

Localization of the continuous allergenic sites of ragweed allergen Ra3 by a comprehensive synthetic strategy

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A comprehensive synthetic approach, previously introduced by this laboratory for the localization of the full profile of the continuous antigenic sites on proteins, was applied here to localize the continuous sites of ragweed allergen, Ra3, that are recognized by human anti-Ra3 IgE antibodies. The following 10 uniform and overlapping peptides 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 with human IgE, established the full profile of allergenic (IgE binding) sites on Ra3. It was found that Ra3 has four continuous allergenic sites. Antibodies prepared against the IgE binding peptides bound to native Ra3. The findings are briefly discussed in relation to other protein antigenic structures and in terms of design of vaccines using synthetic sites.

Allergen Allergenic sites IgE antibodies Ragweed allergen Ra3 Synthetic peptides

1. INTRODUCTION

Desensitization strategies against allergens presently involve immunization protocols that employ purified or crude allergen extracts. Although the mechanism of this protection is not really understood, it is believed to result from elevation, due to allergen inoculation, of the titer of anti-allergen IgG antibodies which can compete for the allergen with anti-allergen IgE antibodies and may alleviate the allergic reaction or decrease its severity. However, frequently immunization with allergens causes side reactions such as anaphylactic shock, delayed Type I IgE-mediated, arthus or systemic reactions. It would, therefore, be desirable to investigate alternatives to present vaccination methods. The possibility of using synthetic peptide vaccines for clinical desensitization is very attractive because it would be expected to cause little or no side reactions. In order for this approach to be successful, the regions on an allergen that bind IgE antibodies need to be

localized and then these synthetic regions can be designed into effective immunogens. However, little is known about the submolecular immune recognition of allergens and, indeed, the full profile of the regions on an allergen that are recognized by IgE antibodies has not been determined. This paper describes the first phase of our studies on this subject, namely the location of the allergenic (IgE binding) sites, using a ragweed allergen as a model.

Ragweed pollen is one of the most common causative agents of seasonal hay fever in the United States. 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]. Ra3 is highly allergenic in about 30–50% of ragweed sensitive individuals [7,8]. 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.

Previously, this laboratory had introduced [9] a

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comprehensive synthetic approach designed to systematically localize the full profile of the continuous sites on a protein. 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 has been applied to localize, for several proteins, the continuous sites recognized by antibodies and by T cells [9–17]. It should be noted that this strategy has been designed specifically for the localization of continuous antigenic sites (for definition of 'continuous' and 'discontinuous' antigenic sites, see [18]). A more detailed discussion of this strategy has already been reported [9,10]. In the present paper we report the application of this strategy to Ra3. Ten overlapping peptides comprising the entire Ra3 molecule have been synthesized and their binding to human IgE antibodies permitted the localization of the allergenic sites to within fairly small regions on the allergen.

2. MATERIALS AND METHODS

Human sera were obtained from 3 individuals with known severe allergy to ragweed. The sera contained 1500–3800 ng IgE antibodies to ragweed allergens/ml. Ra3, rabbit anti-human IgE antiserum and polyvalent rabbit anti-ragweed antiserum were obtained from the Research Resources Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. Antibodies to synthetic peptides were prepared in outbred mice by immunization with an emulsion of the free (i.e. without conjugation to a carrier) peptides (25 µg/mouse) in complete Freund's adjuvant. Each peptide was injected into 4 mice. The mice were boosted by a similar dose of the respective peptide at 3 weeks and thereafter monthly. Test bleeds were obtained at 2-week intervals and the antisera from the 4 mice immunized with a given peptide were pooled. The antisera studied here were from the 98-day bleeds.

The synthetic strategy is shown schematically in fig.1. Solid-phase synthesis of the Ra3 peptides, from the known primary structure of Ra3 [6], was performed by procedures similar to those detailed

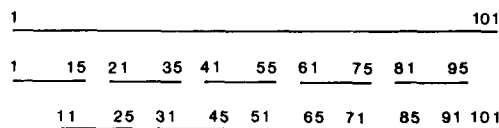


Fig.1. Schematic presentation of the synthetic overlapping peptides strategy that was employed for the delineation of the continuous allergenic sites of Ra3. The strategy relied on the synthesis of the entire polypeptide chain in 15-residue peptides (except for peptide 91–101), each overlapping its 2 adjacent neighbors by 5 residues on both sides.

in [19]. 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) [19,20]. Their purity was checked by peptide mapping [21], and by amino acid analysis of HCl [21] and 3 M toluene-*p*-sulfonic acid [22] hydrolyzates. Amino acid compositions of the purified peptides agreed well with those expected from their covalent structures.

