



Variable binding affinities for allergen suggest a ‘selective competition’ among immunoglobulins in atopic and non-atopic humans

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Abstract

Atopy is a persistent, aberrant humoral response to certain classes of proteins (allergens) characterized by the presence of allergen-specific IgE. Yet, in both atopic and non-atopic individuals, allergen-specific responses involving the IgA and IgG subclasses have been observed, which evidence does not support models suggesting inherited differences in sensitivity to certain protein classes. Using the major ragweed component Amb a 1 as a model allergen, we assessed the humoral responses in three groups of unrelated donors: (A) atopic, ragweed sensitive; (B) atopic, but not ragweed sensitive; (C) non-atopic. As expected, Amb a 1-specific IgE was present in group A only. However, there were essentially no differences in the relative proportions of Amb a 1-specific IgA_{1,2} and IgG_{1–4} among the groups. We also determined the Amb a 1 binding affinities for IgG₁ and IgG₄ in the three groups, and compared these to Amb a 1-specific IgE binding affinities in group A. Group A donors' Amb a 1–IgE had extremely high affinities (10^8 to 10^{11} M^{−1}), but their Amb a 1–IgG₁ and Amb a 1–IgG₄ affinities were significantly lower (10^7 to 10^{10} M^{−1}). The average IgG₄ binding affinities in groups B and C were slightly higher than that of IgG₄ in group A, although not statistically significant. However, the IgG₁ affinity for Amb a 1 among group C, non-atopic donors was significantly elevated and comparable to the IgE affinity observed in group A, ragweed atopics. Inhibition studies with allergen-specific IgE-free serum showed that all isotypes recognized the major epitopes seen by IgE. These results suggest that there may be a “selective competition” among isotypes for allergens that is driven by the ability to produce high affinity, allergen-specific immunoglobulins.

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1. Introduction

An atopic immune response is mechanistically related to the presence of allergen-specific IgE. Although the presence of other immunoglobulin isotypes, specific to allergens, have been reported (Kemeny et al., 1989; Batard et al., 1993a,b), a consensus opinion holds that in non-atopics the immune response is weak compared to that of atopics (Aalberse, 2000). And, the possibility exists that the antibodies produced by atopics and non-atopics recognize different epitopes. The dysfunction in humoral response that is responsible for atopy may thus be related to an inherited selectivity in response to the allergen. Support for this model of atopy derives primarily from measurements of allergen-specific antibody concentrations in serum (Kemeny et al., 1989), although there has never been a satisfactory

correlation between the antibody levels and intensity of clinical symptoms (Witteman et al., 1996).

The primary physiological function of immunoglobulins is to clear antigens from the body. A measure of the effectiveness of this function is the affinity of the immunoglobulin for the antigen (Steward, 1981). After prolonged or repeated exposures to an antigen, an immunoglobulin specific for the antigen will be produced via the mechanisms of isotype chain switching and affinity maturation (Wabl and Steinberg, 1996). A ‘typical’ humoral response favors production of one of the IgG subclasses, specific for the antigen, that is of the highest affinity for the antigen. Thus, upon subsequent antigen exposure, production of lesser amounts of the immunoglobulin will be required for the same physiological effectiveness with a concomitant lowering of the energy burden for its production. Whether or not this basic scenario holds for an atopic response is not well known since immunoglobulin affinities for allergens have been difficult to assess due to the extreme heterogeneity of these

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immunoglobulins, even to highly purified allergens (Van't Hof et al., 1993).

We have recently devised a technique to determine the affinities between purified allergens and immunoglobulins (Pierson et al., 1998) that employs an affinity distribution method (Hunston, 1975). The method details the number of reactions involved between the immunoglobulin and allergen, the average affinity for each reaction and the relative proportion of each reaction to the sum of all reactions involved. In atopic subjects, using the purified allergens Amb a 1 from ragweed and Der p 1 from house dust mite, the reactions between the allergens and allergen-specific IgE are of extremely high affinity (typical values range from 10^8 to 10^{11} M⁻¹) that may be at the highest end of biologically relevant affinities (Foote and Eisen, 1995). The affinity distributions for these purified allergens demonstrate, at most, three specific reactions that, in the case of Der p 1, are associated with immunogenic epitopes (Pierson-Mullany et al., 2001).

We have begun to more clearly delineate mechanistic differences between the atopic and non-atopic humoral responses by characterizing the affinity differences between antibodies to the purified ragweed allergen Amb a 1 in ragweed sensitive and non-atopic humans. The results presented here show that both atopic and non-atopic humans can mount a vigorous response to the allergen based, primarily, upon the affinity of immunoglobulin isotypes for allergen. Atopics produce high affinity IgE toward the allergen, whereas non-atopics produce an equally vigorous response involving IgG₁.

Previous theoretical and experimental work has shown that different populations of B cells, with variable affinities for a limited resource (antigen), will compete with one another for the resource and promote the expansion and survival of one population relative to the other (Freitas et al., 1995; McLean et al., 1997). This suggests a model akin to Darwinian selection in which affinity for the resource (antigen) provides the impetus for selective competition. In this context, our results suggest that the inherited component of atopy is not solely in the propensity to produce allergen-specific IgE. Rather, the mechanistic distinction arises from the production of an immunoglobulin isotype with sufficiently high affinity that can functionally "out compete" other isotypes for the allergen.

