



THE AFFINITY OF ALLERGEN SPECIFIC IgE AND THE COMPETITION BETWEEN IgE AND IgG FOR THE ALLERGEN IN *Amb a V* SENSITIVE INDIVIDUALS

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Abstract—We have calibrated a solid state RAST assay with affinity purified allergen-specific IgE. We then utilized the calibrated assay to measure the average affinity of individual IgE-containing sera in terms of the average association constant $\langle K \rangle$ for purified allergen *Amb a V*. The binding data yielded linear reciprocal plots indicating that the range of affinities of the responding clones was narrow. The range of the average association constant for the IgE-*Amb a V* complex was $0.9\text{--}26 \times 10^{10} \text{ M}^{-1}$. The average affinity of the corresponding IgG response in the same individual, estimated by inhibition studies of IgE binding, was 10^7 M^{-1} in one case and lower than 10^6 M^{-1} in all the other cases. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The probability of developing sensitivity to low levels of allergen and acquiring high levels of allergen-specific IgE is enhanced by a family history of sensitivity. We and other laboratories have investigated the immune response as an inheritable trait (Marsh, 1990; Blumenthal, 1993). The affinity of the allergen-specific antibody has long been considered as a possible variable linked to sensitivity, but the correlation between the average affinity of the antibodies and sensitivity to allergen has not been established.

The polyclonal, multiepitope nature of the humoral response makes the definition of average or maximal affinity difficult, except for mAbs. As a result, investigations of the natural antibody response rely on avidity, a much less rigorous concept (van Dam *et al.*, 1989). However, it is the affinity that remains the expression for a matching process, the outcome of which can well be influenced by inherited factors. The present consensus model of humoral response, ultimately leading to a matching of the epitope structure and the surface of the CDR regions of the antibody, involves at least one possible

restriction point associated with the choice of appropriate sequences from the inherited V(h) library (Alt *et al.*, 1987). Alternatively the development of high affinity clones can instead be associated with the somatic mutation driven maturation of the response (Berek and Zeigner, 1993). Such arguments, however, are mostly based on data from studies of inbred mice and need to be proven to function in the human system.

Human IgE-dependent allergy represents a case where the low level of aeroallergens demands high affinity of antibodies. We can test our basic assumption about the possible inherited advantage of assembling high affinity IgE antibodies by showing in individuals the existence of a wide spread of affinities despite the maturation process that has been going on for years of seasonal exposure to ragweed, our allergen of choice. A correlation of the corresponding IgG response with the achieved IgE affinities would suggest that the maturation process is a major force to shape the ultimate response.

A group of *Amb a V* ragweed allergic individuals was chosen for this study. This purified allergen (MW 4500) was chosen because of its low molecular weight, limiting the number of epitopes recognized simultaneously; and because of the well-defined genetic link between response to *Amb a V* and the presence of the DR-2 allele of the HLA system (Marsh, 1990; Blumenthal, 1993).

This investigation demonstrated that the binding of allergen and IgE antibody in all cases revealed a surprisingly linear behavior when the binding data were plotted as the classical inverse function (Steward and Steensgard, 1983), suggesting a narrow distribution, not

Abbreviations: *Amb a V*, The fifth peptide of allergenic activity isolated from *Ambrosia artemisiifolia* (short ragweed); CDR, complementarity determining region in immunoglobulin sequence; TCR, T-cell receptor; V(h), variable region of the immunoglobulin heavy chain; VDJ, variable-diversity-junction sequence of immunoglobulins; RAST, radioallergoabsorbent test.

of clones but of affinities (van't Hof *et al.*, 1993). As a result, average affinities for the IgE response could be determined with confidence and some estimates of the properties of the heterogenous IgG response through inhibition studies obtained.

MATERIALS AND METHODS

The interaction of divalent antibodies with multi-epitope antigens leads to an undefined stoichiometry in the complexes formed. Except in the extremes of antibody or antigen excess, no dominating species emerges (Steward and Steensgard, 1983). A popular and useful way to avoid stoichiometric complications is to restrict the reactants physically by attaching one of them to a polymer matrix. However, a solid state assay such as the ubiquitous ELISA is difficult to use in affinity determinations due to ambiguous definition of the activity coefficients of the reactants and the results obtained are often described in terms of "activities" in a relative sense (de Savigny and Voller, 1980). The problem of determining effective concentrations in solid state has resulted in the use of indirect ELISA suitable for investigation of monoclonal antibodies (Friguet *et al.*, 1985). However, provided calibration in molar terms against international protein standards can be achieved, any kind of solid state binding assay can be used in affinity determinations.

