

An automated method for determination of antibody affinity distribution functions with nanogram quantities

Lisa Pierson^{a,*}, Sophie Allauzen^b, Malcom Blumenthal^b, Andreas Rosenberg^a

^a *Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN, USA*

^b *Department of Medicine, University of Minnesota, Minneapolis, MN, USA*

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Abstract

We have developed a method to determine the binding affinity distribution functions (the probability density function for affinity constants will be referred to as affinity distribution throughout the text) for human serum antigen specific IgE for their respective allergens. This fully automated method, based on the Access[®] technology of Sanofi Diagnostics Pasteur, is a highly sensitive two-step sandwich immunoassay requiring only nanogram quantities of antibodies. Allergen–antibody binding isotherms can be determined covering a range of 5 magnitudes of ligand (allergen) concentration. An affinity distribution function, describing the polyclonal nature of the antibody response, can be calculated from the binding isotherm. The validity of the method is assessed using mAbs against the purified allergen Der p 1. The resolving power and sensitivity of the method are demonstrated using selected sera from donors with high and low levels of specific IgE for these allergens. We are able to show that heterogeneous IgE populations can be determined using 100 μ l of sera containing 150 pg of specific IgE per ml. Overall, the range of affinities that can be determined by this method is 1×10^6 – 1×10^{11} M⁻¹. © 1998 Elsevier Science B.V.

Keywords: Affinity distribution function; IgE

1. Introduction

It has long been known that allergies are characterized by a polyclonal IgE response to specific allergens. The presence of allergic symptoms generally correlates with the presence of specific IgE. Yet,

it is also known that not all subjects who have elevated levels of total or specific IgE are clinically allergic (Chapman et al., 1983). There appears to be little correlation between levels of specific IgE and presence or severity of symptoms (Haahtela and Jaakonmaki, 1981).

Previous studies demonstrate the biological significance of the effect of affinity on antibody function in antisera of different disease processes (Steward, 1981). The expected variance of antibody affinity is based on the consensus model of cell selection. According to this model, cells are selectively stimulated on the basis of the affinity of their receptors for

Abbreviations: Amb a 1, *Ambrosia artemisiifolia* antigen 1; Der p 1, *Dermatophagoides pteronyssinus* antigen 1; K_A , Affinity in 1/mol; mAb, Monoclonal antibody; RLU, Relative luminescence units

* Corresponding author. Laboratory of Medicine and Pathology, Box 434, 420 Delaware St. SE, Minneapolis, MN 55455. Tel.: +1-612-626-4624; fax: +1-612-624-9188.

antigen. The average antibody–antigen binding affinity increases with time after immunization. Unfortunately, the binding affinity between specific IgE and ligand remains essentially unknown.

The determination of allergen specific IgE has been especially difficult due to the (often) low concentration of human serum IgE and the limited sensitivity of most methods. The presence of IgE in nanogram quantities exceeds the analytical detection limits of most methods that use serum dilution for variation of ligand (antibody) concentration. Further, many studies, possibly due to limits of the methods, do not produce a complete binding isotherm, and only fractions of the complete isotherms are used. Limited use of sections of the total binding isotherm in a Klotz-type analysis (Klotz et al., 1953), developed to describe a homogeneous response, does not give an accurate description of a heterogeneous response of antibody populations of widely varying affinity.

We have developed a fully automated two-step sandwich immunoassay that captures a representative sample from the total serum IgE antibody population. This is a modification of a so-called 'reverse' assay that was first developed for ELISA and RAST for the measurement of IgE (Ceska and Lundkvist, 1972). The reverse strategy avoids the problems of chemical alteration or elimination of antigenic determinants. Highly sensitive chemiluminescent technology (Access[®]) detects the binding of purified, haptenated-allergen at nanogram levels. A complete binding isotherm is generated, which is then used to determine a binding affinity distribution function according to the method developed by Hunston (1975).

Quality control experiments were run to account for the state of equilibrium, binding specificity and nonspecific binding. Limits of sensitivity and limits of affinity determination for the method were calculated, demonstrating the accurate determination of affinities as high as $1 \times 10^{11} \text{ M}^{-1}$. The approach was then used to determine the affinity distribution functions for four Der p 1 specific monoclonal antibodies. These results were compared to results obtained by classical Klotz analysis. Finally, this method was used to examine the affinity distribution for the antigen-specific heterogeneous response of serum IgE.