2. Methods

2.1. Study population

A total of 90 unrelated donors were recruited (age range 7–64 years; mean donor age 29 years; 48 female, 42 male), and categorized into three groups according to atopic status (see further). Non-atopic donors were from the students and staff of the University of Minnesota Medical School and general wellness clinics of the Fairview-University Medical Center, Minneapolis, MN. Unrelated atopic donors,

with or without positive histories of ragweed sensitization, were recruited from the Asthma and Allergy Clinic, Fairview-University Medical Center. None of the atopics were receiving immunotherapy for ragweed or other allergens. All donors provided written, informed consent for participation according to the guidelines of the Institutional Review Board, University of Minnesota Medical School.

2.2. Assessment of atopic status

Each donor was given a battery of tests to assess their sensitization to common allergens. These included monitored answers to a standardized questionnaire regarding allergic symptoms histories, a brief medical exam for allergic symptoms, standardized percutaneous skin prick testing to 14 common allergens, including ragweed extract, and a blood sample for serum immunoglobulin tests (tests described later). A total of 90 donors were selected and divided into three groups (30 donors per group) according to the following criteria.

2.2.1. Group A: atopic, ragweed sensitive

Atopics with ragweed sensitization had positive responses to questionnaire items involving ragweed sensitization, were skin test positive to ragweed, and possibly other allergen extracts, (positive result: wheal area ≥ 9 mm² above negative saline control) and had detectable amounts of Amb a 1-specific IgE in the serum.

2.2.2. Group B: atopic, but not ragweed sensitive

Atopics with positive histories of sensitization to one or more allergens other than ragweed, and no detectable Amb a 1-specific IgE in the serum.

2.2.3. Group C: non-atopic

These donors had no histories of sensitization to any allergen, did not have typical allergic symptomology, were skin test negative to all allergens tested and did not have detectable Amb a 1-specific IgE in the serum.

2.3. Serum immunoglobulin assays

Whole blood was obtained by sterile venipuncture. Serum was separated and stored frozen (-80°C) until assayed. As described here, quantitative or semi-quantitative assays for immunoglobulin concentrations were done based upon availability of commercial standards. Allergen-immunoglobulin binding affinity distributions were done using an assay of our devise (Pierson et al., 1998).

2.3.1. Total IgE

Quantitative serum total IgE determinations were made using the ACCESS Immunoassay System[®] developed by Sanofi Diagnostics Pasteur Inc. (Division of Beckman Coulter, Chaska, MN). Calibration was based upon commercial standards (Beckman Coulter).

2.3.2. Amb a 1-specific IgE and IgG₁

Quantitative assays for allergen-specific IgE and IgG₁ were done using the purified allergen Amb a 1, a major allergen extracted from short ragweed (*Ambrosia artemisiifolia*). Serum concentrations of Amb a 1-specific IgE and IgG₁ were measured by an adaptation of the ACCESS Immunoassay System[®] specific IgE immunoassay (Pierson et al., 1998; Allauzen et al., 1999). ACCESS Immunoassay System[®] is an automated solid phase immunoassay system that utilizes chemiluminescence-based detection (Patterson et al., 1994). Briefly, purified and biotinylated allergen is first attached to an anti-biotin monoclonal antibody (mAb) coated magnetic particles (solid phase support; Dynal Inc., Lake Success, NY). Amb a 1 (M_w 37,800) was purified and biotinylated as described (Pierson et al., 1998). Allergen-specific IgE or IgG₁ from a serum sample is then “captured” by the attached mAb–allergen complex. The amount of captured IgE or IgG₁ is determined using alkaline phosphatase-conjugated equine anti-human IgE or IgG₁ antiserum (Beckman Coulter). Using this step ensures that only IgE or IgG₁ is detected and not other allergen-specific antibody isotypes that may also have been bound by the mAb–allergen complex. A chemiluminescent substrate (Lumi-phos 530[®], Lumigen Inc., Southfield, MI) is added to produce a chemiluminescent signal that is proportional to the amount of captured IgE or IgG₁. The amount of analyte in the sample is determined by a stored, multi-point calibration curve using standards provided by the manufacturer. The lowest limit of detection using this method is 0.02 IU ml⁻¹ (1 IU = 2.4 ng).

Note: The ACCESS Immunoassay System[®] method for determinations of allergen-specific IgE and IgG₁ is not currently commercially available in the US.

2.3.3. Amb a 1-specific immunoglobulin profiles

Due to the unavailability of suitable standards, we devised a semi-quantitative screen for relative concentrations of Amb a 1-specific IgA_{1,2} and IgG_{1–4}. We used an ‘inverse sandwich’ ELISA procedure, in order to exclude competitive reactions between the allergen and multiple allergen-specific isotypes. A 96-well opaque, white polystyrene microtest plate (Costar 3912, Corning) was coated with an optimized dilution of an anti-human IgX ($X = \text{IgA}_{1,2}$ or $\text{IgG}_{1–4}$) mAb. After washing and coating with a blocking protein (purified casein I-Block[™], Tropic Inc.), 100 μl of diluted serum was added. To account for non-specific binding from proteins in the serum samples, parallel determinations were also made by adding diluted sample to wells that did not contain anti-IgX mAbs. After washing, 100 μl of an optimized dilution of purified and biotinylated Amb a 1 (BT-Amb a 1) was added. Details for Amb a 1 purification and biotinylation are described elsewhere (Pierson et al., 1998).

Note: In this step, the optimized dilution represented a “large excess” of allergen. Finally, streptavidin-conjugated alkaline phosphatase (Sigma) was added. A chemiluminescent substrate (Lumi-Phos, Beckman Coulter) was added

to each well, and signal detection was made with a model TR717 Microplate Luminometer (Tropix Inc.). The background (non-specific binding) signal was subtracted from the measured signal to obtain the test result.

