

Evidence of an affinity threshold for IgE-allergen binding in the percutaneous skin test reaction

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Summary

Background Atopy is an aberrant immune response involving allergen-specific IgE production, though serum IgE concentration is not an entirely reliable diagnostic tool, particularly for epidemiological and genetic studies. There is no clear correlation between IgE and other indicators of atopy such as skin prick tests (SPT)s, and physiological associations are difficult to justify in cases with detectable IgE but negative SPT results.

Objective IgE reflects the number of molecules available to produce an atopic response, but the degree of the response is determined by the binding strength (affinity) between receptor-bound IgE and the allergen. We sought to determine if there was an association between binding affinity and SPT results in people with histories of atopy.

Methods Standard SPTs (whole allergen extracts) were administered to people with histories of sensitivities to ragweed and house dust mite. The concentrations and affinities of serum allergen-specific IgEs were determined using the purified allergens Amb a 1 and Der p 1.

Results There was a positive correlation between weal area and allergen-specific IgE among SPT-positive donors. However, for those individuals with detectable amounts of allergen-specific IgE, there was considerable overlap of IgE values between SPT-positive and -negative groups. Among sensitized donors, IgE–allergen interactions were characterized by two or three specific reactions of very high affinity (K_A range 10^8 – 10^{11} M). Negative SPT reactions were associated with lowered IgE binding affinities to major allergens. This delimited two groups with atopic disorders: specific IgE(+)/SPT(+) and specific IgE(+)/SPT(–).

Conclusion The product of antibody affinity and concentration, which we define as antibody capacity ($CAP = K_A \times \text{IgE}$), is more informative with regard to describing allergen sensitivity than antibody concentration alone. Antibody binding capacity provides physiological evidence of atopy in some subjects who do not test positively by common methods and suggests an affinity threshold to produce a positive SPT reaction.

Keywords allergen, IgE, Amb a 1, Der p 1, ragweed, house dust mite, antibody binding affinity, skin prick test

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Introduction

Atopic disorders are adverse IgE-mediated immune responses to ragweed pollen, house dust mites, moulds and other similar allergens. Specific end organ responses, such as those seen with allergic rhinitis or asthma, occur when receptors are cross-linked on the surface of mast cells or basophils with the subsequent release of bioactive mediators (e.g. histamine, leukotrienes). The first step involves the binding of the F_c portion of allergen-specific IgE to 'high affinity' (F_{ce} RI) receptors and is followed by the binding of adjacent receptor-bound IgE molecules with the multivalent allergen.

Epidemiological and genetic studies rely upon evidence of allergen-specific IgE for a definition of atopy. Unfortunately, the presence of specific IgE does not give an accurate indication of the occurrence or severity of atopic disease [1,2], although it is indicative of prior sensitization [3]. Most studies have primarily focused upon determining the quantity of allergen-specific IgE and possible correlations with end organ responses, such as skin test reactions or histamine release [4–6]. However, these analyses are not entirely reliable as they cannot account for cases with relatively large amounts of allergen-specific IgE but no detectable sensitization (e.g. skin test negative) or cases with indications of sensitivity (skin test positive) but no detectable specific IgE in the serum.

The present study was undertaken to investigate additional features of the IgE response in terms of the affinity, concentration and heterogeneity of allergen-specific IgE directed towards two major purified allergens; Amb a 1 (common seasonal

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aeroallergen from ragweed) and Der p 1 (common indoor allergen from dust mites). The antibodies produced against a single allergen may derive from many individual epitope-specific clones. We have developed a method to determine affinity distributions for allergen-specific IgE [7]. Our method allows us to describe, in terms of affinity, a number of concurrent antibody-epitope reactions present *in vivo*. This method is distinct from other, more traditional analyses such as Scatchard's which only determine average affinities, and are more appropriately defined as mean avidities [8,9].

We studied a group of individuals who presented clinical evidence of sensitivity to allergens from dust mite or ragweed and had detectable levels of allergen-specific IgE. We determined the allergen-specific IgE concentrations and their affinity distributions. In addition, we introduced a new variable, antibody capacity, defined as the product of affinity and concentration ($CAP = K_A \times \text{IgE}$), that more clearly defines an individual's ability to respond to a specific allergen. The results suggest an affinity threshold for interaction between IgE and allergen in order to produce a positive skin prick test (SPT) reaction.

Methods

Sample population

We screened 165 members from families with histories of clinical atopy. Each donor was screened for allergic symptoms and the presence of atopy. All donors provided informed written consent according to the policies of the Institutional Review Board of the University of Minnesota.

A history of allergic symptoms was determined from responses to standardized questionnaires. A positive allergic response to dust mites was indicated by the presence of rhinitis upon exposure to house dust. A positive allergic response to ragweed was indicated by the presence of rhinitis during the months of August or September when ragweed pollinates. An atopic response was defined by a positive skin test reaction to ragweed or dust mite extracts and/or detectable amounts of serum allergen-specific IgEs.

Characteristics of the sample population with regard to skin test results and specific IgE determinations are summarized in Table 1. Control subjects are included who were skin test-negative to ragweed and dust mite extracts and did not have detectable amounts of Amb a 1- or Der p 1-specific IgE. Only one individual with a positive skin test reaction to ragweed extract had no detectable amounts of Amb a 1-specific IgE. The study population consisted of 82 females and 83 males ranging in age from 6 to 73 years (mean donor age: 30 years).

Skin testing

Skin prick testing was administered with Duo-Tip Test Applicators (Lincoln Diagnostics Inc., Decatur, IL, USA) using standardized allergen extracts from dust mite (*Dermatophagoides pteronyssinus*) and short ragweed (*Ambrosia artemisiifolia*) (extracts from Bayer Corporation, Elkhart, IN, USA). Positive histamine and negative saline controls were also administered. Weal areas (mm^2) were measured with a planimeter. A positive response was recorded for any weal area $\geq 9 \text{ mm}^2$ above the negative saline control.