2. Materials and methods

2.1. Affinity purification of Der p 1

Der p 1 (m.w. 25,371) is an allergen from house dust mite, species *Dermatophagoides pteronyssinus* (Chapman and Platts-Mills, 1980). Der p 1 was purified from house dust mite spent medium (provided by Bayer, Spokane, WA) using Der p 1 specific mAb 4C1 coupled CNBR-activated Sepharose 4B as described previously (Van der Zee et al., 1988). The 4C1 mAb was kindly provided by Martin Chapman (University of Virginia, Charlottesville, VA). The Der p 1 containing peak was assayed for purity by SDS–PAGE electrophoresis on 15% acrylamide gel under reducing conditions. Protein concentration was determined by Coomassie[®] Plus protein assay (Pierce, Rockford, IL). One milliliter aliquots of 0.35 mg/ml Der p 1 were stored frozen at -20° .

2.2. Amb a 1 purification

Amb a 1 is a protein of m.w. 37,800 from ragweed pollen (*Ambrosia artemisiifolia*). Amb a 1 was purified as described by King et al. (1963). Absorbance was measured at 280 nm and peaks representing forms A, B and C were identified by 12.5% SDS–PAGE. Fractions in peak A were combined and stored frozen at -70° . An error of less than 5% was observed when the protein concentration of Amb a 1, determined by use of the extinction coefficient (King et al., 1963) at 280 nm, was compared to the Coomassie[®] Plus method of protein concentration determination.

2.3. Biotinylation of Der p 1 and Amb a 1

The protocol developed by Pierce (Rockford, IL) (Gretch et al., 1987; Sorensen, 1996) was followed with some modifications. Briefly, 5 mg of NHS-LC-Biotin powder (biotinyl-*N*-hydroxysuccinimide ester; Pierce) was dissolved in 0.25 ml dimethyl formamide solvent to a concentration of 20 mg/ml and vortexed. Biotin was added to the protein in 0.05 M borate buffer pH 8.0 at a molar ratio of 1:3 biotin to protein, then incubated for 2 h at 4°C followed by dialysis against PBS overnight at 4°C . Concentration of biotinylated protein was determined by Coomassie[®] Plus protein assay (Pierce).

2.4. Access[®] immunoassay

The newly developed version of an Access[®] immunoassay is a fully automated sequential two-step immunoenzymatic ('sandwich') assay (Fig. 1). The final concentration of mouse anti-IgE in the particle suspension is approximately 1 $\mu\text{g}/\text{ml}$. Magnetic particles with goat anti-mouse and mouse anti-IgE and diluent are manufactured and supplied by Sanofi Diagnostics Pasteur, Chaska, MN. The concentration of streptavidin–alkaline phosphatase conjugate (Pierce, Rockford, IL) used for this study was 15 ng/ml.

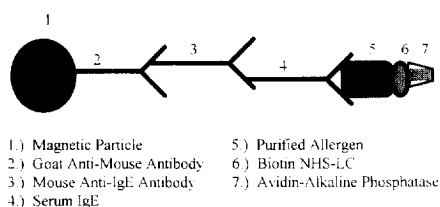
Automated steps for our version of Access[®] immunoassay

Two-step method	Three-step method
Step 1: 50 μl magnetic particles ^a	Step 1: 50 μl magnetic particles ^a
–100 μl antigen ^b	–100 μl 1:10 diluted serum ^a
–100 μl 1:10 diluted serum ^a	–Incubate for 28.8 min
–Incubate for 28.8 min	–Wash
–Wash	Step 2: 100 μl antigen ^b
Step 2: 100 μl conjugate ^a	–Incubate for 28.8 min
–(avidin–alkaline phosphatase)	–Wash
–Incubate for 28.8 min	Step 3: 100 μl conjugate ^a
–Wash	–Wash
–Chemiluminescent detection	–Chemiluminescent detection

The reaction takes place in a reaction vessel at 37° and pH 7.4.

The reagents are agitated and then aspirated from storage vessels at either ^a4° or ^b37°.

A. Sandwich used to obtain binding isotherm for serum IgE:



B. Sandwich used to obtain binding isotherm for Der p 1 specific mAbs:

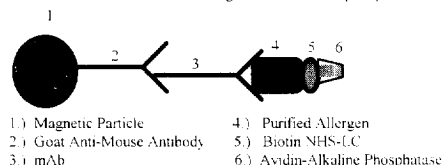


Fig. 1. Outline of the structure of the two-step sandwich immunoassays used for obtaining binding data at increasing concentrations of free antigen.