Although the method of detection for the different isotypes used the same purified allergen and reagents after the capture of the immunoglobulin isotype of interest, commercial standards are only available for IgE and IgG₁ determinations. The concentrations of other isotypes can be estimated, however, by assuming that a similar signal response of relative luminometer units (RLUs) per bound antibody molecule is achieved for each isotype. The concentrations of isotypes other than IgG₁ in Table 2 are thus nominal, and may be subject to small systematic errors. For purposes of this investigation, which centers upon patterns of responses, the numbers are adequate. All test results were within the range of 1 to 1000 ng ml⁻¹ comparable to those reported by other investigators for these allergen-specific isotypes.

2.3.4. Binding affinity distribution functions

Our basic experimental method provides information regarding allergen–antibody (Ag–Ab) binding reactions in vitro (Pierson et al., 1998). Briefly, magnetic particles (Dynal) used in the ACCESS Immunoassay System[®] system are coated with a mouse anti-human, -IgE, -IgG₁ or -IgG₄ mAb. To this is added a serum sample known to contain allergen-specific IgE, IgG₁ or IgG₄, which is “captured” by the particle-bound mAb. BT-Amb a 1 is added to bind only to the specific IgE, IgG₁ or IgG₄ of interest, and detection is achieved by the addition of streptavidin-conjugated alkaline phosphatase (Sigma) and a chemiluminescent substrate to generate a light signal.

Using a range of purified Ag (BT-Amb a 1) concentrations, a binding isotherm is generated: r versus $\log_{10} [\text{Ag}]$, where r = fraction of antibody sites bound at a given $[\text{Ag}]$ 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, IgG₁ or IgG₄. The background was subtracted from the test signal prior to determining the fraction specifically bound, r .

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 (Pierson et al., 1998) using an approach first suggested by Hunston (1975) and extended by 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 an allergen-specific immunoglobulin and the allergen (n), the equilibrium binding affinity constant for each reaction (K_A) and the relative proportion of each reaction to the sum of all reactions involved ($p[K]$). The Data were analyzed

using Mathematica[®] software (Wolfram Research Inc., Champaign, IL). A pamphlet detailing the methods used for determining affinity distribution functions is available, at no cost, to interested investigators.

Note: 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. Multiple peaks represent responses by different antibody clones to these epitopes (Pierson-Mullany et al., 2001). However, its discriminatory utility is limited 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. The application of this method is limited to studies using purified single allergens.

2.3.5. Inhibition tests

We have previously established that the affinity distributions generated by our method represent reactions between allergen-specific immunoglobulins and major allergenic epitopes (Pierson-Mullany et al., 2001). In order to determine if allergen-specific IgGs from non-atopics recognized the same major epitopes as those seen by allergen-specific IgE from atopics, we modified inhibition tests previously used by our group (Kim et al., 1996, 1997).

We randomly selected serum samples from group A atopics known to contain Amb a 1-specific IgE and determined the affinity distributions described earlier. In parallel with these, we also made affinity distribution assays using varying concentrations of BT-Amb a 1 serially diluted with buffer that also contained diluted serum from group C non-atopics (final serum dilutions of 1:10, 1:100 and 1:1000 for each BT-Amb a 1 dilution). Group C donors' sera did not contain Amb a 1-IgE. Comparisons of the results were made to determine whether or not competitive inhibition had occurred.

Although we determined affinity distributions, assuming heterogeneous reaction schemes, our analytic question here

was to determine whether or not Amb a 1-IgG from non-atopic donors recognized the same 'major epitopes' as those recognized by Amb a 1-IgE from atopic donors. Thus, we are interested in average properties of the reaction mixture.

The average association affinity constant for the reactions is:

$$\langle K_A \rangle = \frac{[Ag-Ab]}{[Ag][Ab]}$$

The total allergen concentration is: $[Ag]_{total} = [Ag] + [Ag-Ab]$. After elimination, we rearrange to:

$$\frac{1}{[Ag-Ab]} = \left\{ \frac{1}{(\langle K_A \rangle [Ag]_{total})} \right\} \left\{ \frac{1}{[Ab]} \right\} + \left\{ \frac{1}{[Ag]_{total}} \right\}. \quad (1)$$

The inhibition experiments are designed only to show that the epitopes recognized by IgE are also recognized by other isotypes. Because this allows us to use unseparated, but IgE-free serum as an inhibitor source, we can use RLUs rather than concentrations. Eq. (1) is a linear equation for $1/[Ag-Ab] \propto 1/RLU$ which is proportional to $1/[Ab]$. We expect a linear response for the average reaction properties of the system.

We next introduce the constant for inhibition $\langle K_{In} \rangle = [In-Ag]/[In][Ag]$:

$$\frac{1}{[Ag-Ab]} = \left\{ \frac{(1 + \langle K_{In} \rangle [In])}{(\langle K_A \rangle [Ag]_{total})} \right\} \left\{ \frac{1}{[Ab]} \right\} + \left\{ \frac{1}{[Ag]_{total}} \right\}. \quad (2)$$

For experiments with and without inhibitor, the presence of a strictly competitive inhibitor will lead to two lines (Eqs. (1) and (2)) with different slopes but a common intercept on the Y-axis (Steward and Steensgard, 1983).