In our investigation we used purified allergen attached to a plastic surface as solid state. The actual effective concentration of the epitopes available for binding was determined with allergen-specific IgE by competition with known amounts of free allergen in solution. In addition, the secondary anti-IgE antibody was calibrated using allergen specific isolated IgE, the concentration of which was determined by standard total IgE assay calibrated against World Health Organization standards.

In affinity determinations we assumed that the abnormal activity coefficient for the matrix-bound forms, allergen and allergen antibody complex, are roughly the same and consequently eliminated from calculations. An estimate of the effective allergen concentration gives a factor containing both the number of blocked binding sites and the change of affinity at the available sites. The probable systematic error comes from a possible difference in the activity coefficients for the two matrix bound forms, a much smaller quantity, the limit of which can thus be estimated.

Isolation and purity criteria for Amb a V

Amb a V was isolated from non-defatted short ragweed pollen as previously described (Roebber *et al.*, 1975). The activity of the preparations was monitored by RAST assays using *Amb a V*-specific rabbit antibody raised in this laboratory. The homogeneity of the final product was established by SDS-PAGE and reverse phase chromatography using Pharmacia HR 5/5-18 column.

Preparation of IgG-free, IgE-enriched serum fractions

Kabi Pharmacia Diagnostics AB protein G column was used to remove IgG from the serum samples. The

serum samples were absorbed on the column and the IgE enriched serum fraction eluted with 0.02 M Sodium Phosphate buffer pH 7.0. The sample volume was restored to the original volume of the serum sample and the specific IgE concentration determined by calibrated RAST assay. The IgE-free, IgG-enriched fraction was prepared by elution with 0.1 M Glycine-HCl buffer pH 2.7. The eluate was immediately neutralized by addition of 1M Tris-HCl, pH 9.0. The specific IgG concentrations and the absence of IgG in the IgE-enriched fraction were determined as described by Dunette and Gleich (1981) by double antibody immunoprecipitate method using I^{125} labeled *Amb a V* as tracer. We found that in the case of individual BF the IgE was, as in all other cases, free of IgG, but the IgG fraction contained some IgE. We estimated by analysis that if we assume a 1:1 complex between IgE and IgG, about 5% of the IgG will be carrying IgE. All the other IgG-enriched fractions were shown to be free of IgE on a level of 0.1 mg IgE/ml by calibrated RAST assay.

Measurements of IgE binding to Amb a V and its inhibition by allergen-specific IgG

The measurements were carried at fixed limiting allergen concentration (antibody excess) and with the serum IgE antibody concentration as variable. The concentration of inhibitor IgG remained constant. RAST inhibition assays were carried out between native IgE and IgG from the same patient and between different individuals, as follows:

One-tenth (0.1) ml of *Amb a V* (25ng/ml) solution was added to each well (Immulon 2 plates: Dynatech Laboratories, Inc., Alexandria, VA). The plates were incubated at 37°C for 90 min and then washed with RAST wash solution (normal saline with 0.5% Tween-20) using the Sanofi Diagnostics Pasteur, Inc. model LP 400 plate washer set for three cycles. To block the non-specific binding of immunoglobulin to the well surface, 125 μ l of blocking buffer (1% BSA and 0.01 Na₂N₃ in 0.02M PBS, pH 7.2) was added to each well and the plate incubated at 37°C for 1 hr. The plate was then washed again for three cycles with RAST wash solution. We divided the treated wells into two groups: Group I wells used to determine the dilution profile of IgE received 100 μ l of native IgE enriched serum diluted 1:2, 1:4, 1:8, 1:16 and 1:32 with chicken serum. Group II consisted of wells to which we added a mixture of native IgE diluted in the same way but with a fixed amount of IgG enriched serum diluted 1:8 with chicken serum added. The plates were incubated at 37°C for 2 hr and washed with RAST wash solution for three cycles. Next, we added 100 μ l of Anti-IgE I^{125} (Phadebas RAST, Kabi Pharmacia Diagnostics AB, Uppsala) to each well, the plates were incubated at 37°C for 2 hr and then washed with RAST wash solution three cycles on LP 400. The specifically bound radioactivity (CPM = amount of bound antibody expressed as counts per min of the radioactive tag) was counted by Gammacounter (Gamma 4000 for I^{125} , Beckman Instruments, Inc., IL). The CPM used represents the average of triplicate determinations.

