



Characterization of polyclonal allergen-specific IgE responses by affinity distributions[☆]

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Abstract

Polyclonal IgE responses have been previously characterized by allergen-specific antibody levels and by identification of amino acid sequences related to immunodominant epitopes. However, the binding affinities related to these antibody families are not well known. Using sera from donors with known sensitivities to ragweed or house dust mite allergens, we studied the binding reactions between the purified allergens Amb a 1 and Der p 1 and allergen-specific IgE's by determining affinity distribution functions. The distributions of binding affinities only exhibited a few dominant reactions indicated by peaks in an affinity distribution display. In all the donors tested, there were two dominant peaks and in 2/3 of the cases there was a third peak for both Amb a 1 and Der p 1. We further characterized the polyclonal interactions between IgE and Der p 1 by inhibiting the specific binding of IgE using peptide fragments known to be constituents of Der p 1 epitopes. Each peptide inhibited only a single peak in the affinity distributions. It would appear that the peaks in the affinity distribution represent antibodies directed to single epitopes. These results suggest that in our atopic population the response is surprisingly uniform. The bulk of the IgE response (70–80%) is of high affinity (10^8 – 10^{11} M⁻¹) and directed towards a few epitopes. The relative affinities towards epitopes seem to be determined by the structure of the epitope and not variations of individuals' immune responses. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Compared with other immunoglobulin-mediated, humoral reactions, very little is known about the pathophysiological details of atopic disorders. This is due in large part to the extremely low concentrations of IgE's (< 0.05% of total serum Ig's) and the minute allergen load (estimated to be on the order of nanograms/hour) required to elicit atopic reactions. The characterization of humoral responses with respect to their heterogeneity and affinity for antigen is primarily known from studies

with mouse serum pools and monoclonal antibodies. Much less is known about a polyclonal humoral response for human serum IgE.

Previously, the human polyclonal response has only been accessible to ELISA-based studies that can only determine average properties (Muller, 1980; Friguet et al., 1985). However, affinity is based on a dynamic system where the affinity changes with time (Berek and Milstein, 1987). Therefore, reliable data are absent to answer the question whether the response tends towards extreme polyclonality with a multitude of epitopes recognized (Jemmerson, 1996) or towards a few immunodominant epitopes recognized universally (Atassi et al., 1996). The specific IgE response to allergens has been shown to be of very high affinity (Prah and Nexø, 1982; Kim et al., 1996). The missing paramete-

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ters for describing the system, in most cases, are the relationships of affinity and the degree of polyclonality.

To investigate these questions we used a highly sensitive, chemiluminescence-based assay system to study the binding reactions between allergens and IgE's (Pierson et al., 1998). Our method characterizes allergen-IgE binding reactions in terms of affinity distributions. Using purified allergens from ragweed (Amb a 1; common seasonal aeroallergen) and house dust mite (Der p 1; common indoor allergen), the affinity distribution functions allow us to characterize the heterogeneity of the responses by estimating the number of allergen-IgE reactions, the binding constants of the reactions and the relative contribution of each reaction to the sum of all reactions.

We studied sera from donors with known sensitivities to ragweed or dust mites. The affinity distributions showed limited heterogeneity in the IgE responses to purified allergens. To further characterize this heterogeneity, we performed inhibition experiments using three peptide sequences known to be parts of individual epitopes of the protein allergen Der p 1. The results suggest that sensitized individuals respond and produce specific IgE to only a few immunodominant epitopes and that the epitope specific antibodies exhibit, as a rule, high affinities (10^8 – 10^{11} M⁻¹).

2. Materials and methods

2.1. Sample population

A total of 165 patients receiving treatments at the Allergy Clinic of the Fairview-University Medical Center, University of Minnesota, were screened for responses to common allergens both by standard skin prick testing and the presence of detectable amounts of serum allergen-specific IgE. The sample population consisted of 82 females and 83 males ranging in age from 6 to 73 years (mean donor age, 30 years). All the donors provided written, informed consent according to the guidelines of the Institutional Review Board of the University of Minnesota.

2.1.1. Skin prick testing

Skin prick tests were administered with Duo-Tip Applicators (Lincoln Diagnostics, Inc., Decatur, IL) using standardized extracts of common allergens: house dust mite (*Dermatophagoides pteronyssinus*) and short ragweed (*Ambrosia artemisiifolia*) (extracts from Bayer Corp., Elkhart, IN). Positive (histamine) and negative (saline) controls were also administered. Wheal and flare areas (mm²) were measured by planimetry and a positive response was recorded for any wheal area ≥ 9 mm² above negative saline control.