2.4. Monoclonal antibodies (mAbs)

All mAbs used (except anti-biotin) were anti-human

Procedure	mAb	Species	Clone/code	Supplier ^a
(A) Amb a 1-IgE or Amb a 1-IgG ₁ (ACCESS Immunoassay System [®])	α-Biotin	Mouse	Bio-8	Biodesign
	α-IgE-ALP	Goat	A80-108AP-3	Immunology Consultants
	α-IgG ₁ -ALP	Mouse	HP-6069	Immunology Consultants
(B) Ig profiles (ELISA)	α-IgA ₁	Sheep	AU087	Binding Site
	α-IgA ₂	Sheep	AU088	Binding Site
	α-IgG ₁	Mouse	HP-6001	Sigma
	α-IgG ₂	Mouse	HP-6002	Sigma
	α-IgG ₃	Mouse	HP-6050	Sigma
	α-IgG ₄	Mouse	HP-6025	Sigma
(C) Affinity distributions (ACCESS Immunoassay System [®])	α-IgE	Mouse	GE-1	Sigma
	α-IgG ₁	Mouse	8C/6-39	Sigma
	α-IgG ₄	Mouse	HP-6025	Sigma

^a Binding Site, San Diego, CA; Biodesign, Kennebuck, ME; Immunology Consultants, Sherwood, OR; Sigma, St. Louis, MO.

3. Results

3.1. Basic comparisons between atopic and non-atopic subjects

Comparisons were made between the three subject groups for general atopic history and status: (A) atopic, ragweed sensitive; (B) atopic, not ragweed sensitive; (C) non-atopic. Table 1 gives the basic comparisons between the atopic and non-atopic subject groups with regard to skin test results and relevant serum immunoglobulin concentrations. As expected, the mean total IgE concentration among non-atopics was lower than that of the two atopic groups, although there was considerable overlap of these values between groups. Roughly 15–20% of the non-atopics had ‘elevated’ total IgE (>100 IU ml $^{-1}$) in agreement with similar assessments made previously (Marsh et al., 1974). The group B atopics and group C non-atopics did not have detectable Amb a 1-specific IgE, however.

All subjects in the three groups had detectable amounts of Amb a 1-specific IgG $_1$. However, although the mean IgG $_1$ value was lower among non-atopics, there was no clear demarcation between the subject groups based upon the range of values observed. In addition, there is no known clinical outcomes correlate with IgG $_1$, or other non-IgE isotypes.

In agreement with previous work, the atopic and non-atopic groups may be distinguished based upon standard clinical assessments (allergic symptoms history, positive skin tests, allergen-specific IgE). But, these results provide no useful information regarding the vigor of the humoral responses mounted against the allergen by atopics and non-atopics, and whether or not the responses are mechanistically different.

3.2. Amb a 1-specific immunoglobulin profiles

Sera from the three groups were screened for the relative amounts of Amb a 1-specific IgA $_{1,2}$ and IgG $_{1-4}$. Due to a lack of suitable standards for all immunoglobulin isotypes, we used nominal results for all isotypes based upon IgG $_1$ calibrations in the same analytical system. There was a positive correlation between the quantitative ACCESS Immunoassay System[®] method and the semi-quantitative ‘reverse sandwich’ ELISA screen for Amb a 1–IgG $_1$ values ($r = 0.74$; $P < 0.05$), although estimates of absolute values using the semi-quantitative method were slightly higher. However, our intent was to compare humoral response “patterns” between three groups who differed in atopic status and specific sensitivity to ragweed allergen (Amb a 1), and not to establish normative, quantitative values for the allergen-specific isotypes. Table 2 summarizes the comparisons among the three donor groups.

For each isotype, analysis of variance (ANOVA) was used to compare the results between groups. Save for some minor statistical differences, there were essentially no differences in the relative amounts of the IgG subclasses among the three donor groups. However, there were pronounced differences in the relative amounts of the IgA subclasses found in group A compared to groups B and C.

We also determined the distributions of proportionate amounts of each isotype in each donor group. This was done in order to determine if, within the bounds of comparable response magnitudes, there might be significant differences in the ‘fractional compositions’ of the responses between groups. For each donor, we summed the amounts of all Amb a 1-specific immunoglobulins and calculated the relative proportion for each isotype. The mean proportions, reflecting the fractional make-up of the responses,

Table 1
Assessments of atopic status

	Group A ^a	Group B	Group C
Clinical allergy Hx			
Ragweed	All positive	All negative	All negative
Other allergen	Varied	All positive	All negative
Skin test results			
Ragweed	All positive	All negative	All negative
Other allergen	Varied	Positive to 1 or more	All negative
Total IgE (ng ml $^{-1}$) ^b			
Mean	1260	885	305
S.E.M.	108.6	54.6	44.8
Amb a 1–IgE (ng ml $^{-1}$) ^b			
Mean	27.6	None detected	None detected
Range	0.05–317		
Amb a 1–IgG $_1$ (ng ml $^{-1}$) ^b			
Mean	88.6	20.0	19.5
Range	0.1–1171	0.2–410	0.05–320

^a $N = 30$ per group; group A: atopic, ragweed sensitive; group B: atopic, not ragweed sensitive; group C: non-atopic.

^b Quantitative results based upon commercial standards (Beckman Coulter).

Table 2
Estimated Amb a 1-specific Ig concentrations

Isotype		Donor groups			Comparisons	
		Group A	Group B	Group C	F-ratio	P-value
IgG ₁	Mean	118.71 ^a	63.53	69.01	0.71 ^b	0.496
	S.E.M.	55.95	24.30	17.00		
IgG ₂	Mean	11.61	14.74	18.17	3.03	0.051
	S.E.M.	1.27	1.48	2.63		
IgG ₃	Mean	10.38	12.19	19.20	6.78	0.001
	S.E.M.	1.48	1.35	2.38		
IgG ₄	Mean	12.86	13.28	19.34	2.84	0.06
	S.E.M.	1.64	1.39	2.96		
IgA ₁	Mean	108.67	59.65	73.34	5.80	0.004
	S.E.M.	14.67	4.58	10.21		
IgA ₂	Mean	120.15	60.95	75.55	7.28	0.001
	S.E.M.	15.01	6.65	11.55		

Value for IgG₁ in group A based upon calibration standard. All other values are relative to this standard and, thus, are nominal.