Table 1. Study Population

Skin test	Specific IgE***	n
Ragweed (+)	anti-Amb a 1 11.40 (3.24) [0.02, 131.2]	73
Ragweed (-)	anti-Amb a 1 0.12 (0.06) [0.02, 1.44]	27
Dust mite (+)	anti-Der p 1 12.79 (5.44) [0.02, 120.0]	31
Dust mite (-)	anti-Der p 1 1.44 (0.38) [0.41, 5.26]	14
Ragweed (-)/ dust mite (-)	No anti-Amb a 1 & No anti-Der p 1	20
Total =		165

***Mean specific IgE values (IU/mL) with SEMs in parentheses. Minimum and maximum IgE values (IU/mL) are given in square brackets.

This method is consistent with current diagnostic procedures for evaluating allergen sensitization. In contrast to other methods, such as intradermal testing, the SPT is more specific, although perhaps less sensitive. Intradermal testing may be more sensitive, due to greater control over the concentration of allergen administered, but it is less specific (M. N. Blumenthal, personal communication).

The decision to use ragweed and house dust mite extracts for skin testing, as opposed to purified allergens for affinity determinations to be described later, was based on our investigative goal: to ascertain whether there exists among the population of skin test-negative donors a subpopulation that escapes detection due, possibly, to altered binding affinity for allergen by IgE. Skin testing with purified allergens would be unwarranted for our purposes, as it would exclude individuals who may be positive to other allergen components in the extracts and, thus, already classified as atopic.

Measurement of specific IgE concentrations

Serum concentrations of Amb a 1- and Der p 1-specific serum IgE were measured by an adaptation of the Access[®] specific IgE immunoassay [7,10]. Access[®] is an automated solid phase immunoassay system that utilizes chemiluminescence based detection (Sanofi/Beckman Coulter, Chaska, MN, USA) [11]. Briefly, purified and biotinylated allergen is first attached to anti-biotin mAb-coated magnetic particles (solid phase support; Dynal, Inc., Lake Success, NY, USA). Amb a 1 (m.w. 37 800), a major allergen from ragweed pollen, and Der p 1 (m.w. 25 270), a major allergen from house dust mite were purified and biotinylated as described in Pierson et al. [7]. Allergen-specific IgE from a serum sample is then 'captured' by the attached mAb-allergen complex. The amount of captured IgE is determined using alkaline phosphatase-conjugated equine anti-human IgE antiserum (Beckman Coulter). Using this step ensures that only IgE is detected and not other allergen-specific antibody isotypes, like IgG, that may also have been bound by the mAb-allergen complex. A chemiluminescent substrate (Lumi-phos 530[®], Lumigen, Inc., Southfield, MI, USA) is added to produce a chemiluminescent signal that is

proportional to the amount of captured IgE. The amount of analyte in the sample is determined by a stored, multi-point calibration curve. The lowest limit of detection using this method is 0.02 IU/mL (1 IU = 2.4 ng)

It should be noted that this procedure for the determination of Amb a 1- and Der p 1-specific IgE is not commercially available in the USA.

Negative serum controls and sensitivity validation

To demonstrate the specificity and sensitivity of the methods, we processed serum samples known to contain Der p 1- or Amb a 1-specific IgE [7]. Briefly, these procedures were as follows. For a negative control, we depleted a serum sample of IgE by affinity chromatography using an anti-IgE mAb. The anti-IgE mAb was coupled to CNBr-activated Sepharose 4B, which was then used to absorb IgE from the serum sample. Unbound proteins were detected in the column eluent by ultraviolet light absorption at 280 nm. The eluent was then tested for both total IgE and allergen-specific IgE.

Serum was also fractionated by molecular weight using a column of Sephacryl S-300. The presence or absence of IgE and/or IgG in the eluent fractions was confirmed by Western blotting using the appropriate mAbs against the immunoglobulin isotypes. By locating the eluent fraction that only contained IgE, we tested the lower sensitivity limit of the assay using serial dilutions of this preparation.

IgE-allergen binding affinity distribution functions

Our basic experimental method provides information regarding allergen-antibody (Ag-Ab) binding reactions *in vitro* [7]. The detection method is slightly different from that used for determining antibody concentrations. Briefly, magnetic particles (Dynal) are coated with a mouse anti-human, anti-IgE mAb. To this is added a serum sample known to contain allergen-specific IgE which is 'captured' by the particle-bound mAb. Biotinylated purified allergen is added to bind only to the specific IgE of interest, and detection is achieved by the addition of streptavidin-conjugated alkaline phosphatase (Sigma, St Louis, MO, USA) which reacts upon a chemiluminescent substrate to generate a light signal.

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 analyse this problem [7] using an approach first suggested by Hunston [12] and Thakur et al. [13].

The method employs the following 5-step algorithm to derive the desired information. (A pamphlet detailing the methods used for determining affinity distribution functions is available, at no cost, to interested investigators.) The data were analysed using Mathematica® software (Wolfram Research, Inc., Champaign, IL, USA).

Step 1: Determine the fraction of bound allergen for varying allergen concentrations. Using a range of purified Ag concentrations, a binding isotherm is generated: r vs. Log_{10} Ag, where r = fraction of antibody sites bound at a given Ag concentration. To eliminate 'background noise' from the positive test signal, due possibly to non-specific binding of the Ag, parallel determinations were made for each Ag concentration using

conditions that did not include Ag-specific IgE. The background was subtracted from the test signal prior to determining the fraction of specifically bound r .

It is important that a 'complete' binding isotherm is generated in order to insure 100% maximal (saturation) binding. In practice, as recommended by Thakur et al. [13], at least 10 concentrations of Ag are used spanning 3.5–4 decades on a log molar scale.

Step 2: Plot the fraction of bound allergen r against the Log_{10} Ag concentrations. In theory, this will yield a sigmoid-shaped isotherm.

Step 3: Find the 'best fit' of the isotherm data. This provides an arbitrary function to be used in transforming data. In practice, best results are achieved using a cubic spline fit that gives a continuous function through all data points.

Step 4: Determine the affinity distribution function using the transformation function described in Hunston [12], Thakur et al. [13] or Pierson et al. [7].

Step 5: Plot the results of the affinity distribution. The information from the binding isotherm is transformed to derive an affinity distribution function. These functions identify the number of reactions involved between IgEs and the allergens (n), the equilibrium binding affinity constants for each reaction (K_A), and the relative proportion of each reaction to the sum of all reactions involved ($N(K)$) (see example in Fig. 2 with $N(K)$ on the ordinate and $\text{Log}_{10} K_A$ on the abscissa).