2.2. Allergen-specific IgE determinations

Serum concentrations of allergen-specific IgE's were determined using an adaptation of the Access Immunoassay System[®] specific IgE test developed by Sanofi Diagnostics Pasteur, Inc. (Division of Beckman Instruments, Chaska, MN) (Pierson et al., 1998). Access[®] is a fully automated, solid phase immunoassay system that utilizes chemiluminescence-based detection. Unlike ELISA-based methods in which allergen is first attached to a solid phase followed by capture of the antibodies of interest from serum, the Access[®] method uses monoclonal antibodies (mAb's) directed against the immunoglobulin isotype of interest.

Briefly, a mouse anti-human IgE mAb is attached to a magnetic bead (solid phase support) followed by adding serum from which the IgE antibodies are captured. Allergen-specific IgE's are detected by adding biotinylated allergens to the reaction mixture, and a positive signal is generated by the addition of streptavidin conjugated alkaline phosphatase (ALP). ALP enzymatically acts upon a phosphorous-based substrate to produce a chemiluminescent signal. The allergens were purified and biotinylated as previously described (Pierson et al., 1998). This method precludes interference from other immunoglobulin isotypes as only the isotype of interest is 'captured'. Also, the problems of allergen conformation changes and 'hidden epitopes' are eliminated as the allergen is free in solution.

2.3. Affinity distribution functions

Our basic experimental method provides information regarding allergen–antibody (Ag–Ab) binding reactions in vitro (Pierson et al., 1998). Briefly, using a range of purified Ag concentrations, a binding isotherm is generated, r versus Log₁₀ [Ag]; where r = fraction of antibody sites bound at a given [Ag] concentration. Because the purified allergens may have multiple epitopes, one or more specific Ag–Ab reactions may contribute to the resultant binding curve. However, the number (n) of reactions that contributed is not known or assumed a priori. We have developed a method to analyze this problem using an approach first suggested by (Hunston, 1975; Thakur et al., 1980).

The information from the binding isotherm is transformed to derive an affinity distribution function. These functions identify the number of reactions involved between IgE's and the allergens (n), the equilibrium binding affinity constants for each reaction (K) and the relative proportion of each reaction to the sum of all reactions involved ($N[K]$). The data were analyzed using Mathematica[®] software (Wolfram Research, Inc., Champaign, IL).

A pamphlet detailing the methods used for determin-

ing affinity distribution functions is available, at no cost, to interested investigators.

2.4. Inhibition using *Der p 1* peptide sequences

Der p 1 is a cysteine protease comprised of 222 amino acids. The *Der p 1* cDNA sequence (Chua et al., 1988) and a structural model (Topham et al., 1994) have been established indicating the location of five highly conformation-dependent B-cell epitopes (Lombardero et al., 1990). As described more fully in Section 3 (see also Fig. 3), three of these peptides were produced by Alpha Diagnostic International (San Antonio, TX).

The peptide inhibition studies, for the investigation of epitope recognition for eight individuals, were performed by adding each of the peptides at a constant ratio of 1:1, peptide: *Der p 1*, for each dilution of *Der p 1* allergen used in generating a binding isotherm. Additional peptide inhibition studies to investigate the possibility of steric hindrance were performed by adding the peptides at concentration ratios of *Der p 1* to peptide of 1:0.25, 1:0.5, 1:1 and 1:2 at each binding point. (The peptides ranged from 1700 to 3900 Da giving average molar ratios of approximately 1:5, 1:10, 1:25 and 1:50 in favor of the peptide). Each inhibition experiment was run simultaneously with a control that did not contain a peptide. The affinity distributions with and without the (putative) inhibiting peptide were compared, and any inhibition that resulted was reported as a percent decrease of a particular 'affinity peak' relative to the control.

3. Results

3.1. Affinity regions for *Der p 1* and *Amb a 1* specific IgE

Of the 165 donors screened for sensitivity to common allergens, 76 (46%) had positive skin test reactions to ragweed extract and 35 (21%) had positive skin test reactions to dust mite extract. The donors with positive skin test reactions also had detectable amounts of serum allergen-specific IgE's. For ragweed-sensitive donors the average concentration of *Amb a 1*-specific IgE was 11.8 IU ml⁻¹ (S.E. = 3.30). For dust mite-sensitive donors the average concentration of *Der p 1*-specific IgE was 17.5 IU ml⁻¹ (S.E. = 6.27). (1 IU ml⁻¹ = 2.4 ng ml⁻¹).