^a Values expressed as units of 'bound alkaline phosphatase (ALP)' (ng ml⁻¹).

^b ANOVA results between groups per isotype.

for each donor group are shown in the histogram of Fig. 1. Based upon these determinations, there were no significant differences between the three groups by ANOVA for each isotype.

By simple linear regression, for each donor group there was a comparable inverse relationship between the proportions of the IgA subclasses compared to the IgG subclasses (group A, $r = -0.74$, $P < 0.05$; group B, $r = -0.96$, $P < 0.01$; group C, $r = -0.79$, $P < 0.05$; all groups combined, $r = -0.78$, $P < 0.05$). On an individual basis, higher proportionate amounts of specific IgAs correspond to lower proportionate amounts of specific IgGs. Thus, there would appear to be a similar compensatory mechanism in all three

groups regarding the relative amounts of IgA or IgG that will be produced against allergen.

3.3. Comparisons of affinity distributions

Fig. 2 shows a typical affinity distribution for the reactions between an allergen-specific immunoglobulin and the purified allergen Amb a 1. The three key features in this figure are the number of reactions involved (n), the most likely affinity constant (K) for each reaction (highest point of each 'affinity peak') and the relative proportion (p) of each reaction (height of each peak relative to the other peaks). As demonstrated by Thakur et al. (1980), experimental error of 10% or greater will significantly influence the accuracy of these parameter estimations. Using the automated ACCESS Immunoassay System[®] system, experimental error is typically $\leq 4\%$ (Pierson et al., 1998). Based upon multiple determinations, individual variation is also within this range, and does not significantly contribute to the error for comparisons between donor groups.

Table 3 summarizes the key findings with regard to the average affinity values for the immunodominant peaks detected among group A atopics (IgE, IgG₁ and IgG₄) and groups B and C (IgG₁ and IgG₄ only). As previously shown (Pierson et al., 1998; Pierson-Mullany et al., 2001), atopics mount an extremely high affinity IgE response against allergens. Typical values for affinity constants are in the range 10^8 to 10^{11} M⁻¹. As shown here, they also produce specific IgG₁ and IgG₄, but the vigor of IgG₁ and IgG₄ responses are 'shifted' to lower average affinities (values in the range 10^7 to 10^{10} M⁻¹). Interestingly, all three groups produce IgG₄ with comparable affinities for the allergen.

As a procedural note, the affinity distribution method based upon chemiluminescent detection of allergen–antibody interactions is valid in the range of affinities of about $10^{6.5}$ to $10^{11.5}$ M⁻¹. The 'high affinity limit', corresponding to the

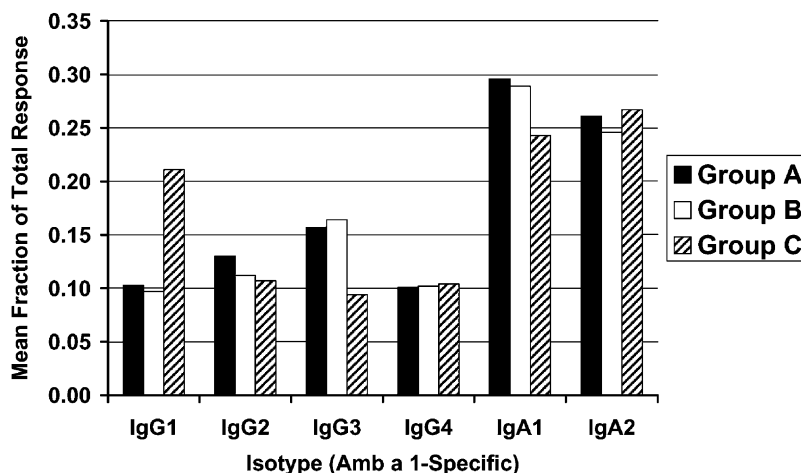


Fig. 1. Amb a 1-specific immunoglobulin isotype profiles. Proportions were found for each donor by summing all values for Amb a 1-specific immunoglobulins (except IgE), dividing the result for each immunoglobulin by this sum and averaging the values for each donor group. Values on Y-axis are dimensionless (group A: atopic, ragweed sensitive; group B: atopic, not ragweed sensitive; group C: non-atopic).

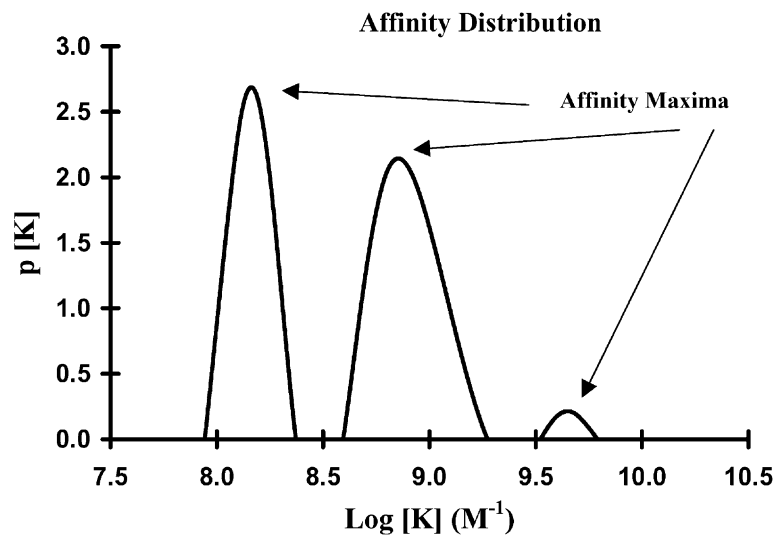


Fig. 2. A representative affinity distribution. The three primary features are the number of reactions involved between Amb a 1 allergen and an antibody (IgE, IgG₁ or IgG₄), the relative contribution, $p[K]$, of each reaction reflected by the height of the affinity maxima (indicated by arrows) and the affinity constant associated with each affinity maximum.