To determine the nature of the inhibition observed when using IgG inhibiting specific IgE binding we used plots of the reciprocal of CPM bound plotted against the dilution factor. The CPM bound is proportional to the amount of antibody bound and the dilution factor proportional to free antibody concentration in arbitrary units, provided that the antibody is in excess. Such qualitative plots have the advantage over the usual plots showing percent inhibition because they tell us whether the interaction between the two antibody isotypes is truly competitive. The plots also tell us directly whether the IgE preparation is homogeneous or represents a wide distribution of clonal products of varying affinity. In the latter case the reciprocal plots show prominent curvature. As thoroughly discussed by Steward and Steensgard (1983), however, the appropriate plot for such a case, the Scatchard plot, cannot be used easily for inhibition studies.

The calibration for quantitative RAST assays

The standard for *Amb a V* specific IgE was calibrated in ng/ml by affinity isolation of the specific IgE from serum and its analysis by total IgE methodology calibrated against WHO standard for IgE.

Briefly, the column for affinity chromatography was prepared mixing 0.35 mg *Amb a V* dissolved in carbonate buffer of pH 9.6 and 3 ml of Reactigel (Pierce, Inc.). The mixture was tumbled in a cold room for 36 hr. An excess of ethanolamine was added resulting in a pH of 9.0 and the mixture tumbled overnight. The gel was then washed with RAST buffer (0.05 M NaHP04; 0.08 M NaCl; 5% NaN3; 5% tween 20; 5% BSA) pH 7.4. Excess air was removed from the Reactigel by subjecting the mixture to vacuum produced by an aspirator for 30 min. The gel beads were then packed in a column of 30 × 10 mm. Ten ml of serum from an individual known to be sensitive to *Amb a V* by skin test and showing a high level of *Amb a V*-specific IgE was passed through a 45 × 8 mm column of Protein G (Kabi Pharmacia Diagnostics) to remove all IgG. The Protein-G had been washed extensively with NaH P04 pH 7.4 buffer and subjected to vacuum by aspirator for 30 min before packing. The IgG-free serum was next filtered through a 0.45 µ syringe filter and passed through the *Amb a V* affinity column with RAST buffer of pH 7.4. The IgE content of the serum was determined before and after the passage through the affinity column. About 20% of the IgE was *Amb a V*-specific and retained on the column in this specific case. The affinity column was eluted with RAST buffer that had been adjusted to pH 11.5 according to Schellenberg and Adkinson (1975). The eluate was neutralized immediately and its IgE content determined with Sanofi Diagnostics Pasteur ACCESS® methodology calibrated to the WHO IgE standard. The purified IgE serum containing known amounts of *Amb a V*-specific IgE was used to calibrate an internal serum standard for *Amb a V*-specific IgE.

AFFINITY DETERMINATIONS

The problem of the number of epitopes per molecule is important in studies of inhibition, competition, and the

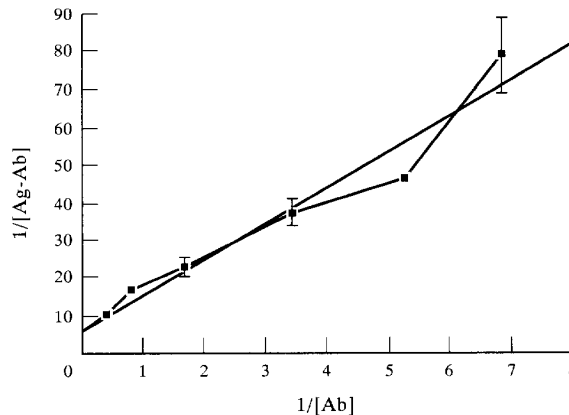


Fig. 1. Binding of the IgE enriched antibody population from individual DA to *Amb a V* attached to plastic. Nominal concentration of *Amb a V* 10 ng/well. Effective concentration 0.14 ng/per well. Experiment carried out at pH 7.4 with reconstituted serum samples diluted with chicken serum, temperature 37°C. A reciprocal plot of bound vs free antibody concentration in mM.

