then dialysis against 0.01 M phosphate buffer, pH 7.5, were repeated. Finally, the solution was concentrated by ultrafiltration.

The solution was dialyzed against 0.01 M phosphate buffer, pH 7.5, and applied on a column (4×23 cm) of DEAE-cellulose DE-52 (Whatman, Maidstone, Kent, GB) which was pre-equilibrated and eluted with the same buffer. The unretarded peak, which constituted the fraction containing basic

allergens, was pooled and freeze-dried. The column was subjected to a gradient to elute the other allergens, which are not the subject of this study.

A portion (500 mg) of the freeze-dried 'basic fraction' was dissolved in 0.05 M NH_4HCO_3 (4 ml) and applied on a column (2.5×96 cm) of Sephadex G-50 (Pharmacia Fine Chemicals, Piscataway, NJ) which was eluted with the same solvent. The fractions (4.2 ml) were monitored by reading their absorbance at 280 nm. Aliquots (50 μl) of the effluent fractions were also used in a solid-phase plate radioimmune assay, described by Sakata and Atassi [17] as modified by Schmitz et al. [18], using mouse anti-Ra3 antibodies. Authentic Ra3, for the preparation of the initial mouse anti-Ra3 antisera, was obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, (NIH, Bethesda, MD). Fractions indicated to contain Ra3 (Fig. 1) were pooled, freeze-dried, redissolved in 0.05 M NH_4HCO_3 (3 ml) and subjected to refiltration on two columns (2.5×95 cm each, connected in series), one of Sephadex G-50 and the other of Sephadex G-100, which were eluted with the same solvent. Fractions containing Ra3 were pooled, and re-chromatographed two more times on similar columns (see Fig. 2). The sample was checked by polyacrylamide gel electrophoresis at pH 8.3, solid-phase radioimmune assay and immunoelectrophoresis, and was found to be identical to authentic Ra3 from NIH. Recovery of pure Ra3 was 160 mg from 1870 mg of 'basic fraction'. The latter was obtained from 200 g of pollen.

2.2 Antisera

Mouse antibodies against Ra3 were elicited by immunization of each mouse in the hind footpads and i.m. with an emulsion of equal volumes of a phosphate-buffered saline solution (50 μl) of Ra3 (50 μg) and complete Freund's adjuvant (CFA; 50 μl) containing *Mycobacterium tuberculosis*, strain H37Ra (Difco Laboratories, Detroit, MI). The mice were boosted by the same route 3 weeks after the first injection using a similar dose of Ra3 in CFA. Thereafter, the mice were boosted monthly. Rabbits were immunized by the same schedule, except that they received 200 μg Ra3 per immunization. Sera

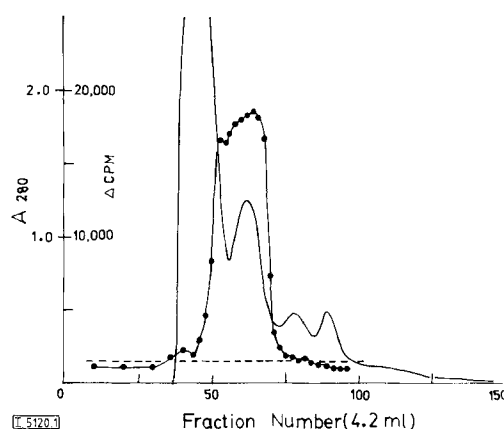


Figure 1. Gel filtration profile of the "basic fraction" of the ragweed extract on Sephadex G-50. The column (2.5×96 cm) was eluted with 0.05 M NH_4HCO_3 . The effluent fractions (4.2 ml) were monitored by reading their absorbance at 280 nm (—) and by the cpm of antibody bound (●) in a solid-phase radioimmune assay using mouse (outbred) anti-Ra3 antisera. For details see Sect. 2.1.

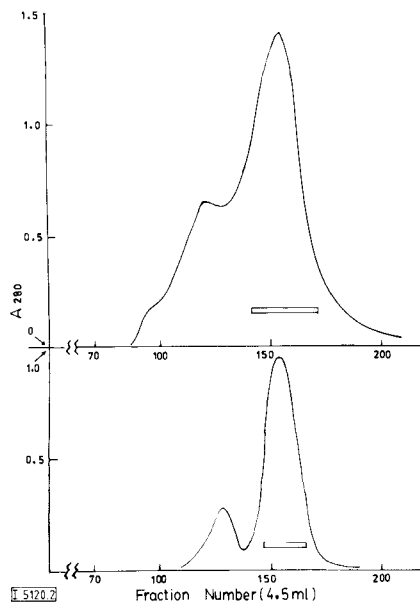


Figure 2. Gel filtration of the Ra3-containing peak from Fig. 1 on two columns (2.5×95 cm each; connected in series), one of Sephadex G-50 and the other of Sephadex G-100. The columns were eluted with 0.05 M NH_4HCO_3 and the effluent fractions were monitored by their absorbance at 280 nm. The fractions indicated in the upper panel were pooled, freeze-dried, redissolved in 0.05 M NH_4HCO_3 (3 ml) and reapplied on the same two columns (lower panel).