lowest added allergen concentrations is at the lowest limit of detection for the luminometer. The 'low affinity limit', corresponding to the highest concentrations of added allergen is prone to serious background error due to non-specific binding effects by the biotinylated allergen. We mention this as we also attempted to determine affinities for the Amb a 1–IgA subclasses. A random selection of 30 serum samples gave no determinable affinity constants, suggesting that the specific IgAs are of extremely low binding affinity, or at least appreciably lower than IgE or IgG subclasses.

In contrast, group C non-atopics mount a vigorous immunoglobulin, high affinity response to allergen that uniquely involves IgG₁. According to the results in Table 3,

the high affinity IgG₁ response among group C non-atopics is comparable to the high affinity IgE response of group A atopics. However, comparisons between groups based upon concentrations alone or affinities alone do not reflect the functional capabilities of the antibodies. To more clearly show this comparison, we introduce a parameter reflective of the overall binding capacity of the immunoglobulins in these subject groups.

The binding capacity is defined as the mathematical product of the immunoglobulin concentration and the binding affinity: $CAP \equiv [Ig] \times \sum_{i=1}^n p_i \times K_i$, where $[Ig]$ is the concentration of the particular immunoglobulin, n the number of specific reactions, p_i the relative proportion of the i th

Table 3
Mean affinity values for Amb a 1–Ig binding

Isotype	Group A		Group B		Group C	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
IgE						
log $[K_1]^a$	8.49	0.102	Not defined		Not defined	
log $[K_2]$	8.99	0.126	Not defined		Not defined	
log $[K_3]$	9.66	0.111	Not defined		Not defined	
IgG ₁						
log $[K_1]$	7.04	0.130	7.66	0.135	9.17	0.157
log $[K_2]$	7.82	0.153	8.84	0.126	9.99	0.120
log $[K_3]$	8.37	0.099	9.64	0.128	10.14	0.108
IgG ₄						
log $[K_1]$	7.64	0.131	7.98	0.124	8.06	0.133
log $[K_2]$	8.49	0.148	8.84	0.128	8.84	0.108
log $[K_3]$	9.24	0.133	9.64	0.131	9.64	0.119

Note: In group A, mean IgE affinities are higher than mean IgG₁ affinities. Also, mean IgG₁ affinities from group C are higher than mean IgG₁ affinities from group A. However, there is no apparent difference in mean affinities by comparing group A IgE and group C IgG₁.

^a Base 10 logarithms of affinity maxima (see Fig. 2); units: 1 mol^{-1} .

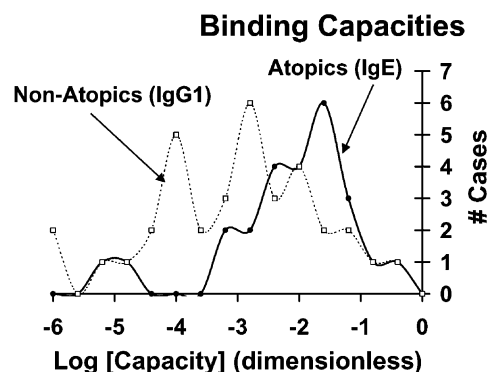


Fig. 3. Comparison of IgG₁ (non-atopics) and IgE (atopics) binding capacities for Amb a 1. As described in Section 3, the binding capacity is the mathematical product of the antibody concentration (units of mol l⁻¹) and the antibody binding affinity (units of l mol⁻¹). The number of individuals in groups A and C are plotted as a function of their response binding capacity.

reaction and K_i is the affinity constant of the i th reaction. With [Ig] given in units of M l⁻¹ and K given in units of l M⁻¹, the parameter capacity is dimensionless.

Fig. 3 shows the binding capacities of IgG₁ among group C non-atopics and the binding capacities of IgE among group A atopics. Among non-atopics, the mean value of the log [capacity] for IgG₁ was -3.27 (S.E.M. = 0.222), while among atopics the mean value of the log [capacity] for IgE was -1.90 (S.E.M. = 0.151). There was no significant difference between these mean values by t -test ($P < 0.001$). With regard to this parameter, there is virtually no difference between the two groups. Although not shown, as reflected by the results in Tables 1 and 3, the IgG₁ capacities among group A atopics are appreciably lower than those depicted in Fig. 3. Thus, it would appear that atopics have an attenuated IgG₁ response to allergen that has been supplanted by a vigorous IgE response.

3.4. Inhibition tests

Typical affinity distribution results (Fig. 2) represent reactions of immunoglobulins with major allergenic epitopes (Pierson-Mullany et al., 2001). We did inhibition tests in order to determine if the Amb a 1-IgGs among group C non-atopics recognized the same major epitopes seen by Amb a 1-IgE among group A atopics. As described in Section 2, we randomly selected serum samples from group A donors, with detectable amounts of Amb a 1-IgE, and determined the average affinity properties with and without serum from group C donors (no detectable Amb a 1-IgE).