calibration experiments. For determination of affinity in the form of the first moment, the average association constant, carried out in this investigation, the problem is not essential, the epitope number appears only as an apparently higher amount of allergen on the plate accounted for in the experimentally determined intercept. The actual IgE affinity determinations were carried out in a similar manner to those described for inhibition studies, except that each serum dilution used was analysed in quadruplicate to lessen the scatter of the data points. The error bars in Fig. 1 indicate the scatter. The experiments designed to determine the inhibition constant for IgG were identical to the inhibition studies described except that each data point again represents quadruplicate determinations. The free antibody concentration was derived from the total antibody added by subtraction of the amount bound. It should be pointed out that the extrapolated values of intercepts are related to the effective concentration of epitopes seen by the antibodies. This concentration is much less than the nominal concentration of antigen attached to the solid state. Although the knowledge of the absolute effective allergen concentration is not essential for affinity determination we have to be convinced that it remains constant under experimental conditions. The effective concentration can be determined by competition with allergen in solution (Niewola *et al.*, 1983; Hogg *et al.*, 1987). We found that although less than 50% of the nominal concentration, it remained remarkably constant if the times and conditions for coating the plastic wells with allergen remained constant.

Statistical weighting factors for analysis of the affinity data

The standard deviation of the individual points are plotted in Fig. 1, representing the data set for one of the sera. The data sets for the three others not shown were

quite similar, with the exception of the serum BF where the scatter was somewhat larger.

The straight line fitted through the points was calculated by linear regression using the customary y^4 weighting factor (Wilkinson, 1961), for reciprocal plots. We have here assumed that the error for the first four points that totally dominate the data are comparable.

The calculation of association constant $\langle K_{\text{ass}} \rangle$ of IgE for *Amb a V*.

1. The binding experiments were performed with IgE enriched fraction at various dilutions (1:2, 1:4, 1:8 and 1:16).
2. After determining the concentration of applied Ab, Ag-Ab complex and free Ab using our calibration factor, the inverse of Ag-Ab complex and free Ab in nM were plotted on the y and x axis of the graph, respectively. The points were fitted to a straight line as described above. The total concentration of antibody bound to *Amb a V* can now be read from the drawing and $K_{\text{ass}} = \langle K_{\text{ass}} \rangle$ calculated from the slope as follows;

$$\text{slope} = \frac{1}{K_{\text{ass}}} \frac{1}{[Ag]_{\text{tot}}} \quad (1)$$

RESULTS

Determination of the average association constant for the IgE enriched serum fraction

The apparent association constant for the four IgE enriched sera were calculated from analysis of data by reciprocal plots. Although the reciprocal plot is the simplest and visually most satisfying way to display binding data it has many disadvantages. The most disturbing is the effect of the reciprocal transform on the error. Our data, however, are of sufficient quality that the error problem can be taken care of by appropriate statistical weights. The precision in determining the slope of the line is adequate, because we are not dealing with totally homogeneous antibody. The antibody population represents a sum of many clones, thus the association constant is an average for the population. As judged from the linearity of the data in Fig. 1, the expected deviations at high antibody concentration, an indication of broad distribution of constants did not appear. Thus, we are dealing with a relatively narrow distribution of antibodies of quite high affinity. For such cases, until a full envelope of the distribution in the form of probability density function is determined (Sturgill and Biltonen, 1976; Thakur *et al.*, 1980); the average constant, the first moment, gives an adequate description of the antibody population. The nature of the data used can be seen in Fig. 2 which represents on a log scale the four first, dominating points of each of the four data sets. The logarithmic scale is necessary to bring the wide variations of antibody concentrations on a comparable scale. Naturally the data will not fit a linear equation in this form and extrapolation is not possible. This brings us to the point of the values for the y -intercept representing the