It should be noted that the strength of the affinity distribution method is the capability to detect multiple reactions that may occur simultaneously, which is the case with purified allergens that may consist of multiple allergenic epitopes. However, its discriminatory utility is lost in extremely complex reaction schemes, which would be the case with allergen extracts that consist of a number of major allergens each with their own epitopes.

Results

Concentrations of Amb a 1- and Der p 1-specific IgEs

We confirmed the specificity and sensitivity of the method using serum known to contain allergen-specific IgE. As described in Methods, we removed IgE from serum samples and tested this with the assay. No signal above background was detected, indicating that only IgE and not some other immunoglobulin isotype (e.g. IgG) produced a signal. We also fractionated serum by molecular weight, and found a fraction that contained only IgE (confirmed by Western blotting, data not shown). Using serial dilutions of this fraction, we could reliably detect allergen-specific IgE down to 0.02 IU/mL (≈ 0.05 ng/mL).

To verify that those with positive SPT reactions to allergen extracts included responses to the major allergen components, we compared the logarithms of the respective weal areas to the logarithms of the serum allergen-specific IgE concentrations. In the case of Amb a 1, the correlation coefficient was: $r = 0.66$ ($P < 0.001$). For Der p 1 the correlation coefficient was: $r = 0.47$ ($P < 0.01$). Of course, we cannot account for responses to other allergens that may be present in the extracts by this method. However, there is clear evidence of response to the major components among SPT-positive donors. Those with negative SPTs do not respond to any component in the extracts

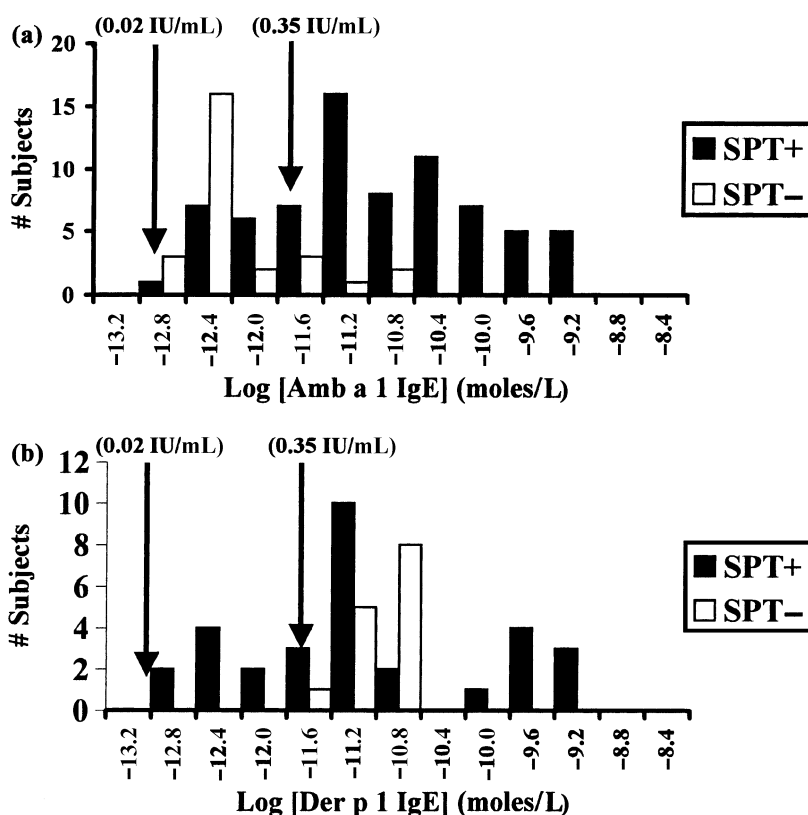


Fig. 1. Allergen-specific IgE concentrations. (a) Distribution for 100 individuals with detectable Amb a 1-specific IgE levels. (b) Distribution for 45 individuals with detectable Der p 1-specific IgE levels. The arrow indicating a value of 0.35 IU/mL is the current, albeit arbitrary, cut-off for detectable specific IgE using allergen extracts. The arrow indicating a value of 0.02 IU/mL is the lowest, reproducible detection cut-off using purified allergens and Access[®].

although, as described below, some of these people mount an atopic immune response to allergens as evidenced by the presence of allergen-specific IgE in the serum.

Figure 1 shows frequency histograms for the allergen-specific IgE concentrations plotted on a logarithmic molar scale (units of moles/L) using a value for IgE m.w. = 190 000. Using the Access[®] method, the lowest detectable concentration is 0.02 IU/mL (≈ 0.05 ng/mL ≈ -12.60 log molar units) indicated by arrows in the figure. The non-sensitized control group did not have detectable amounts of Amb a 1- or Der p 1-specific IgE. We are able to reliably detect allergen-specific IgEs at a concentration well below the generally accepted, albeit quite arbitrary, value of 0.35 IU/mL (≈ 0.84 ng/mL ≈ -11.35 log molar units; indicated by arrows in the figure) which is based upon detection procedures using whole allergen extracts [3].

In Fig. 1, the results given as solid bars are from donors with positive skin test reactions. The open bars are the results for the donors who had detectable amounts of specific IgE but were skin test-negative.

The Amb a 1-specific IgE values for those with positive skin test reactions to ragweed extract had a range of -12.9 to -8.8 log IgE units with an average of -10.85 log IgE (≈ 11.4 IU/mL) and SEM = 0.122. The skin test-negative donors to ragweed extract had Amb a 1-specific IgE values with a range of -12.9 to -10.7 log IgE with an average of -12.26 log IgE (≈ 0.12 IU/mL) and SEM = 0.094. The range of Der p 1-IgE concentrations for dust mite skin test-positives was -12.9 to -8.8 with an average value of -11.63 log IgE (≈ 12.8 IU/mL) and SEM = 0.211. For skin test-negatives the range was -11.3 to -10.2 IgE with an average value of -10.79 (≈ 1.44 IU/mL) and SEM = 0.089.

As expected, those donors with positive skin test reactions tended to have higher concentrations of specific IgE than those donors with negative skin tests. However, there was considerable overlap between the skin test positive and skin test negative groups without a clear demarcation between them. Comparison of the mean values between SPT-positive and SPT-negative groups was not statistically significant by *t*-test ($P = 0.05$) for both Amb a 1 and Der p 1.