For purposes of this analysis, we were only interested in average affinity properties of the reaction mixtures. The affinity distributions are derived from a mathematical transformation of an allergen–antibody binding isotherm (Hunston, 1975; Thakur et al., 1980). Rather than using this transformation, we determined the average properties without inhibitor (Eq. (1) in Section 2) or with inhibitor (Eq. (2) in Section 2) using the results directly obtained from the binding isotherms. Inhibitor was in the form of dilutions of serum without detectable Amb a 1-IgE (dilutions of 1:10, 1:100 and 1:1000 were used).

Fig. 4 shows a representative example of the results (one of five experiments). Plots of $1/\text{RLU}$ versus $1/\text{concentration}$ gave straight lines with and without the inhibiting serum dilutions. (RLU derived from subtracting a background signal from a test signal. Concentration = concentration of added biotinylated allergen in units of ng ml⁻¹.) This representation does not imply that the detailed, heterogeneous reactions between the purified allergen, with multiple epitopes, and antibodies can be parameterized by a single, average affinity constant. However, if the individual reactions involving all of the 'major epitopes' were inhibited, then this would be reflected in an apparent inhibition of the average affinity.

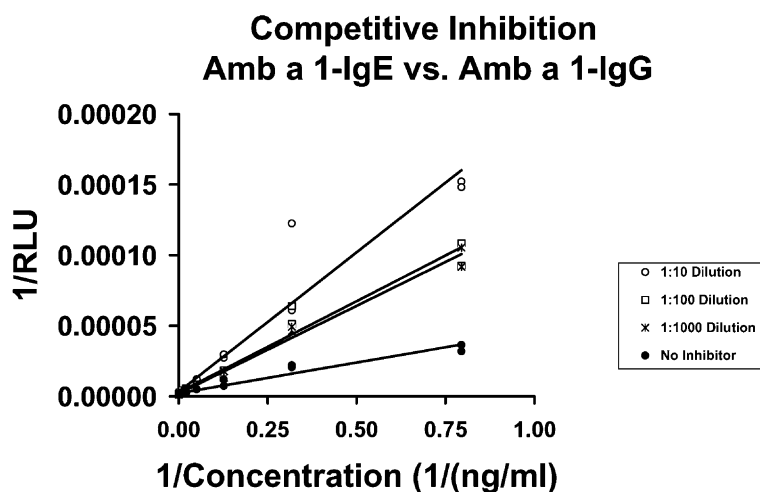


Fig. 4. Relative binding inhibition of Amb a 1-IgE by Amb a 1-IgG. Plots of the inverses of $1/\text{RLU}$ vs. $1/\text{concentration}$ (concentration = concentration of added allergen in ng ml⁻¹) without added inhibiting serum and with added dilutions of inhibiting serum (dilutions of 1:1000, 1:100 and 1:10). Representative results of five experiments.

For the straight lines in Fig. 4, the slope with no added inhibitor was 4.34×10^{-5} ($R^2 = 0.95$; $P < 0.001$), where the slope is the inverse of the average affinity constant, $\langle K \rangle$ (arbitrary units of 1/concentration). The slopes with added inhibiting serum were: 1:1000 dilution, slope = 1.24×10^{-4} ($R^2 = 0.99$; $P < 0.001$); 1:100 dilution, slope = 1.30×10^{-4} ($R^2 = 0.97$; $P < 0.001$); 1:10 dilution, slope = 1.98×10^{-4} ($R^2 = 0.93$; $P < 0.001$).

All results gave good statistical significance for the assumption of linearity. Also, comparing the slope without added inhibitor serum to the slopes with different serum dilutions containing suspected inhibitor were statistically different, indicating that there was a competition for allergen between Amb a 1-IgE from an atopic donor and Amb a 1-IgG from a non-atopic donor.

More importantly, however, all lines converged to the same point on the Y-axis. Without inhibitor, intercept = 2.12×10^{-6} (S.E. = 5.1×10^{-7}); 1:1000 dilution, intercept = 1.82×10^{-6} (S.E. = 5.0×10^{-7}); 1:100 dilution, intercept = 2.62×10^{-6} (S.E. = 1.3×10^{-6}); 1:10 dilution, intercept = 3.24×10^{-6} (S.E. = 2.87×10^{-7}). Statistical comparisons of these results (intercept without inhibitor to intercepts with inhibitor) were not significantly different by *t*-test ($P = 0.05$). It would appear that there is a competitive inhibition between the Amb a 1-IgGs from non-atopic donors and the Amb a 1-IgE from atopic donors for the major epitopes on the purified allergen.

4. Discussion

The major finding of this study is that both atopic (ragweed sensitive) and non-atopic humans can mount a vigorous, high binding affinity immunoglobulin response to allergen. Among non-atopics the response primarily centers around IgG₁, whereas among atopics the focus is upon IgE. Thus, a mechanistic distinction can be made: non-atopics mount a high affinity IgG₁ response to allergen while atopics mount a high affinity IgE response. This observation is in contrast to previous studies, based solely upon concentrations, in which it has been assumed that non-atopics mount limited, if any, response to allergen.

The results of inhibition studies (representative example in Fig. 4) indicate that the IgGs from non-atopics recognize the same major epitopes on the purified allergen as the Amb a 1-IgE from atopics. We cannot rule out the possibility that other minor epitopes may be involved, and that the 'recognition patterns' for these are different between atopics and non-atopics (Batard et al., 1993a,b). However, if these minor epitopes are involved, either their concentrations are so low or their binding affinities are so low as to make them undetectable in our experimental set-up. In any event, based upon the average properties of the most significant epitopes recognized, it would appear that the immunoglobulins produced against allergens by atopics and non-atopics are primarily directed against the same moieties.