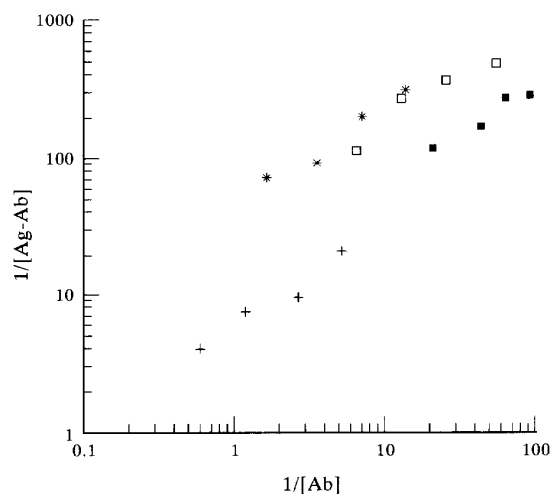


Fig. 2. The reciprocal plots of binding data for IgE enriched serum from four individuals, see Table 1, all determined under conditions specified for Fig. 1. The scales on both axes are logarithmic to accommodate the wide range of values.

(Individuals)			
□ —	□ —	□	KN
■ —	■ —	■	BF
+ —	+ —	+	DA
* —	* —	*	CM

inverse of the antibody molarity after saturating the allergen sample. If we were dealing with a single possible epitope, we would expect the reciprocal plots for all the individuals to produce the same intercept. In our case we cannot make such a prior assumption. The values for the intercept were: DA 5 ± 3 ; CM 32 ± 8 ; KN 47 ± 30 ; BF 69 ± 10 nM $\exp -1$.

It seems that the three enriched sera, with the exception of DA, show a good correlation. The average is 40 ± 15 . It is quite unlikely that the DA value would represent scatter. Two consecutive independent determinations with different blood samples and different enrichment experiments produced an average intercept of 5.5 ± 2 . It is thus likely that DA, the lowest affinity sera, recognizes a higher number of epitopes than the other sera. This is further supported by saturation experiments with pooled (10 patients) sera. The saturation value 4.8 is close to the value observed in the case of DA.

At this point, we have to discuss the problem of the decision to consider the binding process of allergen to antibody in these conditions as approximate to a monovalent reaction. We argue that with an effective amount of 0.14 ng of *Amb a V* per well, we can model the surface of the 5 mm diameter plate and *Amb a V* as a sphere of 45 Å diameter, and conclude that the average distance between two allergens is twice the distance of the reach of the two arms of IgE (Posner *et al.*, 1991). We are doing well up to the concentration of 0.5 ng per well before the binding of two allergens by one IgE molecule becomes probable. We are also operating in the regime of antibody excess. Calculations of ligand binding show that at total saturation below 50% the univalent complex dominates. The data obtained at relatively low saturation, at low

Table 1. Characteristics of individuals tested

	Age, Sex	HLA DR	<i>Amb a V</i> -specific IgE (ng/ml)	<i>Amb a V</i> -specific IgG (ng/ml)	$\langle K_{\text{ass}} \rangle^*$ -to- IgG (M^{-1})	$\langle K_{\text{ass}} \rangle$ -to- IgE ($\times 10^{10} \text{M}^{-1}$)
BF	27, F	4:7	10.4	2772	$< 10^6$	26 ± 5
DA	21, M	2:1	691.8	12,114	$\langle 1 \times 10^7 \rangle$	0.9 ± 0.5
CM	24, M	2:2	221.6	1300	$< 10^6$	3.0 ± 3
KN	46, F	2:7	59.9	1850	$< 10^6$	4 ± 2

* $\langle K_{\text{ass}} \rangle$ = an average estimate from inhibition studies.

allergen density can be used in calculation of K_{ass} , representing the average association constant $\langle K_{\text{ass}} \rangle$.

We have compiled the association constants obtained in Table 1. When comparing the original levels of IgE *Amb a V*-specific antibody and the association constants here determined, a relationship appears which was first mentioned in connection with IgG antibodies to nuclease (Berzofsky *et al.*, 1978). We have in Fig. 3 plotted the IgE levels as a function of affinity expressed as $\langle K_{\text{ass}} \rangle \text{ nM}^{-1}$.

Competition between *Amb a V*-specific IgE and IgG

We next determined the competitive behavior of *Amb a V*-specific IgG fraction towards the IgE enriched preparations.

Because we compare two different isotypes in this experiment, our efforts to keep the inhibiting IgG concentration approximately at the same level as the IgE may not be as effective as comparing IgE with IgE. The inherent problem is that the precision of IgG determinations is lower than the precision experienced in IgE determinations. This is due to the high background from the very high concentration of bystander IgG.