Affinities for Amb a 1- and Der p 1-specific IgEs

Affinity distribution functions [12,13] were determined for all donors with detectable amounts of specific IgE. Figure 2 shows representative affinity distributions derived from these functions for Amb a 1- and Der p 1-specific IgE plotted on a logarithmic scale in units of l/mole (Figs 2a and b). These distributions are a series of peaks, where the width of each peak is indicative of the range of affinities included in that particular peak.

This method determines three key features of allergen-antibody interactions: the number of reactions involved (n), the equilibrium binding constant of each reaction (K_A) and the relative proportion of each reaction to the sum of all reactions ($N(K)$). The highest points of each peak, indicating the most likely reaction affinities, are referred to as affinity maxima. The values corresponding to these maxima are used to determine the parameters K_A and $N(K)$.

For example, the affinity maxima (K_A)s in Fig. 2a are at 1.45×10^8 M, 7.13×10^8 M and 6.50×10^9 M. Each maximum represents a proportion of the total response. When normalized, the area under each peak indicates the probability of finding a responding antibody clone in that particular range of

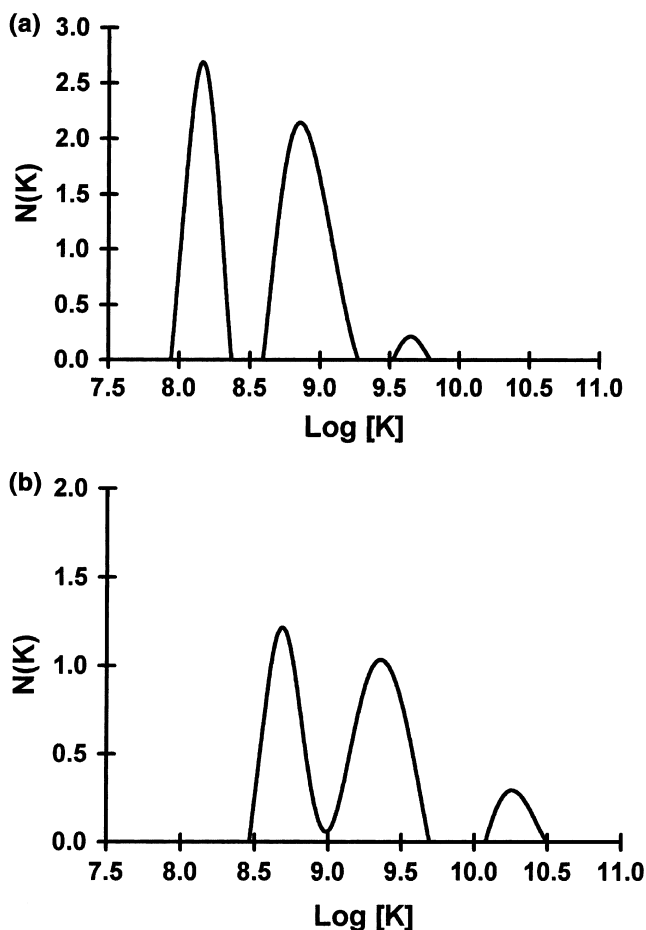


Fig. 2. Representative affinity distributions for Amb a 1- and Der p 1-specific IgE. Three features are evident in these distributions: the number of allergen-antibody reactions (n), the affinity of each reaction (K given in units of l/M on a log scale), and the relative proportion of each reaction to the sum of all reactions ($N(K)$ in dimensionless units) determined by the highest point (affinity maximum) for each peak. (a) Affinity distribution for Amb a 1-specific IgE. Three affinity maxima and their relative contributions are: 1.45×10^9 M (53.25%); 7.13×10^8 M (42.50%); and 6.5×10^9 M (4.25%). (b) Affinity distribution function for Der p 1-specific IgE with three affinity maxima at 4.84×10^8 M (47.80%); 2.27×10^9 M (40.62%); and 1.80×10^{10} M (11.58%).

affinities (ΔK_A). The maxima in Fig. 2a, from left to right, represent $N(K)$ s of 53.25%, 42.50% and 4.25% of the total response, respectively.

We have recently shown that, in the case of Der p 1, each of the affinity maxima corresponds to a specific response to an allergenic epitope found on the allergen [14]. This was confirmed by binding inhibition studies using peptides known to be incorporated in these epitopes. Although comparable analyses are not yet possible for Amb a 1, it seems reasonable to assume that each of the peaks in these distributions also correspond to specific epitopes.

These results also highlight the importance of using purified allergens rather than allergen extracts in the affinity determinations. The number of allergens present in the extracts, along with the possibility of multiple epitopes for each allergen, would tax the discriminatory power of the method. Overlap between affinity 'peaks' would make it impossible to distinguish individual reactions.

Table 2. Affinity distribution statistics (Amb a 1-specific IgE)

	Affinity* SPT (+) Log (K)	SPT (-) Log (K)	P-value‡	Relative peak height† SPT (+) N (K)	SPT (-) N (K)	P-value
Reaction #1§						
Mean	8.49	7.31	< 0.001	0.71	0.63	< 0.025
Std dev.	0.751	0.539		0.147	0.163	
n	73	27		73	27	
Reaction #2						
Mean	8.99	7.55	< 0.001	0.23	0.22	N.S.
Std dev.	0.969	0.946		0.110	0.107	
n	73	27		73	27	
Reaction #3						
Mean	9.66	8.18	< 0.001	0.06	0.06	N.S.
Std dev.	0.868	0.853		0.707	0.054	
n	49	16		49	16	

*Most probable affinity value for a given reaction (Log (K) in Fig. 2).
†Relative proportion of a given reaction ($N(K)$ in Fig. 2). ‡P-value from comparison of means by *t*-test (N.S. = not significant, $P=0.05$).
§Distribution number of reaction, from left to right (examples in Fig. 2).
SPT = Skin prick test reaction: positive (+) or negative (-). Std dev. = Standard deviation.