were collected prior to immunization and at 10-day intervals and kept frozen until use. The antisera used in the present studies were the 50-day bleed from a rabbit and a pool of the 50-day bleeds from two outbred mice (RM8-4 and RM8-5). Human sera were obtained from 3 individuals with known severe allergy to ragweed.

2.3 Synthesis and purification of the peptides

The peptides were synthesized by the solid-phase method. The Merrifield resin (0.8 mequiv. of Cl/g) and the N^α -t-butoxycarbonyl amino acid derivatives were from Vega Fox Biochemicals (Tucson, AZ). The side chain protecting groups used were arginine, nitroguanidinium; serine and threonine, *O*-benzylether; tyrosine, 2,6-dichlorobenzylether; histidine, im-dinitrophenyl; lysine, ϵ -chlorocarbobenzyloxy; aspartic and glutamic acids, β - and γ -benzylesters, while the *t*-butoxycarbonyl was employed for the protection of all α -amino groups. The purity of all the amino acid derivatives was confirmed by thin layer chromatography. All organic solvents employed for peptide synthesis were of spectroscopic grade and were again dehydrated and further purified by fractional distillation.

Solid-phase synthesis [19] of the Ra3 peptides (see Fig. 4), from the known primary structure of Ra3 [6] was performed by procedures similar to those described in detail for the synthesis of myoglobin antigenic sites [20, 21]. In the present work, the *t*-boc group was removed by treatment with trifluoroacetic acid in CH_2Cl_2 . The peptides were cleaved from the resin by HF at -10°C for 30 min. The dinitrophenyl protecting group on the imidazole side chain was removed by thiolation. The peptides were purified by gel filtration on Sephadex G-25, followed by ion-exchange chromatography on CM-Sephadex (C-50) or DEAE-Sephadex (A-50) as previously described [20–22]. Peptide purity was checked by pep-

tide mapping [23], and by amino acid analysis of acid hydrolysates [23]. For the determination of tryptophan, peptides were hydrolyzed with 3 M toluene-*p*-sulfonic acid containing 0.2% 3-(2-aminoethyl)-indole, as described by Liu and Chang [24].

2.4 Preparation and radiolabeling of antibodies

The IgG fractions of antisera from rabbit, two outbred mice and human were prepared as previously described [25, 26] and labeled with ^{125}I (Amersham Corp., Arlington Heights, IL) by a chloramine-T procedure [27]. Unbound ^{125}I was separated from the radiolabeled samples by gel filtration on Sephadex G-15. Protein-linked ^{125}I was assayed by precipitation with 10% trichloroacetic acid (v/v). The specific radioactivities of the labeled IgG fractions were 17 – 23 $\mu\text{Ci}/\mu\text{g}$ = 629 – 851 $\text{kBq}/\mu\text{g}$.

2.5 Preparation of Sepharose immunosorbents

The proteins (Ra3, or unrelated control proteins human adult hemoglobin and bovine serum albumin) and the synthetic peptides were coupled to CNBr-activated [28] Sepharose CL-4B (Pharmacia) under the optimum conditions previously described [29]. The amounts of proteins and peptides used in the coupling reaction were 2.0 mg and 0.9 mg per ml packed Sepharose, respectively. The extent of coupling was quantitated by amino acid analysis of each adsorbent (50 μl) hydrolyzed in constant boiling HCl in vacuum-sealed tubes. Analysis on triplicate samples indicated a substitution of 1.0 – 1.2 mg and 0.4 – 0.5 mg per ml packed volume for proteins and peptides, respectively.

2.6 Determination of antibody binding

To determine the immunochemical activities of the protein and peptide fractions, quantitative immunosorbent titrations of fixed amounts of ^{125}I -labeled immune IgG and various amounts of peptide or protein adsorbents were used as previously described for myoglobin antigenic sites [29]. Nonspecific adsorption was determined by titrating equivalent volumes of uncoupled Sepharose, hemoglobin-Sepharose, bovine serum albumin-Sepharose and Sepharose adsorbents of two unrelated synthetic peptides of hemoglobin α chain of equal size to the Ra3 peptides (*i.e.* α 1–15 and 91–105, [10, 26]) under identical conditions. The values of ^{125}I -labeled immune IgG bound by the various adsorbents and reported in Table 2 were corrected for this nonspecific binding.