Our comparison of the competitive capabilities between these two populations is thus somewhat ambiguous. We can for each IgE clone have a corresponding IgG clone, but as such a clone represents only a small fraction of the total IgG response, its effects may not be seen if we dilute the total specific IgG level to that of IgE. We decided for these reasons to test the inhibiting capacity of IgG at concentrations found in serum, and as the responders were all chosen for their very high IgG

response, the absence of inhibition cannot be due to insufficient levels of specific IgG. More detailed studies of the affinity properties of the IgG population have to wait until solid state binding assays of IgG of high precision are available. Figures 4 and 5 show the nature of positive and negative responses assembled in Table 2.

The calculation of IgG inhibition constant

We can determine with reasonable precision the concentration of *Amb a V*-specific IgG. This allows us to circumvent to some degree the inhomogeneity and low affinity in IgG response in assessing the relative affinity of IgG antibodies. We chose for the experiment the IgE and IgE-enriched fractions from individual DA. We carried out three successive determinations of the association constant with IgE-enriched sera from DA. However, in the last two experiments we added two different dilutions of the IgG fraction.

The binding equilibria in this case can now be described by:

$$K_{\text{ass}} = \frac{[AbAg]}{[Ab][Ag]} - K_i = \frac{[InAg]}{[In][Ag]} K_{\text{ass}} = \langle K_{\text{ass}} \rangle \quad (2)$$

where $[Ab]$ = free antibody, $[Ag]$ = free allergen, $[Ag]_{\text{tot}}$ =

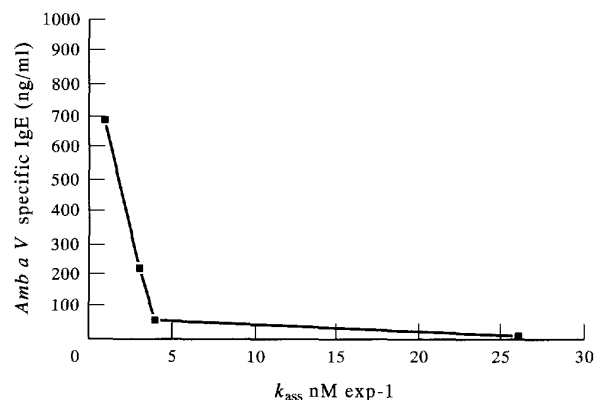


Fig. 3. The level of *Amb a V*-specific IgE ng/ml in individuals plotted as a function of the affinity of each IgE preparation.

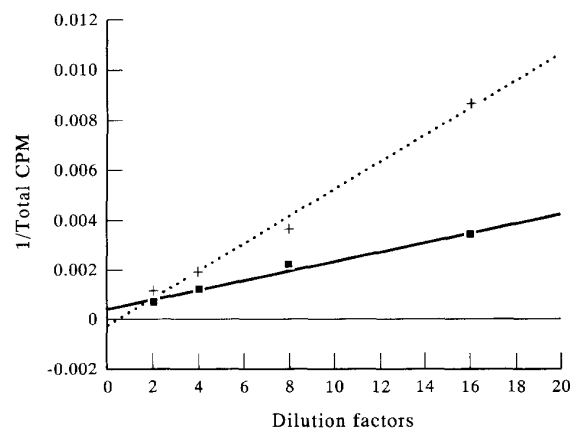


Fig. 4. The inhibition of the binding to *Amb a V* of IgE enriched serum fraction from individual KN by 1:2 dilution of the reconstituted IgG fraction from the same individual. Conditions are same as described for Fig. 1. Amount bound expressed in CPM and free antibody concentration in terms of the dilution factor.