Figure 3 shows frequency histograms for the maxima from all donors who had specific IgE towards ragweed (Fig. 3a) and dust mite (Fig. 3b) allergens. Similar to the representative distributions shown in Fig. 2, there were two affinity maxima for each donor tested, and in about 2/3 of the cases, a third maximum was detected. Donors with positive skin test reactions (solid bars in Fig. 3) had, on average, higher binding affinities than those with negative skin test results (open bars in Fig. 3). Because the control population did not have measurable specific IgEs, affinity distribution functions for this group were undefined.

The donors with Amb a 1- and Der p 1-specific IgE always had two binding reactions and in about 2/3 of the cases three separate reactions regardless of their results for skin test reactivity. For skin test-negative donors, the mean binding affinity for each reaction maxima is 'shifted' to a lower value relative to the results for the skin test-positive donors. Table 2 gives comparisons of the mean affinity values corresponding to each of the two (or three) maxima for ragweed skin test-positive and skin test-negative subjects.

Comparisons of these mean values by Student's *t*-tests were significantly different ($P < 0.001$). Interestingly, the relative proportion of each corresponding maximum is similar for both donor groups. Comparisons based upon skin test reactions to dust mite are given in Table 3. Again, the mean affinity values for skin test-negative subjects are 'shifted' to lower values than the values for skin test-positives. Additionally, there is far less overlap between the affinity distributions for the skin test-negative and -positive groups than there is for IgE concentrations (Fig. 1)

We plotted affinity vs. IgE concentration to investigate a possible relationship between the affinity and concentration of allergen-specific IgE (Fig. 4). Because there was more than one binding reaction in each case, the total concentration of specific

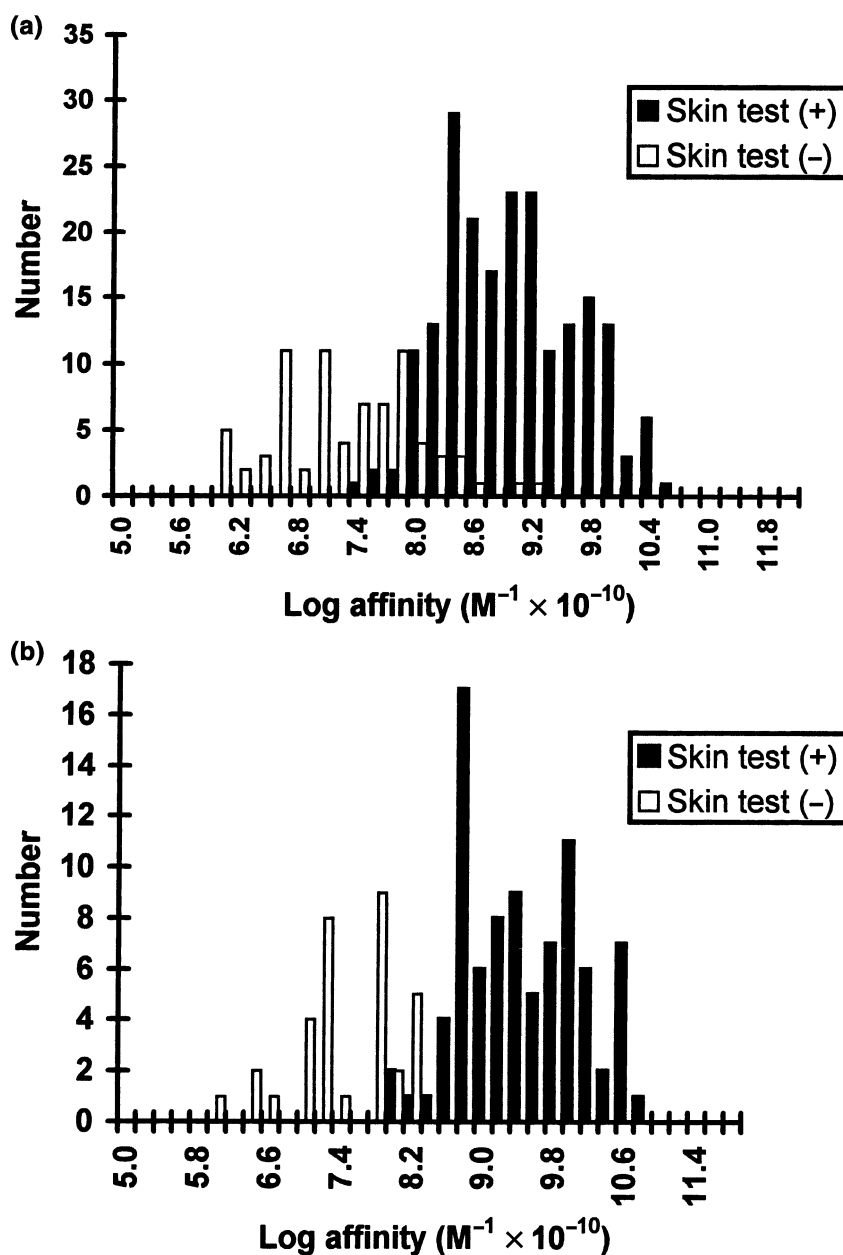


Fig. 3. Affinity maxima histograms. (a) Affinity maxima for Amb a 1-specific IgE from 73 individuals with positive skin tests (solid bars) and the maxima from 27 individuals with negative skin tests (open bars). Summary statistics can be found in Table 2. (b) Affinity maxima for Der p 1-specific IgE from 31 individuals with positive skin tests (solid bars) and the maxima from 14 individuals with negative skin tests (open bars). Summary statistics can be found in Table 3.

IgE was multiplied by the relative proportion of each particular peak to the total. These proportions were obtained from normalized affinity distributions. Plotted on a log-log scale, there appears to be an inverse relationship between IgE and affinity.

Antibody capacity

It is apparent that there can be considerable variability among individuals with regard to serum concentrations of allergen-specific IgEs and the binding affinities of these IgEs for allergens. The interpretive question becomes one of how to describe the interrelationship between these variables in a biologically meaningful way. A biological, end organ response such as histamine release from mast cells is a function of both of these modalities. Concentration relates to the number of molecules available to produce a response, while binding affinity relates to the strength of the binding reaction.