The specificity of antibody binding to peptide adsorbents was confirmed by inhibition experiments. In these studies, the appropriate adsorbents and labeled IgG fractions were allowed to react as above in the presence of either Ra3 (0.5 mg/ml) or unrelated proteins, human hemoglobin A and bovine serum albumin (1 mg/ml). After correction for nonspecific binding the amount of radioactivity bound in the presence of Ra3 (inhibited values) was expressed as a percentage of that bound in the absence of inhibitor (uninhibited values) and compared to the effects of the unrelated proteins.

3 Results

3.1 Synthesis, purification and characterization of the peptides

The synthetic strategy is shown schematically in Fig. 3, and the primary structures of the peptides are given in Fig. 4. After

cleavage and deprotection, each peptide gave by ion-exchange chromatography a major chromatographic component. The latter migrated as a single spot in high-voltage paper electrophoresis, and was confirmed by amino acid analysis to be the desired product. The results of the amino acid analyses of the deprotected and purified synthetic peptides are given in Table 1. The compositions found for these peptides agree well with the expected values.

3.2 Binding of IgG antibodies to peptide and protein adsorbents

Quantitative immunosorbent titrations were performed using fixed amounts of ^{125} I-labeled immune IgG and increasing amounts of adsorbents. The immune IgG fractions were isolated from anti-Ra3 antisera of rabbit, mouse (outbred) and human, and their binding profiles determined to all the overlapping peptides. Fig. 5 shows an example of a titration with ^{125} I-labeled mouse anti-Ra3 antibodies (RM8-4) and Table 2 summarizes the binding values in the plateau region, with rabbit, outbred mouse and human antisera. Table 2 also summarizes recently reported [30] binding results of human IgE antibodies to Ra3 and its synthetic peptides.

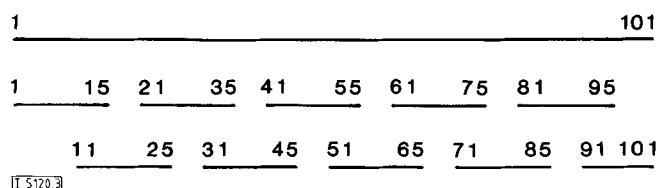


Figure 3. Schematic presentation of the synthetic overlapping peptide strategy that was employed for the delineation of the continuous antigenic sites of Ra3. The strategy relied on the synthesis of the entire polypeptide chain in 15-residue peptides (except for peptide 91-101), each overlapping its two adjacent neighbors by 5 residues on both sides.

Table 1. Summary of the amino acid compositions of the synthetic peptides^{a)}

Amino acid	Peptide	1-15	11-25	21-35	31-45	41-55	51-65	61-75	71-85	81-95	91-101
Asp		-	1.03 (1)	-	2.21 (2)	2.12 (2)	2.29 (2)	3.19 (3)	1.07 (1)	1.02 (1)	1.22 (1)
Thr		-	-	2.02 (2)	3.80 (4)	1.86 (2)	-	-	1.98 (2)	1.95 (2)	-
Ser		-	1.09 (1)	0.86 (1)	-	1.04 (1)	-	-	1.15 (1)	0.87 (1)	-
Glu		1.08 (1)	1.08 (1)	2.02 (2)	0.89 (1)	2.79 (3)	3.03 (3)	1.03 (1)	-	1.16 (1)	0.96 (1)
Pro		1.03 (1)	1.09 (1)	-	-	-	-	3.16 (3)	2.89 (3)	0.91 (1)	0.74 (1)
Gly		5.09 (5)	2.91 (3)	-	1.18 (1)	1.10 (1)	1.23 (1)	1.97 (2)	3.12 (3)	0.96 (1)	0.92 (1)
Ala		-	1.93 (2)	3.13 (3)	-	1.23 (1)	2.97 (3)	0.89 (1)	-	1.00 (1)	2.06 (2)
Val		1.90 (2)	-	-	1.28 (1)	2.29 (2)	0.93 (1)	-	-	1.09 (1)	2.00 (2)
Ile		-	-	-	-	-	0.82 (1)	1.70 (2)	-	0.95 (1)	-
Leu		2.12 (2)	2.22 (2)	0.92 (1)	1.05 (1)	-	-	0.96 (1)	1.88 (2)	-	-
Tyr		0.82 (1)	0.89 (1)	0.99 (1)	-	-	-	-	-	-	-
Phe		-	-	1.00 (1)	2.69 (3)	0.80 (1)	-	-	1.13 (1)	2.03 (2)	1.08 (1)
His		-	-	-	-	-	0.92 (1)	-	1.00 (1)	1.00 (1)	-
Lys		1.99 (2)	1.19 (1)	0.94 (1)	0.95 (1)	-	1.17 (1)	0.90 (1)	-	2.16 (2)	1.03 (1)
Trp		0.88 (1)	1.09 (1)	1.00 (1)	1.13 (1)	0.82 (1)	0.90 (1)	-	-	-	-
Arg		-	0.89 (1)	1.73 (2)	-	1.13 (1)	1.01 (1)	0.98 (1)	1.00 (1)	-	1.08 (1)