For sensitized donors, the affinity distribution functions were determined for *Amb a 1* or *Der p 1* specific IgE. Fig. 1 shows representative affinity distribution functions for *Amb a 1* (Fig. 1A) and *Der p 1* (Fig. 1B) from two different individuals. The height of each peak represents the relative proportion of a reaction with a particular binding affinity to the overall sum of reactions. When normalized, the area under each peak indicates the probability of finding a responding antibody clone in that

particular range of affinities. The highest point, or most probable affinity, represents an 'affinity maximum' for a particular Ag–Ab reaction.

For example, the affinity maxima in Fig. 1A are at $1.45 \times 10^8 \text{ M}^{-1}$, $7.13 \times 10^8 \text{ M}^{-1}$ and $6.50 \times 10^9 \text{ M}^{-1}$. The corresponding proportions of these maxima are 53.3, 42.5 and 4.3%. For all sensitized donors, we invariably found two distinct affinity maxima and in about 2/3 of the cases we found three, whether for *Amb a 1* or *Der p 1*.

Histograms of the accumulated (normalized) results of these analyses are shown in Fig. 2A for *Amb a 1* affinity maxima and Fig. 2B for *Der p 1* affinity maxima. While not immediately apparent from these histograms, there are three distinct affinity maxima 'regions' for both *Amb a 1* and *Der p 1*. The summary statistics for these affinity regions are given in Table 1.

Comparisons of the mean affinity values for these regions by Student's *t*-tests for *Amb a 1* and *Der p 1* show that there are three distinct regions for each. Also, the relative proportions of each affinity maxima are different; the region with lowest affinity contributes a higher proportion to the overall results while, conversely, the region with highest affinity contributes the least to the total.

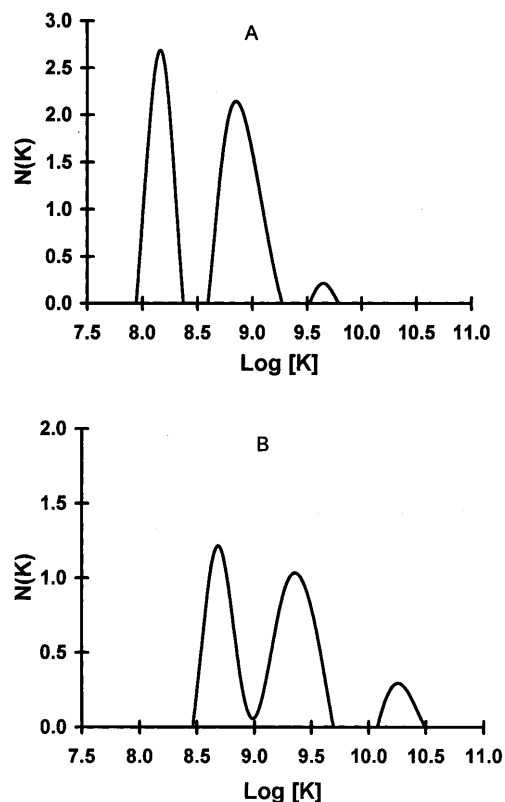


Fig. 1. Representative affinity distributions. Ordinate, $N(K)$, is relative proportional contribution of each particular reaction with a given affinity constant, K , plotted as a logarithmic scale on the abscissa. (A) *Amb a 1*-specific IgE and (B) *Der p 1*-specific IgE. The highest point of each peak represents an affinity maximum.