The IgG fractions of rabbit anti-IgE antisera were prepared as described in [20] and were labelled with ^{125}I (Amersham, Arlington Heights, IL) by a chloramine-T procedure [23]. Unbound ^{125}I was separated from the radiolabelled samples by gel filtration on Sephadex G-15 (Pharmacia, Piscataway, NJ). The proteins [Ra3, or unrelated control proteins human adult hemoglobin (Hb) and bovine serum albumin (BSA)] and the synthetic peptides were coupled to CNBr-activated Sepharose CL 4B (Pharmacia) under the optimum conditions previously described [24].

The binding of human IgE antibodies to the Sepharose adsorbents was determined by the double antibody quantitative immunoabsorbent titration assay [25] using human serum prediluted 1/1000 (v/v) with PBS containing 0.1% BSA, followed by ^{125}I -labelled immune IgG of rabbit anti-human IgE antiserum. Non-specific adsorption was determined by titrating equal volumes of uncoupled Sepharose, BSA-Sepharose, Hb-Sepharose and Sepharose adsorbents of 2 unrelated synthetic Hb peptides of equal size (i.e. α 1–15 and 91–105 [9,10]). The specificity of antibody binding to peptide adsorbents was confirmed by inhibition experiments. In these studies, the appropriate adsorbents and labelled IgG fractions were allowed to react as above in the presence of either Ra3 (0.5 mg/ml) or unrelated proteins, Hb

and BSA (1 mg/ml). After correction for non-specific 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. Antibodies in mouse antipeptide antisera were assayed for binding to Ra3 and peptide by a plate assay [26]. To guard against removal of peptide during washing of the plates, the peptides were used in the plate assay as conjugates onto succinyl BSA. The coupling procedure has been described [27].

3. RESULTS AND DISCUSSION

The results of quantitative immunoabsorbent titrations are summarized in table 1, which gives the maximum binding values of human IgE an-

tibodies to Ra3 and its synthetic peptides. Several peptide adsorbents showed very little (below 3%) or no binding activity of IgE antibodies throughout the titration range. These were peptides 41-55, 61-75, 81-95 and 91-101. On the other hand, the other peptides exhibited considerable antibody binding capacity. The binding of antibodies to these peptide-adsorbents was completely inhibited by the addition of free uncoupled Ra3 to the reaction (not shown) but not by unrelated proteins (BSA, Hb). Also, none of the peptides bound labelled IgG from pre-immune sera of mouse and rabbit or antibodies to unrelated proteins (BSA, Hb 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 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 below). Thus adjacent overlapping peptides that have similar activities may be expressing identical specificities (e.g. peptides 21-35 and 31-45 each absorbs the activity towards the other). Finally, antisera raised against the active peptides 1-15, 21-35, 31-45, 51-65 and 71-85 were examined for their ability to bind to native Ra3. The results are summarized in table 2. In each case, antipeptide antibodies bound to native allergen.

The antibody binding activities of the synthetic peptides have permitted the localization of the full allergenic profile of Ra3. The finding that several of the peptides exhibited very low (below 3%) antibody-binding activity (table 1) indicated the presence of very low antibody responses to regions outside the 4 immunodominant sites. These antibodies were difficult to quantitate because of their presence in very low amounts. Thus, regions of the Ra3 that are apparently inactive may be recognized by amounts of antibodies that are below detection levels by these sensitive methods. Similar results have been obtained with other proteins [10,12,28,29] where low antibody levels (below 1-2%) were observed for regions outside the immunodominant antigenic sites and these have been termed as 'general background' responses [30].