a) The values represent the average of 4 or more replicate analyses at 22, 48 and 72 h. The values of serine and threonine were obtained by extrapolation to zero hydrolysis time. The values expected from the structures of the respective peptide (see Fig. 4) are given in parentheses.

SYNTHETIC OVERLAPPING PEPTIDES OF Ra3

PEPTIDE	
1-15:	Gly-Lys-Val-Tyr-Leu-Val-Gly-Gly-Pro-Glu-Leu-Gly-Gly-Trp-Lys
11-25:	Leu-Gly-Gly-Trp-Lys-Leu-Gln-Ser-Asp-Pro-Arg-Ala-Tyr-Ala-Leu
21-35:	Arg-Ala-Tyr-Ala-Leu-Trp-Ser-Ala-Arg-Gln-Gln-Phe-Lys-Thr-Thr
31-45:	Gln-Phe-Lys-Thr-Thr-Asp-Val-Leu-Trp-Phe-Asp-Phe-Thr-Thr-Gly
41-55:	Asp-Phe-Thr-Thr-Gly-Glu-Asp-Ser-Val-Ala-Glu-Val-Trp-Arg-Glu
51-65:	Glu-Val-Trp-Arg-Glu-Glu-Ala-Tyr-His-Ala-Ala-Asp-Ile-Lys-Asp
61-75:	Ala-Asp-Ile-Lys-Asp-Pro-Ile-Arg-Leu-Glu-Pro-Gly-Gly-Pro-Asp
71-85:	Pro-Gly-Gly-Pro-Asp-Arg-Phe-Thr-Leu-Leu-Thr-Pro-Gly-Ser-His
81-95:	Thr-Pro-Gly-Ser-His-Phe-Ile-Ala-Thr-Lys-Asp-Gln-Lys-Phe-Val
91-101:	Asp-Gln-Lys-Phe-Val-Ala-Ala-Val-Pro-Gly-Arg

Figure 4. Primary structures of the synthetic overlapping Ra3 peptides. The numbers refer to the position of the residues in Ra3. The underlined parts represent the regions on a peptide that overlap with the preceding and following peptides. Note that cys-61, 88 and 97 were replaced by alanine in peptides 51-65, 61-75 and 91-101. For details, see Sect. 3.1.

Several peptide adsorbents showed very little (below 3%) or no binding activity throughout the titration range. These were for the rabbit, mouse and human IgG antibodies peptides 41-55, 61-75, 81-95 and 91-101. However, the other peptides exhibited considerable antibody-binding capacity and were therefore unambiguously active. Essentially the same set of peptides was active with rabbit, mouse and human IgG antibodies. These peptides were 1-15, 11-25, 21-35, 31-45, 51-65 and 71-85.

The titration curves for adsorbents of Ra3 rose more sharply and attained a maximal value at lower amounts than those of the immunosorbents of the peptides. This is consistent with the observation that peptides, even though active, will have predominantly random conformations and thus diminished affinities for their antibodies [23]. Further, a considerable