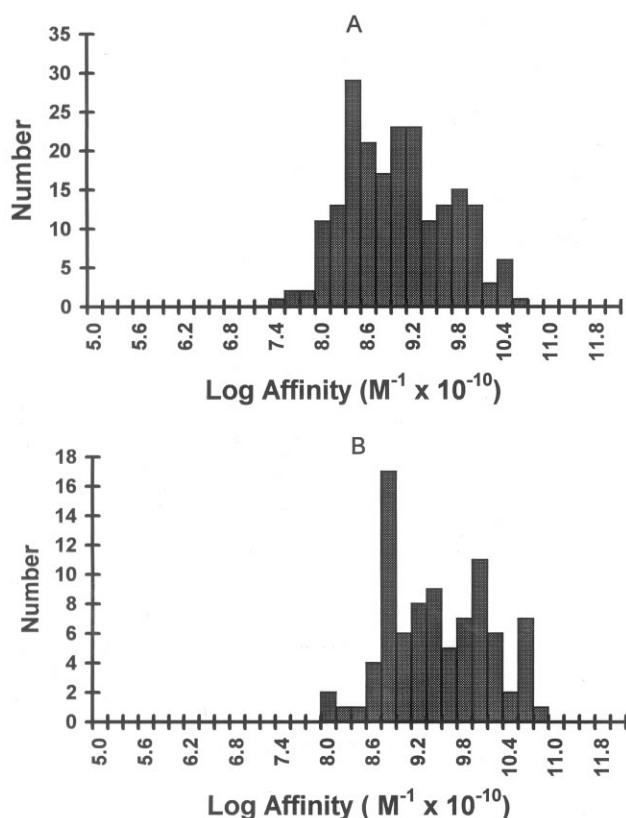


Fig. 2. Affinity distribution regions. (A) Amb a 1-specific IgE and (B) Der p 1-specific IgE. Histograms were derived using the affinity maxima from normalized affinity distributions for donors who were sensitized to, and had detectable serum concentrations of, the respective allergens. Summary statistics for the 'affinity regions' are given in Table 1.

Comparisons of the affinity maxima regions for Amb a 1 and Der p 1 show that the Der p 1 reactions have slightly higher affinities, on average, than those for Amb a 1. Yet, the relative proportions of the three affinity maxima regions for each allergen are similar.

3.2. Immunodominant epitopes for Der p 1

Fig. 3 shows the amino acid sequence with 5 peptide sequences underlined and designated P1–P5 (EO1–EO5) known to be associated with human B cell epitopes (EO1 is the first epitope according to the cDNA sequence and so forth) (Lombardero et al., 1990). We attempted to inhibit the binding of Der p 1-specific IgE to the Der p 1 allergen using three synthesized peptides (P1/EO1, P2/EO2 and P5/EO5). For controls, we also attempted binding inhibition using lysozyme and BSA.

Lysozyme and BSA at 1 and 10 $\mu g\ ml^{-1}$ ($> 10 \times$ and $100 \times$ [Der p 1] concentration) had no inhibitory effect, although lysozyme 'shifted' the entire binding affinity distribution 'to the right' (i.e. towards higher affinity). In contrast, each of the peptides, added at equal concentrations with Der p 1 allergen, inhibited

only a single affinity maximum for eight individuals tested, as described later.

We first conducted a series of experiments to exclude the possibility that steric hindrance, rather than competition by the peptides, resulted in decreased binding. Der p 1 was inhibited with peptides by using a reaction mixture with concentration ratios of free Der p 1 allergen to peptide at 1:0.25, 1:0.5, 1:1, and 1:2 at each binding point. Fig. 4 shows double-reciprocal plots for the %inhibition versus peptide concentration for peptides P1 and P2. The linearity of these results argue for competitive binding by the peptides.

It is important to point out that changes of the affinity distributions upon inhibition reflect relative changes in the contributions by antibodies of different affinities and do not reflect the absolute numbers of sites blocked. This must be calculated from the total amount of ligand bound at very high inhibitor concentration and the appropriate fraction of affinities seen in the distributions.

The affinity distribution for Der p 1 without the addition of a peptide is given as a solid line in Fig. 5A. The distribution in the presence of the peptide P5 (corresponding to epitope EO5) is shown as a dotted line. The only affinity peak that is appreciably affected is the one with lowest affinity where, in this case, there is about 45% inhibition. The results for the eight dust mite-sensitive donors tested for inhibition are summarized in Table 2.

The three peptides tested (P1, P2 and P5) each affected a single peak with an average inhibition of about 20%. The percent decrease varies for each individual for a particular epitope affinity range. The average values of the three affinity peaks that were inhibited by the peptides correspond to the average affinity values for the Der p 1 'affinity regions' summarized in Table 1.

We also studied the binding of Der p 1 using a combination of peptides P1 and P5 in an attempt to inhibit two affinity peaks simultaneously (Fig. 5B). There was over 50% inhibition in binding for the entire distribution compared with the original distribution without added peptides. The peak with highest affinity is clearly inhibited by the P1 peptide (epitope EO1). However, the inhibition with two peptides simultaneously is more complex.