Diluent: 10 mM Tris, 150 mM NaCl, 0.5 mM EDTA; pH 7.4.

The Access system uses the substrate Lumi-Phos[®] 530 (Lumigen, Southfield, MI) which is dephosphorylated by the conjugate alkaline phosphatase, resulting in the release of light. The light produced is proportional to the amount of analyte present in the sample and is read by a luminometer and reported as relative luminometer units (RLUs).

For this study, we used the two-step method which is faster and increases precision by having fewer steps. Binding results did not differ between the two different methods (data not shown) as expected if the antigen is in large excess. If we suspect the presence of any blocking antibodies, the three-step method is necessary.

2.5. Total serum IgE determination

Total IgE was determined using the Access[®] Immunoassay System (Sanofi Diagnostic Pasteur, Chaska) (Patterson et al., 1994).

2.6. Der p 1 specific IgE determination

The amount Der p 1 specific IgE¹ was determined using the Access[®] Immunoassay System (Sanofi Diagnostics Pasteur, Chaska, MN). The Access[®] specific IgE assay is a sequential two-step immunoenzymatic assay. The assay is performed by combining serum, haptenated purified Der p 1, and paramagnetic particles coated with anti-hapten antibodies. Then a 30-min incubation is followed by magnetic separation, washing and addition of alkaline phosphatase (ALP) conjugated equine anti-human IgE antiserum. Chemiluminescent substrate (Lumi-Phos[®] 530, Lumigen, Southfield, MI) is added following a 30-min incubation with the conjugate. The light production is proportional to the amount of specific IgE in the sample. The amount of analyte in the sample is determined by means of a stored, multipoint calibration curve.

¹ This procedure for determination of Der p 1 specific IgE is not commercially available in the US.

2.7. Negative control

We depleted a serum sample of IgE by affinity chromatography using anti-IgE mAb. Eighty nanograms of anti-IgE mAb (provided by Sanofi Diagnostics Pasteur) was coupled to 2.0 g of CNBr-activated Sepharose 4B as described for coupling of Der p I specific 4C1 mAb. Affinity chromatography was run under conditions similar to those used for Der p I purification. Briefly, 6.0 ml of serum containing 16.2 ng/ml total IgE was absorbed onto the anti-IgE coupled Sepharose column. The eluent was monitored at 280 nm, and the fractions in the protein containing peak were combined, concentrated to 6.0 ml by 100,000 MW cutoff Diaflo ultrafiltration membrane. The eluent contained no IgE as determined by the Access[®] total IgE assay.

3. Results

3.1. Binding Isotherm

A binding isotherm is obtained by measuring the amount of antigen bound, expressed as RLUs, at

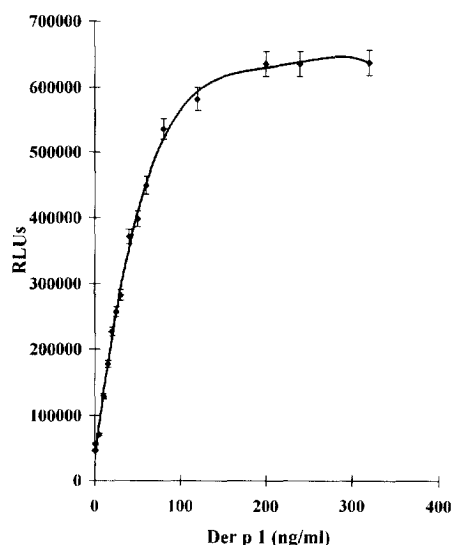


Fig. 2. The binding of Der p I to human IgE. Access[®] instrument readings in RLUs (relative luminometer units) are plotted as a function of increasing concentrations of Der p I.