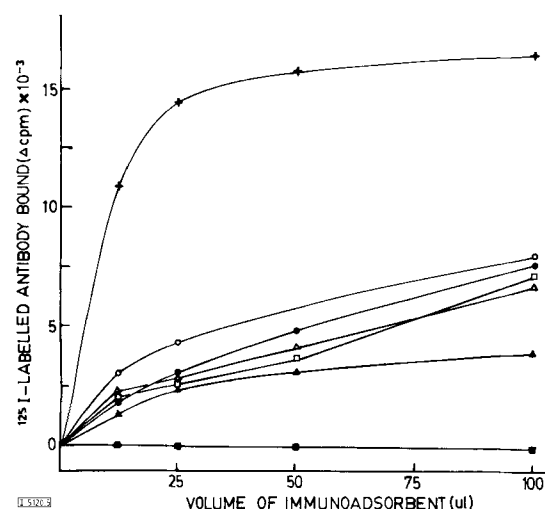


Figure 5. An example of a quantitative radioimmunosorbent titration of mouse anti-Ra3 antibodies with peptide adsorbents. For the titration, fixed amounts of ^{125}I -labeled anti-Ra3 immune IgG (1.95×10^5 cpm), from outbred mice RM8-4, were titrated with increasing amounts of adsorbent of Ra3 and the 10 synthetic overlapping Ra3 peptides. Ra3 (+); peptide 21–35 (○); peptide 1–15 (●); peptide 51–65 (□); peptide 31–45 (Δ); peptide 71–85 (▲); and peptides 41–55, 61–75, 81–95 and 91–101 (■). Each point represents the average of 6 replicate analyses which varied $\pm 1.3\%$ or less. For details see Sect. 3.2.

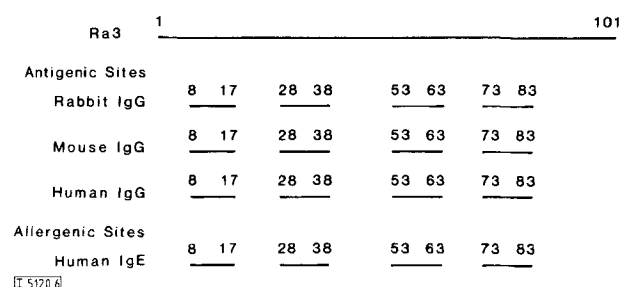


Figure 6. Schematic diagram showing the chain of Ra3, and its antigenic sites that have been localized with rabbit, mouse and human IgG antibodies to Ra3, as well as by human IgE antibodies. The numbers refer to the locations of the residues in the Ra3 chain. It is not implied that the antigenic sites comprise the entire size of the regions shown, but rather that they fall within these regions. For details see Sect. 4.

proportion of a peptide on the immunosorbent is expected to have been rendered inactive due to attachment of the peptide via side chains that are also essential for binding with antibody, and to steric inaccessibility [29, 31]. The binding of antibodies to these peptide-adsorbents, however, was completely inhibited by the addition of free uncoupled Ra3 to the reaction (results not shown) but not by unrelated proteins (bovine serum albumin, human adult hemoglobin A). Also, none of the peptides bound labeled IgG from pre-immune sera of mouse and rabbit or antibodies to unrelated proteins (bovine serum albumin, human hemoglobin A and sperm whale myoglobin). These findings indicated unambiguously that antibodies reacting with the peptide adsorbents are specific for Ra3.

It should be cautioned that the calculated sum of the activities of the peptides with each of the antisera must be considered in

Table 2. Binding of anti-Ra3 antibodies to synthetic Ra3 peptides^{a)}

Protein or peptide adsorbent	Antibodies bound to (Δcpm) from various antisera				
	Human IgE anti-bodies ^{b)}	Rabbit IgG anti-bodies	Mouse (outbred)		
			IgG (RM8-4)	IgG (RM8-5)	
Ra3	17 360	13 030	16 550	16 750	23 860
1– 15	2 590	5 160	6 720	7 600	6 850
11– 25	920	890	1 370	1 660	1 490
21– 35	4 580	2 610	5 950	8 000	5 090
31– 45	4 150	3 920	4 220	6 850	6 250
41– 55	580	0	0	0	0
51– 65	5 340	1 900	2 420	7 210	6 550
61– 75	800	0	0	0	0
71– 85	3 970	1 290	1 070	4 130	4 710
81– 95	310	0	0	300	320
91–101	0	0	0	0	0

- a) The results in this Table summarize the maximum binding values obtained in the plateau by quantitative radioimmunosorbent titrations. The values represent the average of 3 or more replicate analyses which varied $\pm 1.4\%$ or less. A fixed amount of radiolabeled immune IgG (2×10^5 cpm) was used in these titrations. Human IgE binding was determined by a double antibody assay using ^{125}I -labeled immune IgG (10^5 cpm) from rabbit anti-human IgE antiserum. Baseline control antigens were bovine serum albumin, human hemoglobin, sperm whale myoglobin and hemoglobin synthetic peptide $\alpha 1$ –15. Values not significantly > 0 by Student's t-test ($p < 0.05$) are reported as 0.
- b) The binding data with human IgE antibodies are from [30].

the context of the structures of the peptides and their overlaps, which were designed to detect antigenic sites located in the overlapping regions between adjacent peptides (see Sect. 4). Thus, adjacent overlapping peptides that have similar activities may be expressing identical specificities.