Instead of the three discernible peaks in the original distribution, there is one high affinity peak inhibited by P1 and one broadly distributed peak of low affinity with two apparent 'shoulders'. The low affinity shoulder may be a peak that was inhibited or undetected in the original affinity distribution. The high affinity shoulder would be less than 5% in the original distribution. This is on the borderline of peak detection by our method, yet it is not unexpected that there may be additional peaks of similar affinities hidden within the three main peaks of the distributions. In any event, we

Table 1
Summary statistics for affinity maxima positions and percent contributions^a

	Maximum # 1 log [K] (l/mole)	%1 N(K)	Maximum # 2 log [K] (l/mole)	%2 N(K)	Maximum # 3 log [K] (l/mole)	%3 N(K)
<i>Amb a 1</i>						
<i>Maxima</i>						
Mean	8.7	71	9.2	23	9.9	6
S.D.	0.56	15	0.69	11	0.61	7
N	87	87	87	87	53	53
<i>t</i> -Test comparisons	Maxima	<i>P</i> -value	Proportion	<i>P</i> -value		
	# 1 vs. # 2	<0.001	# 1 vs. # 2	<0.001		
	# 1 vs. # 3	<0.001	# 1 vs. # 3	<0.001		
	# 2 vs. # 3	<0.001	# 2 vs. # 3	<0.001		
<i>Der p 1</i>						
<i>Maxima</i>						
Mean	9.0	72	9.5	29	10.2	5
S.D.	0.58	18	0.56	26	0.53	8
N	27	27	27	27	16	16
<i>t</i> -Test comparisons	Maxima	<i>P</i> -value	Proportion	<i>P</i> -value		
	# 1 vs. # 2	<0.001	# 1 vs. # 2	<0.001		
	# 1 vs. # 3	<0.001	# 1 vs. # 3	<0.001		
	# 2 vs. # 3	<0.001	# 2 vs. # 3	<0.001		
<i>t</i> -Test comparisons	Maxima	<i>P</i> -value	Proportion	<i>P</i> -value		
	# 1	<0.001	# 1	0.022		
Amb a 1 vs	# 2	<0.001	# 2	0.018		
Der p 1	# 3	<0.001	# 3	0.020		

^a Maxima from Amb a 1 and Der p 1 specific IgE affinity distributions.

have evidence here for the production of three distinct specific IgE's to Der p 1 epitopes for individuals sensitive to dust mite allergen that can be identified by three different affinities.

4. Discussion

The polyclonal IgE response in a study population of sensitized donors is characterized by two or three affinity maxima peaks. These distributions occupy the high end of possible affinities that approach a so-called natural limit for affinity (Foote and Eisen, 1995). And, the affinity distributions invariably showed two or three major affinity peaks for both Amb a 1 and Der p 1. This suggests the presence of at least two, and possibly three, Amb a 1 and Der p 1 responding IgE clones, or groups of clones.

Each affinity maximum may represent the response to a single epitope or the response to multiple epitopes by antibodies with similar affinities. The first possibility suggests that these responses result from the recognition of two or three immunodominant epitopes. The affinities of these antibodies may represent the highest achievable affinities for specific epitopes and their struc-

tural configurations. We have not yet found any individual whose response is characterized by a single peak in the affinity distribution. In preliminary studies we have found that IgG1 specific for Der p 1 has a distinctly different distribution, with more affinity peaks on average, indicating that it is possible to observe a wider range of affinities using this method (data not shown).

The intent of these studies was to characterize the polyclonal response in terms of affinity and not to attempt to identify epitopes, for which purpose the method is not suitable. Other studies have been used to identify fragments of antigens recognized by human serum antibodies or monoclonal antibodies raised in

TNACSINGNAPAEIDLRMRVTPIRMQGGCGSCWAFSGVAATESAYLAHRNQS
P1(E01) ▲ ▲▲
LDLAEQELVDCASOHGCHGDTPRGIEYIOHNGVVOESYRYVAREQSCRRPNA
P2(E02) P3(E03)
QRFGISNYCQIYPNANKIREALAAQQTTHAIAVIIKIDLDADRHYDGRTHIQRDNGY
P4(E04)
HAVNIVGYNSAOGVDYWIWRNSWDTNWGDNGYGYFAANIDLMMIEYPYVYJL
P5(E05) ▲▲▲

Fig. 3. Amino acid sequence for the Der p 1 protein. Peptide sequences of B cell epitopes are underlined and labeled P1–P5 (corresponding to epitopes EO1–EO5). Peptides P1 (EO1), P2 (EO2) and P5 (EO5) were used for binding inhibition studies. (▲) = active site of Der p 1 (Chua et al., 1988).