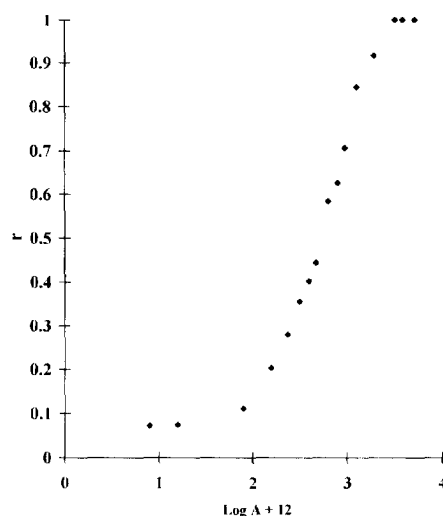


Fig. 3. Binding isotherm obtained from data in Fig. 2. RLUs have been converted to degree of saturation (r) and plotted against $\log A + 12$, where A is concentration of Der p I in mol/l.

increasing concentrations of free antigen until the point of saturation is reached (Fig. 2). The molarity of antigen is expressed as $\log A + 12$ for ease of data manipulation. The data are plotted as r vs. $\log A + 12$ where A = molarity of free antigen, which is equivalent to total antigen for this method, and r = degree of saturation (Fig. 3). The data are then smoothed by the best fitting polynomial function to be described in a following section. All experiments are run in duplicate to minimize error. An experiment was run twice with monoclonals and serum using different aliquoted dilutions of antigen and conjugate with both demonstrating excellent reproducibility (data not shown) with little scatter of the data points. The estimated error, expressed as percent of total RLUs, is less than 5% which is at the lowest antigen concentration (0.25 ng/ml). The estimated random error was calculated for a binding isotherm with triplicate values collected at 15 points of antigen concentration, and was determined to be constant by the Bartlett test (Neter and Wasserman, 1974). The error is minimized due to the automation of the method also, human pipetting errors of antigen and conjugate dilutions are negligible if these reagents are kept in excess. Minimal scatter of the

data increases both the ease of the smoothing process and the reliability of the smoothed data as a true reflection of the antibody/ligand interaction. A wide range of ligand concentration is necessary to accurately reflect the features of the binding isotherm. It has been previously shown to be impossible to reconstruct the entire distribution with fewer than 10 points (Thakur et al., 1980). A minimum of 10 points, equal to 200 μ l serum, covering four to five magnitudes of ligand concentration were used for each binding isotherm.

3.2. Negative control

The RLUs for IgE depleted serum (*vida supra*) and by serum containing no measurable Der p 1 specific IgE were determined at increasing concentrations of Der p 1. The RLU values for the negative controls at increasing concentrations of antigen were linear when plotted as RLUs as a function of antigen concentration. The range of RLUs for the negative control were 16,000 at 5 ng/ml Der p 1 to 10,000 RLUs at 400 ng/ml Der p 1. The nonspecific binding of allergen observed for the IgE depleted serum was fitted by linear regression and subtracted from the RLU values measured at corresponding values of Der p 1 concentration used in all the binding experiments. The similarity of binding produced by the IgE depleted serum and natural serum containing no Der p 1 specific IgE show that the low MW proteins, excluded by the concentration procedure used to eliminate IgE, do not increase the background. The background is highly reproducible; however, it changes for each sandwich configuration and should be redetermined when the sandwich configuration changes.

3.3. Inhibition assay

The specificity of the assay was determined by inhibiting the binding of haptenated antigen to anti-Der p 1 mAb 4Cl by unlabeled Der p 1 (data not shown). The assay was performed by combining a constant amount of antigen with increasing concentrations of unlabeled antigen. Fifty percent inhibition was achieved at a 1:1 ratio of labeled antigen and unlabeled antigen, which shows a negligible effect of biotinylation on the antigenicity of Der p 1.

3.4. Approach to equilibrium

Integration of the kinetic equation $dAB/dt = k_1[A][B] - k_2[AB]$ under the condition of antigen excess leads to the equation:

$$\ln([AB]_{eq}/([AB]_{eq} - [AB])) = (k_1 B_0 + k_2)t \quad (1)$$

where $[AB]_{eq}$ is the complex concentration at equilibrium, B_0 is initial concentration of the ligand, and t is the time. To verify that equilibrium has been reached at the 30-min incubation time, we measured the amount bound $[AB]$ at two different time points (10 and 20 min). Eq. (1) was used to calculate the expected amount bound at equilibrium $[AB]_{eq}$ and compared to the amount found experimentally using anti-Der p 1 mAb 4Cl (Fig. 4). This establishes that equilibrium has been reached during the 30-min incubation time. As an experimental alternative, we can use two shorter incubation times and the calculated equilibrium concentration. This allows binding studies at low temperatures and high molecular weight ligands.