4 Discussion

For a protein of an unknown antigenic structure, two architectural alternatives for its antigenic sites (continuous or discontinuous [32]) should be anticipated. The sites may occupy continuous segments of the protein chain (e.g. the continuous antigenic sites of myoglobin [33]) or may be formed by residues that are not contiguous in sequence but come in close spatial proximity in the three-dimensional structure (e.g. the discontinuous antigenic sites of lysozyme [34]). Continuous antigenic sites were inferred very early (for recent review of these early studies, see [35]), and the first complete antigenic structure of a protein (*i.e.* myoglobin) revealed 5 continuous sites [33]. Therefore, contrary to recent impressions [36–39], continuous sites are not a new discovery. Discontinuous sites, although proposed relatively early [23], were first discovered in lysozyme and confirmed unequivocally by surface-simulation synthesis [34, 40, 41]. The antigenic structures of myoglobin and lysozyme were each determined by a multi-approach intricate chemical and synthetic strategy which is labor intensive and requires considerable chemical expertise [33, 42]. To simplify the localization of continuous antigenic sites, this laboratory introduced [9] a comprehensive synthetic strategy, specifically intended for the localization of the full profile of the continuous sites of immunological activity on a protein. It

consists of the synthesis of a series of consecutive overlapping peptides that have uniform size and overlaps, and encompass the entire protein chain (for more details, see [9, 10]).

The Ra3 peptides designed here (Fig. 3) consisted of 15 residues (except for peptide 91–101) and overlapped by 5 residues. For the localization, the antigenic sites were considered to consist of 7 residues, and, at least 5 of these residues are required for binding to antibody [33]. Antibody-binding activities in adjacent peptides may localize in the overlap regions and such similar activities were confirmed by adsorbent experiments. Four antigenic sites were localized on Ra3 by IgG antibodies from human, rabbit and mouse. Significantly, the same regions were also recognized by human IgE antibodies [30]. The sites reside in, but do not necessarily include all of the regions shown in Fig. 6. It should be emphasized that the precise boundaries of the sites are yet to be determined, and that they are intentionally enlarged here to avoid errors stemming from narrow assignments at this stage. Further synthetic studies, overlapping within the assigned regions, are required to define the precise boundaries, upon which small boundary shifts should not be unexpected [20, 21, 33]. It should also be expected that the regions assigned to the antigenic sites are influenced, to various degrees, by spatially close, or even by more distant residues in native Ra3. It is shown that antigenic sites exist in a steric and electronic equilibrium with neighboring residues [43, 44], and their reactivities are affected by changes or perturbations in environmental, and even more distant residues [45].

The finding that several of the peptides exhibited very low (below 3%) antibody-binding activity (Table 2) indicated the presence of trace antibody responses to regions outside the 4 sites localized above. These antibodies, which were difficult to quantitate because they existed in trace amounts, accounted for less than 1–2% of the total anti-Ra3 antibodies. Thus, apparently inactive regions of Ra3, interspersed among the immunodominant sites, may be recognized by amounts of antibodies that are below detection levels by these sensitive methods. This would appear to be a general phenomenon for proteins and has been observed with hemoglobin α chain [10] and β chain [12], myoglobin [33] and lysozyme [34]. In these proteins, a general background response [46] to regions outside their antigenic sites accounted for 0.1–1.4% of the total anti-protein antibodies. It has been proposed [46] that such trace antibody responses can be expanded by the hybridoma technology [47]. Indeed, monoclonal antibodies to myoglobin [48, 49] and to lysozyme [50] have been reported whose submolecular specificities are not detectable in the anti-protein antisera. It has been cautioned [46] that localization of protein antigenic sites, if done by monoclonal antibodies, should be carefully evaluated with regard to *in vivo* relevance. The existence of such specificities in the antisera of the parent animal should be confirmed.

The present findings show unambiguously that Ra3 has discrete antigenic sites which, although varying in immunodominance from serum to serum, are similarly recognized by human, rabbit and mouse IgG antibodies. Significantly, these are the same sites that are recognized by human IgE antibodies [30]. This is in agreement with our conclusions (from myoglobin [33, 45] lysozyme [34], serum albumin [31, 51, 52], the α chain [10] and the β chain [11, 12] of human hemoglobin and influenza virus hemagglutinin [53, 54]) that the regions of protein antigenic sites are not dependent on the immunized

species [55, 56], but rather are determined by their conformational locations [56]. Thus, the present and the previous findings do not support the recent assertions [57] that protein antigenic sites are not discrete areas on a protein but represent a "continuum of recognition" over the entire protein molecule, that they vary with the host species and even with individual animals within a given species and that the very idea of localizing and synthesizing antigenic sites is a meaningless endeavor [57]. These proposals are a resurrection of an erroneous and outdated impression of antigenicity, especially in view of the massive amount of recent information on the localization and synthesis of antigenic sites of a number of proteins, viruses, bacteria, etc.