We introduce a variable, antibody capacity, defined as affinity $\times$ concentration ($K_A \times \text{IgE}$). It is derived from binding equilibrium as follows:

$$K_A = [\text{PL}]/[\text{P}][\text{L}] \quad (1)$$

where P = protein (IgE antibody) and L = ligand (allergen). The total ligand (allergen) in the reaction mix is: $L_{\text{total}} = [\text{PL}] + [\text{L}]$. The fractional saturation of bound ligand (allergen) is: $\alpha \equiv [\text{PL}]/L_{\text{total}} = [\text{PL}]/\{[\text{PL}] + [\text{L}]\}$. After some straightforward algebra, the following relationship is found:

$$K_A \times [\text{P}] = \alpha/(1 - \alpha) = [\text{PL}]/[\text{L}] \quad (2)$$

$\alpha \equiv$ fractional saturation is always < 1 since under equilibrium conditions $[\text{PL}]$ never equals L_{total} . With affinity (K_A) given in units of l/M and IgE given in units of M/l , the parameter of

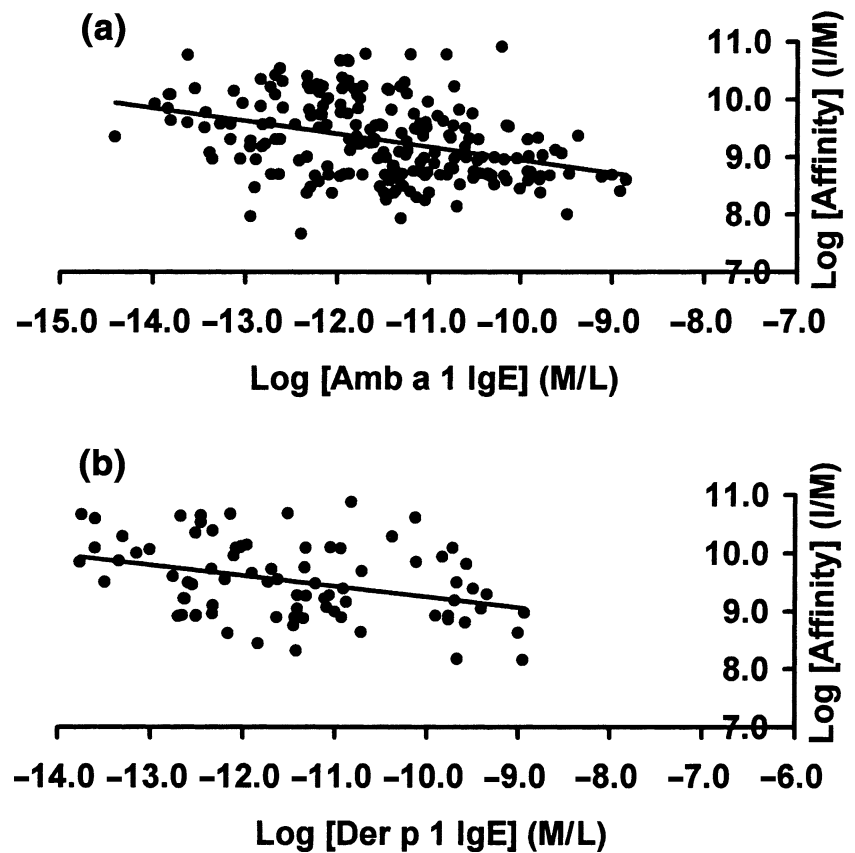


Fig. 4. The affinity maxima for Amb a 1- and Der p 1-specific IgE plotted against the concentrations of IgE corresponding to each affinity peak.

Table 3. Affinity distribution statistics (Der p 1-specific IgE)

	Affinity* SPT (+) Log (K)	SPT (−) Log (K)	P-value‡	Relative peak height† SPT (+) N (K)	SPT (−) N (K)	P-value
Reaction #1§						
Mean	9.09	7.13	< 0.001	0.72	0.83	N.S.
Std dev.	0.482	0.215		0.179	0.161	
n	34	14		34	14	
Reaction #2						
Mean	9.60	7.80	< 0.001	0.23	0.15	N.S.
Std dev.	0.483	0.412		0.135	0.11	
n	34	14		34	14	
Reaction #3						
Mean	10.33	8.41	< 0.001	0.05	0.02	N.S.
Std dev.	0.390	0.321		0.082	0.150	
n	19	8		19	8	

*Most probable affinity value for a given reaction (Log (K) in Fig. 2).
 †Relative proportion of a given reaction (N (K) in Fig. 2). ‡P-value from comparison of means by *t*-test (N.S. = not significant, *P* = 0.05).
 §Distribution number of reaction, from left to right (examples in Fig. 2). SPT = Skin prick test reaction: positive (+) or negative (−). Std dev = Standard deviation.

capacity is dimensionless. Capacity defines the relative degree of saturation achievable for a given ligand concentration and affinity.

Figure 3 illustrates the distributions of the affinity maxima for individuals with positive and negative skin tests. Each individual had two or three affinity maxima for Amb a 1 or Der p 1. The two or three capacities associated with the affinity maxima were added to obtain total capacities for each individual and plotted on a log scale:

$$\text{Total CAP} \equiv [\text{IgE}] \times \sum_{i=1}^n K_i, \quad (3)$$

where IgE is the allergen-specific IgE concentration and K_i and $N(K)_i$ are the affinity constant and proportion of the i^{th} reaction, respectively. Figure 5 shows the total capacities for the individuals with Amb a 1- and Der p 1-specific IgE, with and without positive skin tests. As with the affinities (Fig. 3), the binding capacities for skin test-negative subjects were generally lower than for skin test-positive subjects. The mean capacities for Der p 1 were slightly higher than those for Amb a 1.

For subjects with detectable amounts of Amb a 1-specific IgE, the mean log value for capacity was -1.64 (SEM = 0.123) for skin test-positive subjects and the mean log value for capacity was -4.30 (SEM = 0.203) for skin test-negative subjects. The mean values for total capacities between skin test-positives and skin test-negatives were significantly different by Student's *t*-test ($P < 0.001$).

For subjects with Der p 1 IgE, the mean log capacity among skin test positive subjects was -2.17 (SEM = 0.268) and the mean log capacity for skin test-negatives was -3.48 (SEM = 0.143). These mean values were also significantly different by *t*-test ($P < 0.001$).