■ — without IgG
+ — with IgG

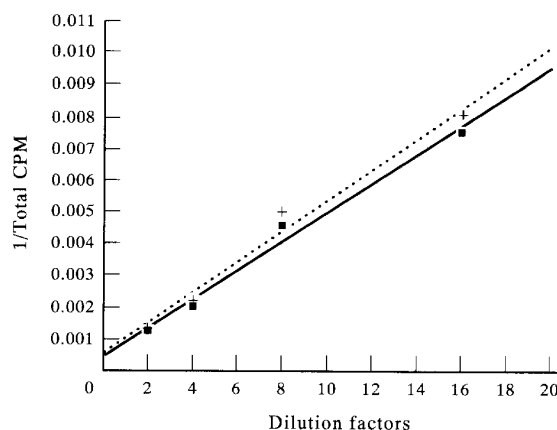


Fig. 5. The absence of inhibition of the binding of IgE enriched serum from individual CM to *Amb a V* on solid support by 1:2 diluted reconstituted IgG fraction from the same individual.

Conditions are same as described for Fig. 4.

■ — ■ — ■ — without IgG
+ — + — + with IgG

Table 2. The result of mutual RAST inhibition between native IgE and IgG fraction

	IgG Fraction			
	BF	DA	CM	KN
BF	—	—	—	ND
DA	—	+	ND	—
CM	ND	ND	—	ND
KN	ND	—	ND	+

+ = Inhibited, — = not inhibited, ND = not done.

total allergen equivalents; which after introducing concentration restriction leads to:

$$\frac{1}{[AbAg]} = \frac{(1 + K_{in}[In])}{K_{ass}[Ag]_{tot}} \cdot \frac{1}{[Ab]} + \frac{1}{[Ag]_{tot}} \quad (3)$$

The slope of line with inhibitor is

$$K^1 = \frac{(1 + K_{in}[In])}{K_{ass}[Ag]_{tot}} \quad (4)$$

and without inhibitor

$$K = \frac{1}{K_{ass}[Ag]_{tot}} \quad (5)$$

$$K^1 = K(1 + K_e[In]) \quad (6)$$

with $[In]$, K known and K^1 determined, K_{in} can be calculated.

We know the concentration of IgG, IgE and the association constant for IgE binding which allows us to calculate the average binding constant for IgG in the form of an inhibition constant. It is still simpler to calculate it from the ratio of slopes (4) and (5) estimated in whatever units we have measured it. The $\langle K_{ass} \rangle$ for the IgG from DA is $1 \cdot 10^7$, nearly 100-fold less than the affinity of IgE

from the same individual. The error in this determination is of course quite large.

Similar inhibition experiments with individual CM revealed no measurable inhibition at these concentrations. This allows us only to estimate the upper limit of the affinity of IgG for *Amb a V*. Such estimates for individual CM, KN, and BF are included in Table 1.

DISCUSSION

There are two major forces defining the affinity and specificity pattern of the humoral response to an antigen. First the choice and combination of germline V,D,J sequences resulting in a CDR region, complementary to the epitope surface. The second force is the continuous somatic point-mutation (Berek and Milstein, 1987) producing a sequence of better and better fits between these two surfaces. In assessing the relative importance of these two factors, specifically in response to the development of IgE response, we often assume that the very large size of the V(h) library (Schroeder *et al.*, 1990; Freitas *et al.*, 1990) for the V,D,J segments provides such a variety of germline sequences that the inherited choice of such sequences should not provide a restriction point for IgE and antibody response to an allergen. We have also learned from animal studies of IgG response that given enough time and multiple exposure to antigen the original low and high responding strains show in the end a very similar response (Berzofsky *et al.*, 1978). If these observations on inbred mice strains are to be extrapolated to the human IgE system, we would expect that individuals sensitive to a small allergen (restricted number of epitopes) should after years of regular exposure (repeated immunizations) develop a very similar IgE response as far as affinity goes. We can further expand our model and argue that in case the well-known IgM to IgE switch is not sensitive to the affinity of the antibody site the affinity and epitope specificity seen in IgE response should be reflected in the IgG response, albeit with a broader affinity distribution resulting possibly from the reported higher mutation rate in the IgG clones when compared to IgM (Gearhart *et al.*, 1981). These are the problems of IgE response we have tried to address in this investigation.

We chose the four individuals among *Amb a V* responsive individuals with a good IgE and IgG response. The HLA-D allele complement of the individuals had already been determined. The linkage between *Amb a V* sensitivity and the DR-2 allele is the most pronounced of the allergen-HLA linkages reported (Marsh, 1990; Blumenthal, 1993). We have one monozygous, two heterozygous, and one non-DR-2 responders.