The Ra3 antigenic sites are not predictable from the empirical, hydrophilicity profile approach [58]. Indeed, the region 88–93 was predicted [58] to be an antigenic site because it has the highest average hydrophilicity in the protein. The present findings clearly show that this prediction is not validated. The antigenic structures of myoglobin [33] and lysozyme [34] were the first indication that protein antigenic sites are exposed and that their antigenicity is determined by their conformational locations [56]. However, not every exposed region is an antigenic site. Exposure is not a sufficient criterion for antigenicity [33, 34], and antigenic sites are not necessarily in highly hydrophilic locations [10, 53]. Hydrophobic interactions make major contributions to the binding energy [33, 53, 59]. This subject has been discussed in detail elsewhere [53, 60].

In conclusion, we have systematically screened the entire Ra3 molecule by a comprehensive synthetic strategy and localized the full profile of the continuous sites recognized by IgG antibodies in rabbit, outbred mouse and human antisera. The profile is independent of the immunized species. Furthermore, the sites reside in the same regions that are recognized by human IgE antibodies in ragweed-sensitive individuals. This is the first allergen for which the full IgG and IgE recognition profiles have been determined.

Received June 3, 1985; in revised form September 19, 1985.

5 References

- 1 King, T. P., Norman, P. S. and Connell, J. T., *Biochemistry* 1964. 3: 458.
- 2 King, T. P., Norman, P. S. and Lichtenstein, L. M., *Biochemistry* 1967. 6: 1922.
- 3 Underdown, B. J. and Goodfriend, L., *Biochemistry* 1969. 8: 980.
- 4 Griffiths, B. W. and Burnet, R., *Can. J. Biochem.* 1971. 49: 396.
- 5 Goodfriend, L. and Lapkoff, C., *Int. Arch. Allergy Appl. Immunol.* 1974. 46: 215.
- 6 Klapper, D. G., Goodfriend, L. and Capra, J. D., *Biochemistry* 1980. 19: 5729.
- 7 Lichtenstein, L. M., Roebber, M. and Goodfriend, L., *J. Allergy Clin. Immunol.* 1973. 51: 285.
- 8 Adolphson, C. R., Goodfriend, L. and Gleich, G. J., *J. Allergy Clin. Immunol.* 1978. 62: 197.
- 9 Kazim, A. L. and Atassi, M. Z., *Biochem. J.* 1980. 191: 261.
- 10 Kazim, A. L. and Atassi, M. Z., *Biochem. J.* 1982. 203: 201.
- 11 Yoshioka, N. and Atassi, M. Z., *5th Int. Congr. Immunol. Abstr.* 1983. 232: 0023.
- 12 Yoshioka, N. and Atassi, M. Z., *Biochem. J.* 1986, in press.
- 13 Bixler, G. S. and Atassi, M. Z., *Immunol. Commun.* 1983. 12: 593.
- 14 Bixler, G. S. and Atassi, M. Z., *J. Immunogenet.* 1984. 11: 339.