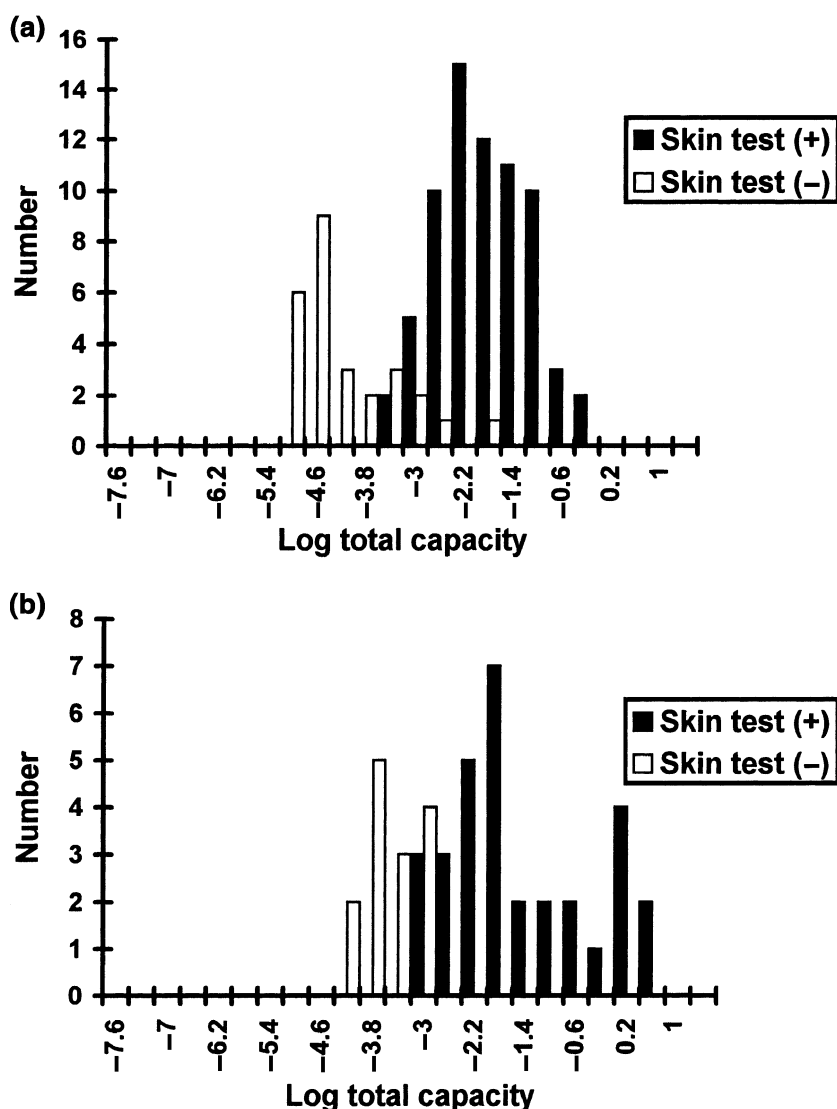


Fig. 5. Distribution of total capacity. (a) Amb a 1 specific-IgE total capacities for 73 individuals with positive skin tests (solid bars) and 27 individuals with negative skin tests (open bars). (b) Der p 1-specific IgE total capacities for 31 individuals with positive skin tests (solid bars) and 14 individuals with negative skin tests (open bars). Total capacity calculation given by equation 3 in Results.

Finally, there is a much clearer demarcation between donors who were skin test-positive and those who were skin test-negative using capacity rather than IgE concentration alone. In this limited study sample, a log capacity value around -3.0 units appears to demarcate between the skin test-positive groups and skin test-negative groups.

Discussion

Atopic diseases are complex disorders involving adverse immune responses to highly heterogeneous allergenic stimuli. Currently, diagnoses of atopy in clinical practice or epidemiological or genetic studies rely almost exclusively upon evidence of allergen-specific IgE production. This is typically manifest as an adverse skin reaction to a standardized allergen challenge or detectable amounts of serum allergen-specific IgE. However, evaluations that rely upon IgE concentrations alone are limited in characterizing the heterogeneous nature of (potential) responses to complex allergen components because equal levels of IgE may have different effects due to differences in binding affinity.

The serum-specific IgE concentrations for the individuals studied here ranged from 0.02 to over 120 IU/mL (≈ 0.05 to over 300 ng/mL). The lower detection limit generally used for clinical reports is 0.35 IU/mL [3] which, however, refers to total response to multiple allergens present in allergen extracts. The purified proteins Amb a 1 and Der p 1 are the major allergens found in ragweed and dust mite extracts, respectively [15]. There were significant statistical correlations between Log (weal area) and Log (specific IgE), indicating that the SPT-positive donors were responding to the major allergens Amb a 1 or Der p 1.

Using purified allergens and a highly sensitive chemiluminiscent detection system, we chose a cut-off of 0.02 IU/mL. The assay produces a linear response when measuring increasing amounts of IgE, and there is a proportional increase in signal with the addition of known amounts of IgE in the form of IgE dilutions. Using IgE-depleted serum gave no detectable signal with this method. Additionally, after fractionation of serum, the signal provided by an IgE-rich fraction gave a clear response to as low as 0.02 IU/mL. Thus, we can reliably measure IgE concentrations below the arbitrary cut-off value of 0.35 IU/mL.

Figure 1 shows that by using a cut-off value of 0.02 IU/mL with purified allergens instead of the 0.35 IU/mL used for extracts, we pick up an additional 41% and 25% of the study population as positive for Amb a 1- and Der p 1-specific IgE, respectively. Please note that this is not intended to be an epidemiological report to establish new cut-off values for specific IgE concentrations, as the number of subjects sampled is far too small. Also, although the standardized extracts used for skin testing are commonly used for evaluation of allergen sensitization, they do not always provide unequivocal evidence of what allergenic component a person is sensitized to, or to what degree. Rather, the data in Fig. 1 are of interest in the context of our study of affinity distributions within a small atopic population.