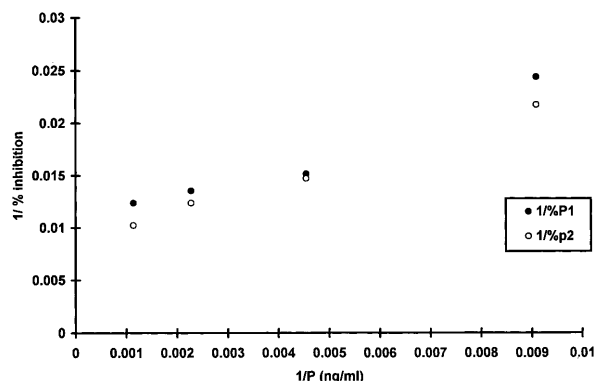


Fig. 4. Competitive binding by Der p 1 peptides. Double-reciprocal plots for the decrease in binding affinity with increasing concentrations of peptides P1 and P2.

other species. Der p 1, for example, has been shown to have multiple epitopes recognized by human serum antibodies. IgE antibody binding to both peptide fragments (Greene et al., 1991; Greene and Thomas, 1992;

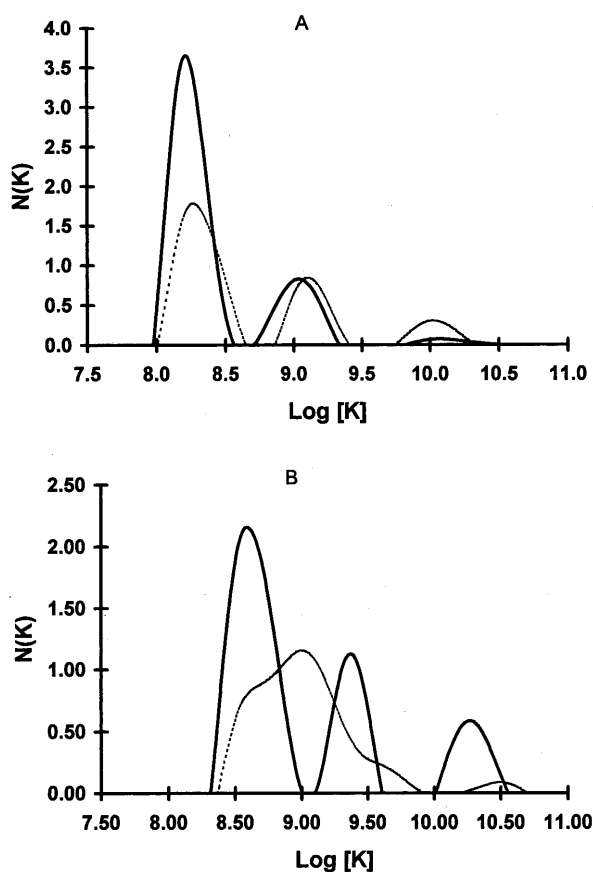


Fig. 5. The effect of binding inhibition by peptides on affinity distributions. (A) Solid line shows a Der p 1 affinity distribution in the absence of competitive peptide binding and the dashed line shows the affinity distribution in the presence of peptide P5. (B) Solid line shows a Der p 1 affinity distribution in the absence of competitive peptide binding and the dashed line shows the effect of the addition of peptides P1 and P5.

Duchateau et al., 1997) and to structurally intact antigens (Topham et al., 1994) have been shown. In our study, peptide fragments from identified epitopes were used to investigate the pattern of the IgE antibody affinity to allergen in a population of atopic individuals.