- 15 Bixler, G. S., Yoshida, T. and Atassi, M. Z., *Exp. Clin. Immunogenet.* 1984. 1: 99.
- 16 Bixler, G. S., Yoshida, T. and Atassi, M. Z., *J. Immunogenet.* 1984. 11: 327.
- 17 Sakata, S. and Atassi, M. Z., *Mol. Immunol.* 1981. 18: 961.
- 18 Schmitz, H. E., Atassi, H. and Atassi, M. Z., *Mol. Immunol.* 1982. 19: 1699.
- 19 Merrifield, R. B., *J. Am. Chem. Soc.* 1963. 85: 2149.
- 20 Koketsu, J. and Atassi, M. Z., *Biochim. Biophys. Acta* 1973. 328: 289.
- 21 Koketsu, J. and Atassi, M. Z., *Immunochemistry* 1974. 11: 1.
- 22 Koketsu, J. and Atassi, M. Z., *Biochim. Biophys. Acta* 1974. 342: 21.
- 23 Atassi, M. Z. and Saplin, B. J., *Biochemistry* 1968. 7: 688.
- 24 Liu, T.-Y. and Chang, Y. H., *J. Biol. Chem.* 1971. 246: 2842.
- 25 Gray, G. D., Mickelson, M. M. and Crim, J. A., *Immunochemistry* 1969. 6: 641.
- 26 Kazim, A. L. and Atassi, M. Z., *Biochem. J.* 1980. 185: 285.
- 27 Hunter, W. M. and Greenwood, F. C., *Nature* 1962. 194: 495.
- 28 March, S. C., Parikh, I. and Cuatrecasas, P., *Anal. Biochem.* 1974. 60: 149.
- 29 Twining, S. S. and Atassi, M. Z., *J. Immunol. Methods* 1979. 30: 139.
- 30 Atassi, H. and Atassi, M. Z., *FEBS Lett.* 1985. 188: 96.
- 31 Atassi, M. Z., Sakata, S. and Kazim, A. L., *Biochem. J.* 1979. 179: 327.
- 32 Atassi, M. Z. and Smith, J. A., *Immunochemistry* 1978. 15: 609.
- 33 Atassi, M. Z., *Immunochemistry* 1975. 12: 423.
- 34 Atassi, M. Z., *Immunochemistry* 1978. 15: 909.
- 35 Atassi, M. Z., *Adv. Exp. Med. Biol.* 1985. 185: 1.
- 36 Lerner, R. A., *Nature* 1982. 299: 592.
- 37 Lerner, R. A., *Adv. Immunol.* 1984. 36: 1.
- 38 Sutcliffe, J. G., Shinnick, T. M., Green, N. and Lerner, R. A., *Science* 1983. 219: 660.
- 39 Wabuke-Bunoti, M. A. N., Taku, A., Fan, D. P., Kent, S. and Webster, R. G., *J. Immunol.* 1984. 133: 2194.
- 40 Atassi, M. Z., Lee, C.-L. and Pai, R. C., *Biochim. Biophys. Acta* 1976. 427: 745.
- 41 Lee, C.-L. and Atassi, M. Z., *Biochem. J.* 1976. 159: 89.
- 42 Atassi, M. Z., in Pressman, D., Tomasi, T. B., Grossberg, A. L. and Rose, N. R. (Eds.), *Specific Receptors of Antibodies, Antigens and Cells*, S. Karger, Basel 1972, p. 118.
- 43 Atassi, M. Z. and Kazim, A. L., *Biochem. J.* 1980. 187: 163.
- 44 Kazim, A. L. and Atassi, M. Z., *Biochem. J.* 1980. 191: 673.
- 45 Twining, S. S., Lehmann, H. and Atassi, M. Z., *Biochem. J.* 1980. 191: 681.
- 46 Atassi, M. Z., *Mol. Cell. Biochem.* 1980. 32: 21.
- 47 Köhler, G. and Milstein, C., *Nature* 1975. 256: 495.
- 48 Schmitz, H. E., Atassi, H. and Atassi, M. Z., *Immunol. Commun.* 1983. 12: 161.
- 49 Atassi, M. Z., in Atassi, M. Z., Van Oss, C. J. and Absolom, D. (Eds.), *Molecular Immunology*, Marcel Dekker, New York 1984, p. 15.
- 50 Metzger, D. W., Ch'ng, L.-K., Miller, A. and Sercarz, E. E., *Eur. J. Immunol.* 1984. 14: 87.
- 51 Sakata, S. and Atassi, M. Z., *Mol. Immunol.* 1980. 17: 139.
- 52 Atassi, M. Z., *Biochim. Biophys. Acta* 1982. 704: 552.
- 53 Atassi, M. Z. and Webster, R., *Proc. Natl. Acad. Sci. USA* 1983. 80: 840.
- 54 Atassi, M. Z. and Kurisaki, J.-I., *Immunol. Commun.* 1984. 13: 539.
- 55 Kazim, A. L. and Atassi, M. Z., *Biochim. Biophys. Acta* 1977. 494: 277.
- 56 Atassi, M. Z. and Kazim, A. L., in Atassi, M. Z. and Stavitsky, A. B. (Eds.), *Immunobiology of Proteins and Peptides*, Vol. 1, Plenum Press, New York 1978, p. 19.
- 57 Benjamin, D. C., Berzofsky, J. A., East, I. J., Gurd, F. R. N., Hannum, C., Leach, S. J., Margoliash, E., Michael, J. G., Miller, A., Prager, E. M., Reichlin, M., Sercarz, E. E., Smith-Gill, S. J., Todd, P. E. and Wilson, A. C., *Annu. Rev. Immunol.* 1984. 2: 67.
- 58 Hopp, T. P. and Woods, K. R., *Proc. Natl. Acad. Sci. USA* 1981. 78: 3824.
- 59 Benjamini, E., in Atassi, M. Z. (Ed.), *Immunochemistry of Proteins*, Vol. 2, Plenum Press, New York 1977, p. 265.
- 60 Atassi, M. Z., *Eur. J. Biochem.* 1984. 145: 1.