The purpose of this investigation is to show that when employed in a diagnostic or epidemiological or genetic context, negative skin test results or low levels of circulating IgE *per se* are not sufficient to support a classification of individuals as non-atopic. We show among individuals who are skin test-negative to dust mite or ragweed extracts that there is a subpopulation with respectable levels of specific IgE who are clinically symptomatic. We also can show that low levels of specific IgE (<0.35 IU/mL) can have a very high capacity response. Both phenomena are due to wide variations of IgE affinity; low affinity in the case of skin test-negative individuals and very high affinity in the case of low IgE levels.

The histograms of affinities for Amb a 1- and Der p 1-specific IgE separate into distinct populations based upon the results of SPTs (Fig. 3). These populations can be provisionally identified as IgE⁽⁺⁾/SPT⁽⁺⁾ and IgE⁽⁺⁾/SPT⁽⁻⁾. It is interesting to note that the majority of individuals with negative skin test to house dust mite extract had measurable concentrations of Der p 1-specific IgE well above the customary 0.35 IU/mL cut-off for specific IgE (Fig. 1). It was the affinity of the response that more clearly separated the skin test-positive individuals from the skin test-negative individuals. It appears that the affinity of the specific IgE reaction may be more indicative of skin test reactivity than IgE concentration.

The concentration and affinity of allergen-specific IgE are clearly related. As shown in the log-log plot of Fig. 4, there is an apparent inverse relationship between these variables. These findings are in contrast to a study using inbred mice by Steward and Petty [16] who did not find an association between affinity and levels of anti-HSA antibodies. The difference in findings may be explained by considering that the study by Steward and Petty measured affinity after one strong exposure to antigens, while our study involves a very long-term exposure process. Our observations, in contrast to those from inbred mice, show that the apparent affinity of the IgE clones is inversely related to the amount of IgE produced by these clones.

The affinity distributions reported here occupy the high end of possible affinities that approaches a so-called natural limit for affinity [17]. The affinity distributions invariably show 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 IgE clones, or groups of clones, responding to Amb a 1 and Der p 1. These data, in agreement with previous results [18], support the notion of development of a response over time to a limited number of epitopes to a minimum needed for cross-linking receptors on bioactive cells.

Although not shown, with a linear plot of affinity vs. IgE concentration, there is an apparent inverse hyperbolic relationship. The lower asymptotic limit of the hyperbolic relationship, around 10^8 M, can be explained by functional limits. An affinity below this value would require unreasonably high IgE concentrations due to competition with IgG molecules [19]. The higher limit at about 10^{11} M is most probably related to the Foote and Eisen limit for antibody affinity [17]. These authors argue that affinities higher than 10^{11} M would not be biologically meaningful, as almost all biological activities can be initiated by reaction affinities at or below this level.

As a procedural note, it should be borne in mind that the affinity results reported here are due to specific interactions between IgE and purified allergen. As noted in the Methods section, due to the use of isotype-specific mAbs for 'capture', only IgE is available for interaction with purified allergen. More importantly, for each affinity distribution determination, parallel assays are made without serum containing IgE. The latter condition accounts for 'non-specific' binding that may occur between the purified allergen and other, non-IgE, components in the reaction mixture.

The use of purified allergen rather than whole extracts for affinity determinations is a necessary trade-off that enables us to characterize the heterogeneous nature of allergen-antibody interactions and allows for determining the important thermodynamic parameters of the reactions [14]. Traditional analyses, such as Scatchard plots, only provide information regarding mean affinities of all possible reactions involved (more appropriately termed avidities) [8,9]. The trade-off results as the discriminatory power of the affinity distribution method is lost in extremely complex reaction mixtures, such as would be the case using allergen extracts.

We have attempted to dissect these complex reactions with whole allergen extracts using binding to affinity columns. However, in our hands, the results were not reproducible and thus unreliable. We are not aware of any other method currently in use that might aid in resolving these complex reaction schemes. In any event, the primary purpose of this investigation was to attempt to provide explanations for some conflicting results (e.g. SPT-negative with detectable specific IgE) that might arise either in a clinical setting or epidemiologic or genetic studies by comparing the results of our affinity determinations to those of standardized allergen preparations. Our focus was not on varying results that might result from varying allergen preparations.

The IgE concentration relates to the number of molecules available to produce a biological response (e.g. histamine release). The affinity relates to binding strength; the higher the affinity, the more likely it is that a particular response will occur. To functionally combine these variables, we introduced the antibody capacity defined as the product of IgE and affinity.

Capacity is a variable that estimates the fraction of receptor-bound IgE molecules that may be bound by ligand. Figure 5 shows the calculated total capacities corresponding to the affinity maxima of Fig. 3. The capacities for skin test-negative donors are, on average, lower than those for skin test-positive donors for both Amb a 1 and Der p 1. The specific IgE total capacity at about -3.0 log units (Fig. 5) appears to delimit the skin test-positive and skin test-negative groups. This cut-off may represent the minimum capacity necessary to produce a measurable skin test reading. The boundary separating the IgE

response capacities of the two groups in comparison to the overlap seen with the specific IgE concentrations of the same individuals, establishes that it is not the IgE concentration alone, but the capacity and affinity that determine the biological response. It is possible that titration of standard subcutaneous skin tests, using purified allergens, may allow a more accurate analysis of the contribution of affinity and concentrations of allergen-specific IgE to skin test reactivity.

The clinical relevance of low affinity IgE remains to be determined. Preliminary evidence suggests that most of the low affinity determinations are found among young children, which could suggest a critical period of time during early life when the affinity response 'matures'. However, we have not yet tested sufficient numbers of samples from paediatric donors to confirm this. In any event, it is possible that within the context of larger population studies, significant subsets of donors may be present who have low affinity IgE associated with negative SPT reactions who must be accounted for.

This study, through the use of affinity distributions, highlights the heterogeneous nature of the interactions between IgE antibodies and allergens in atopic disease. Our ability to test the low level of IgE responses and verify their high capacities by affinity determinations allows us to show that there is a significant proportion of skin test-negative/IgE-positive people among clinically atopic individuals. Specific IgE capacity might be considered before classifying a skin test-negative individual as non-atopic.

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ERRATUM

Evidence of an affinity threshold for IgE-allergen binding in the percutaneous skin test reaction

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On page 113, there was an error in the equation given in the second column. The equation should read:

$$\text{Total CAP} \equiv [\text{IgE}] \times \sum_{i=1}^n K_i * N(K)_i$$