Before discussing the content of Tables 1 and 2, we have to return to the question of affinity, although we discussed our approach thoroughly in the Materials and Methods section. The problem of a response to multi-epitope allergen by multiclonal antibody makes the definition of affinity in terms of molar association constant difficult. A broad distribution of association constants allows the presentation of data only in terms of Scatchard or similar plots (Steward and Steensgard, 1983; Karlsson

et al., 1992). Such plots do not allow estimates of average association constants. To our surprise, all four IgE populations studied showed linear behavior when binding data were plotted in classical inverse plots. This does not mean that the response is not polyclonal. In fact, IgE response has been recently and clearly shown to be polyclonal (van't Hof *et al.*, 1993). The apparent linearity of inverse plots indicates that the distribution of association constants is relatively narrow. Narrow or restricted distribution of antibody clones is nothing new. It was first reported in animal responses (Braun *et al.*, 1969). The authors speculated that the electrophoretically monodisperse response was due to prolonged vigorous immunization with antigens showing limited number of epitopes—conditions quite similar to sensitization with *Amb a V*.

Regardless of the origin of the narrow distribution of association constants it allows us to determine a reliable average affinity. True association constants are available only for monoclonal antibodies. Dilution of the antigen in solid state and an estimate of the limits of its activity coefficient together with the calibration of our secondary antibody against WHO IgE standard allows us to express the average affinity in molar and not relative terms. The values can consequently be compared to the published value of antibody responses in general (Karush, 1978; Steward and Steensgard, 1983). To our knowledge, this is the first time the average affinity of individual IgE responses has been characterized in this manner.

We present in Table 1 the main results of the study. Within the limits of errors as previously discussed, the average affinities for the individual IgE responses show a 30-fold variation. All the values are, as expected, in nanomole range, which is considerably higher than the range reported for most IgG responses (Steward and Steensgard, 1983) although higher values have been reported (Watt *et al.*, 1980). What is interesting to note is that there is no apparent correlation between the presence of the DR-2 allele and the average affinity of antibodies observed. If DR-2 is necessary for a response to *Amb a V*, it is not necessarily linked to presence of high-affinity IgE antibodies. Other factors such as T cell epitope recognition must play an important role. It is of course clear that the limited number of sera studied does not allow the claim that inherited limitations in availability or choice of V(H) segments is more important than somatic mutation. However, when and if confirmed by study of larger populations, such results would support the idea. The inverse relationship between specific IgE levels and affinity reaffirms the observations that IgE levels and disease severity are not necessarily linked.

Table 2 shows the results of using IgG fraction as inhibitors for IgE binding. We concentrated on the most informative comparison inhibition of an individual's IgE by its own IgG. Only in one case could we determine an inhibition constant. The value observed points to very much lower affinity. In view of the high concentrations of IgG used, the limits of average inhibition constants for the three other individuals in Table 1, although speculative, should represent the upper limit. This with the

caveat that IgG in some cases might see another epitope than IgE recognizes. The affinity, measured directly in the case of IgE and indirectly in the case of IgG, is toward epitopes and not *Amb a V per se*. However *Amb a V* was chosen as the smallest allergen with a very small possibility for a variety of non-overlapping epitopes. Consequently the evidence favors the presence of IgG with lower affinity. We have to comment at this point on the behavior of sera BF. In that and only in that case some of the IgE presumably in the form of IgE–IgG complexes was found in the IgG fraction (see Materials and Methods). As the IgE represented binding to only 5% of the IgG it should not influence the observed absence of inhibition by IgG.

Although the data in Tables 1 and 2 are surprising in terms of the absence of maturation of IgG response, direct measurements of the affinity of specific IgG is necessary before any of the tempting conclusions can be drawn. These observations for *Amb a V* agree with the observation for *Der p I*-specific antibodies where it was shown that for every epitope recognized by IgE there was an IgG response but the reciprocal relationship did not occur (Greene *et al.*, 1991).

The final and most intriguing observation is that presented in Fig. 3, the relationship between the concentration and affinity of antibodies, first discussed in context of IgG response in different mouse strains by Berzofsky *et al.* (1978). If observations such as ours are confirmed by further studies, they point to a relationship not linear but corresponding more to the function $xy = \text{constant}$. This means that it is the capacity of antibody to saturate hypothetical binding sites that regulate the antibody production.

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