The average amount of allergen-specific IgG₁ produced by non-atopics is appreciably lower than the IgG₁ from atopics (Table 1). Based upon immunoglobulin concentration alone, it might be suggested that the humoral immune response among non-atopics to an allergen challenge is significantly lowered compared to the response(s) of atopics. However, immunoglobulin concentration only reflects the number of molecules available for a response (response potential), while the combination of the concentration with its associated driving force (affinity) provides the overall functional capability (response capacity). In this regard, the binding capacity of IgG₁ in non-atopics is comparable to the binding capacity of IgE in atopics (Fig. 3).

Interestingly, it would appear that the IgG₁ response of atopics is diminished or attenuated, reflected in the lowered binding affinity (Table 3). It may be that the resultant, diminished binding capacity is insufficient to clear allergen from the system and necessitates an immunoglobulin isotype switch in concert with affinity maturation to higher affinity IgE production (Wabl and Steinberg, 1996).

Alternatively, atopics may be genetically predisposed to obligately produce IgE to certain stimuli (allergens). We previously found that 85% of the immunoglobulin response to ragweed pollen among sensitized adults involved IgE and IgG₄ and/or IgG₂, with the remaining 15% of the response involving IgG₁ and/or IgG₃ (Allauren et al., 1999). In conjunction with a proposed evolutionary duplication of the γ - ϵ - α genes involved with immunoglobulin heavy chain (IgH) production (Flanagan and Rabbits, 1982), these results suggested that there may be two locus control regions (LCRs) that govern IgH; an LCR-1 would govern IgG₁/IgG₃/IgA₁ while an LCR-2 would govern IgG₂/IgG₄/IgE/IgA₂. Studies by other investigators have also suggested this possibility (Batard et al., 1993a,b).

As shown in Table 2 and Fig. 2, at least for the model allergen Amb a 1, there does not appear to be much difference in the relative proportions of allergen-specific immunoglobulins produced by atopics (ragweed sensitive or not) and non-atopics. In fact, save for Amb a 1-specific IgE in group A donors, the responses to Amb a 1 are virtually identical. Thus, if there is an LCR-2 governing a region of IgH genes, it would appear that it is a common feature rather than being unique to sensitized humans. Again, based upon immunoglobulin concentration alone, it is difficult to define a mechanistic distinction between an atopic and a non-atopic humoral response.

It is most likely that a combination of adaptive immune activities (isotype switch with affinity maturation and genetic predisposition) are involved with the dynamics of the atopic humoral response. Clearly, both atopics and non-atopics are able to produce IgE (reflected in the total IgE values; Table 1), although most, if not all, of the IgE in non-atopics is likely to be non-cognate. In a prospective study involving children "at risk" and "not at risk" for atopy, Hattevig et al. (1993) showed that in the earliest years of life (up to 2 years) both groups of children could produce allergen-specific IgE.

The difference between the groups was that the production of cognate IgE among children “not at risk” was transient, while cognate IgE production in the “at risk” group was persistent.

The propensity to develop atopy is not simply reflected in the particular cognate immunoglobulin isotype that is produced. Rather, it would appear to be a unique combination of both the kind (isotype) and vigor (affinity) of the immunoglobulins that dictate the outcome. Membrane bound immunoglobulin is the principal component of the B cell receptor (BCR), and is instrumental in determining the isotype and amount of immunoglobulin that a given B or plasma cell will produce (Alés-Martinez et al., 1991). The ‘competition’ between cells that produce different immunoglobulin isotypes will be dictated by the ‘selective advantage’ of their BCRs.

Theoretical and experimental evidence that ‘competition’ among B cell subsets can occur has been provided in a mouse model of B cell homeostasis during cellular reconstitution after bone marrow irradiation (Freitas et al., 1995; McLean et al., 1997). Using a model from the ecology literature for inter-species competition, these investigators have shown that the proportionate amounts of each B cell population is related to the variable affinity that each population has for interacting with a limiting resource, wherein the resource is required for the survival and expansion of each cell population. The population with the ‘selective competitive edge’ (higher affinity for the resource) has a better probability of survival and expansion. Also, both populations can co-exist in a homeostatic balance, although the proportionate amount of one population is higher.

If we speculate that both atopics and non-atopics have subsets of B cells that may produce allergen-specific IgG or IgE, then the subset most likely to dominate will be the one with the most efficient BCR signaling capacity. The immunoglobulin isotype component of the BCR with the highest affinity for antigen/allergen will be more likely to have a ‘competitive edge’. A higher affinity is mechanistically linked to a longer residence time for a ligated BCR and an increased probability ‘selective advantage’ for immunoglobulin production (Batista and Neuberger, 1998). This mechanism would explain how the B cell clones that produce low affinity IgG₁ among atopics, in the presence of repeated allergen encounter, would lose in competition to high affinity IgE clones.

What may account for this ‘selective advantage’ is not known, although the results given here call into question the proposal that non-atopics have developed a tolerance for allergens (i.e. they are ostensibly unresponsive to allergen challenges). If non-atopics mount an IgG response to allergens of extremely high affinity, they will not require large amounts of allergen-specific IgG in order to have an efficient allergen clearance capacity. Quantities of ng ml⁻¹ or lower may be adequate, although these minute amounts may not be detected by many assay systems currently in use.

The ‘selective advantage’ may be a combination of both genetic and environmental factors involving such things as favored IgH gene activation, transient stress upon the immune system during initial allergen exposure (e.g. viral infections), apoptosis of B cells with low residency BCRs and the like. In any event, it is clear that the factors that favor high affinity immunoglobulin production are intimately involved with the likelihood that an individual will or will not develop atopy.

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