Table 1

Binding of human anti-Ra3 IgE antibodies to synthetic Ra3 peptides

Protein or peptide adsorbent	IgE antibodies bound	
	Δ cpm	% relative to Ra3
Ra3	17360	100
1-15	2590	14.9
11-25	920	5.3
21-35	4580	26.4
31-45	4150	23.9
41-55	430	2.4
51-65	5340	30.8
61-75	485	2.8
71-85	3970	22.9
81-95	310	1.8
91-101	0	0

The results summarize the maximum binding values obtained in the plateau by quantitative radioimmunoabsorbent titrations. The values represent the average of 3 or more replicate analyses which varied $\pm 1.5\%$ or less. Human IgE binding was determined by a double antibody assay using ^{125}I -labelled 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

Table 2
Binding to Ra3 of anti-peptide antibodies

Immunizing peptide	Antibodies bound (Δ cpm)	
	Binding to immunizing peptide	Binding to Ra3
1-15	3119	3313
21-35	6320	6340
31-45	3140	3840
51-65	3340	3510
71-85	18670	17530

Anti-peptide antisera were elicited in 4 outbred mice each by immunization with free (i.e. not coupled to any carrier) peptides. For RIA plate assay, the peptides were used as conjugates on succinylated bovine serum albumin. Mouse anti-peptide antibodies were assayed for binding to Ra3 by a solid-phase plate RIA. The assay involved briefly the following order of steps. A solution of Ra3, peptide conjugates or unrelated control antigens (5 μ g in 50 μ l PBS) was added to each well, plates incubated (1 h, 37°C), washed (5 $\times$ with PBS), blocked with 100 μ l of 2% BSA (1 h, 37°C), washed (5 $\times$ with PBS), incubated (2.5 h, 37°C) with 50 μ l of anti-peptide antiserum (prediluted 1/1000 with PBS/0.1% BSA), washed (5 $\times$ with PBS), incubated (2.5 h, 37°C) with rabbit anti-mouse (IgG + IgM) prediluted 1/1000 with PBS/0.1% BSA, washed (5 $\times$ with PBS), incubated (2 h, room temperature) with 50 μ l 125 I-labelled protein A (2 $\times 10^5$ cpm) in PBS, washed (5 $\times$ with PBS) and finally the wells were cut out and counted on a gamma counter. The results were corrected for non-specific binding (0.3–0.5% of total label) of antibody on plates coated with BSA, Hb or conjugates of Hb peptide α 1–15. Values are expressed as the mean cpm of 125 I-labelled protein A bound, and they varied $\pm 1.4\%$ or less (PBS: 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2)

This is the first allergen for which the full IgE submolecular recognition profile has been determined. The fact that anti-peptide antibodies bound to native Ra3 suggests that these synthetic regions, which are also the target of IgE recognition, may be used in the future as the basis for design of synthetic vaccines. These investigations will be carried out.

The present mapping of the allergenic profile of Ra3 shows that the allergen has discrete sites that are recognized by human IgE antibodies (table 1).

These findings are consistent with what is known about protein antigenic structures [28–31] and do not support the recent proposals that protein antigenic sites are not discrete areas on a protein but rather represent a 'continuum of recognition' over the entire protein molecule and that localization of antigenic sites and their synthesis are a meaningless endeavor [32]. It should be noted that the antigenic sites of Ra3 reported here are not predictable from the empirical approach based on hydrophilicity analysis of the sequence [33]. Indeed, the region 88–93 was predicted [33] to be an antigenic site because it has the highest average hydrophilicity in the protein. Our findings clearly do not confirm this prediction. Although protein antigenic sites are exposed [19,20,28–31], exposure is not a sufficient criterion for antigenicity [28,29]. Furthermore, antigenic sites are not necessarily in highly hydrophilic locations. Hydrophobic interactions provide major contributions to the binding energy [28]. The lack of correspondence between regions of hydrophilic maxima and antigenic site locations in several proteins has been discussed [31,34].

Recent studies from this laboratory on the T-cell recognition of proteins [13–17,35] have led to the conclusion [35,36] that the design of effective vaccines using small synthetic peptides must take into account the role of the T-cell recognition in the responses to protein antigens and the regulation of these responses. Our work is now directed to the localization of the submolecular profile of the regions recognized on Ra3 by T cells.

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