We used three peptides that are associated with known structural epitopes to identify the epitope associated with each affinity peak. We confirmed that the observed affinity peaks corresponded to activity towards separate epitopes of the Der p 1 molecule. In Fig. 3, the peptides labeled P1, P2 and P5 correspond to epitopes E01, E02 and E05. (epitope E04 represents the surface including the enzymatically active site.) Each of these are present and represented by three peaks in the affinity distributions characterizing the Ag–Ab reactions between serum IgE's and the Der p 1 allergen (representative examples shown in Fig. 5). As summarized in Table 2, peptide P1 (epitope E01) inhibits a reaction with a mean affinity of $1.26 \times 10^{10} \text{ M}^{-1}$, peptide P2 (epitope E02) inhibits a reaction with a mean affinity of $2.75 \times 10^9 \text{ M}^{-1}$, and peptide P5 (epitope E05) inhibits a reaction with a mean affinity of $3.16 \times 10^8 \text{ M}^{-1}$. In all cases, only a single affinity peak was affected by the addition of a single peptide.

The representative inhibition results shown in Fig. 5A and summarized in Table 2 were determined using 1:1 molar concentrations of Der p 1 allergen: peptide. Although the results are not shown, by using increasing peptide concentrations we could achieve up to 70–80% inhibition for each affinity peak. Thus, we can account for the majority of the response for each peak. The 20–30% residual binding for each affinity peak could contain responding antibody clones specific for other epitopes. Or the residual may be due to inhibition of the conformational epitopes that can not be achieved with simple peptides. Human serum IgE can bind to at least five confirmed B cell epitopes and other studies have shown Der p 1 specific IgE and IgG recognition using similar peptide fragments (Greene et al., 1991; Greene and Thomas, 1992).

As shown in Fig. 5B, simultaneous addition of two blocking peptides (P1 and P5) at high concentration inhibits over 50% of the overall binding to the two dominant epitopes. Such experiments, however, also reveal the structure of the residual response that normally is overshadowed by the dominant response by 70–80% of the antibodies. We see in Fig. 5B the appearance of an additional peak at about $\log K \approx 9.0$. A more extensive analysis with additional peptide fragments, not attempted in this context, would map and provide affinities to most of the antibody response.

The antibodies represented by peaks of our distribution functions, although directed against single epitopes, do not necessarily represent single clones. It is possible that the same affinity can be produced by two

Table 2

Affinity maxima inhibited and the percent decrease following the addition of peptides 1, 2 and 5, using 1:1 Der p 1:peptide ratios^a

	Maximum affected peptide1 (log K _A)	% decrease	Maximum affected peptide 2 (log K _A)	% decrease	Maximum affected peptide 5 (log K _A)	% decrease
Individual 1	10.1	26	9.03	29	8.20	45
Individual 2	10.2	35	9.40	7	8.50	25
Individual 3	10.1	12	9.40	22	8.80	5
Individual 4	10.2	13	9.65	15	8.40	2
Individual 5	10.0	18	9.60	26	8.30	20
Individual 6	10.0	20	9.50	11	8.40	30
Individual 7	10.0	20	9.40	26	8.80	20
Individual 8	10.2	8	9.50	19	8.70	18
Average	10.1	19	9.44	20	8.50	21
Maxima from Table 1	10.23		9.53		9.00	

^a For comparison, the last row contains the corresponding values of the three affinity maxima regions from Table 1.

quite different VDJ regions, although the probability of constructing similar complimentary surfaces for the epitopes leading to the similar *K*'s is not too large. Thus, until the epitope-specific responses can be dissected further, we can only refer to a limited heterogeneity of response.

The drift toward apparent restricted clonality of response with the attainment of high affinity agrees well with a model of humoral response maturation (Berek and Milstein, 1987) and the observed narrowing of the antibody population upon prolonged immunization (Braun et al., 1969). It would appear that the response to each epitope develops towards a maximum possible affinity related to the structure of the epitope as discussed by (Foote and Eisen, 1995).

The most important of our findings is the observation that the rank and order of the peaks is maintained even if the overall affinity distribution shifts. This shows that in a relatively large population of sensitized donors, the highest affinity is always associated with the same epitope. This excludes the possibility of the affinity being restricted by inherited availability of VH chain fragments. Thus the final affinity is determined by the structure of the allergenic epitope and by variables determined such as allergen level and time of exposure.

Although the epitope specificity for Amb a 1 response was not determined, the character of the overall IgE response is quite similar. There were differences between the antibody responses to Amb a 1 and Der p 1 in terms of the affinity ranges covered and the mean values for the maxima positions. The IgE specific for the year-round allergen Der p 1 had mean values for maxima slightly higher than the seasonal allergen Amb a 1 (Table 1). This could be the result of different exposure schedules to these common allergens. In both cases, the response appears to be towards a limited number of immunodominant epitopes.

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