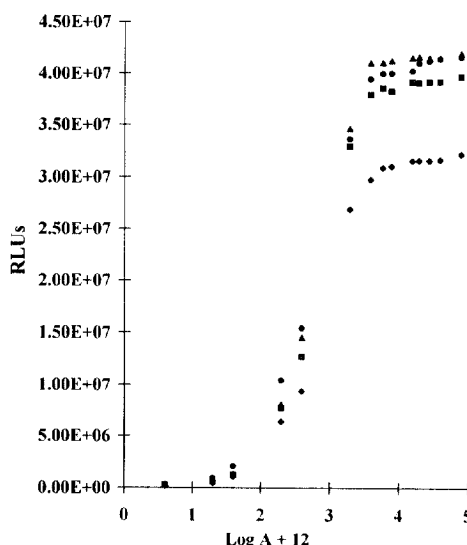


Fig. 4. Approach to the calculated equilibrium by the experimental binding isotherms obtained using Der p 1 specific mAb 4Cl. The expected concentration at equilibrium ($[AB]_{eq}$) was calculated as described in the text from the data of binding isotherms measured at 10- and 20-min incubations. This was compared to experimental data with a 10-, 20- and 30-min incubation time. 10 min ($\diamond$); 20 min ($\blacksquare$); 30 min ($\bullet$); $[AB]_{eq}$ ($\blacktriangle$).

3.5. The question of antigen excess

It is important that the antigen is in excess for accuracy in determining the concentration of free antigen in terms of total antigen, once having established that equilibrium for the system has been reached. For this method, the concentration of the magnetic particles, coating monoclonals, serum antigen specific IgE and bound antigen can be converted to molarity to demonstrate antigen excess in the range of determined affinities. The concentrations were calculated for low levels of Der p 1 specific IgE using 50 μ l of magnetic particles and a K_A of $1 \times 10^{11} \text{ M}^{-1}$ with a 50% saturation level at 0.5 ng/ml Der p 1. One hundred microliters of a 1:10 dilution of serum containing 0.5 ng/ml antigen specific IgE is equal to 2.66×10^{-8} nmol of specific IgE. One hundred microliters of a $2 \times 10^{-11} \text{ M}$ solution of Der p 1 contains 1×10^{-6} nmol of Der p 1. With 50% of antigen binding to two sites per antibody molecule, we are at a 40-fold excess of antigen. The amount of excess increases with lower affinities. This shows that the antigen is in excess for all our binding studies and allows for the determination of affinities up to $1 \times 10^{11} \text{ M}^{-1}$ without correction for difference between free and total antigen.

Since this is a kinetic method, we need to be concerned about maintaining equilibrium at our experimental conditions. A connection for the thermodynamics and kinetics of antigen–antibody binding is expressed by the relationship $K_A = k_{\text{on}}/k_{\text{off}}$ where k_{on} and k_{off} are rate constants for association and dissociation reactions. A first-order rate constant is k_{off} and one can calculate the half-time for dissociation based on $t_{1/2} = \ln 2/k_{\text{off}}$. Related antigens of similar size and other similar physical properties have similar association rates, with the differences in affinity corresponding to differences in dissociation rates (Eisen, 1980). Many studies have shown rates for several peptide, protein/antibody systems to be in the range of 1×10^4 to $1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (Zeder-Lutz et al., 1993). For K_A of $1 \times 10^8 \text{ M}^{-1}$ and higher, this puts the half-life to an hour scale, confirmed by direct measurements of off rates in these experiments. Problems appear when low affinity antibodies, such as mouse mAbs against some polysaccharides, are measured (Cooper et al., 1994), where a K_A of 1×10^6 can result in off rates of

$1 \times 10^{-3} \text{ s}^{-1}$, which can yield half-times in the range of 15 min. To ascertain the lower limit of affinities accessible to our method, we carried out an experiment of reducing the incubation time for conjugate from 28.8 to 3.6 min compensating the binding with an 8-fold increase in the concentration of the conjugate (data not shown). We found for serum IgE with $K_A \cong 1 \times 10^6$ a loss of 6% antigen bound during a 28.8-min incubation in antigen-free solution.

3.6. Determination of affinity distribution function

The overall fraction of antibody sites binding ligand for a heterogeneous system can be expressed by:

$$r = \sum_{j=1}^m \frac{n_j K_j [A]}{1 + K_j [A]} \quad (2)$$

where A represents the molar concentration of free antigen; m represents the number of different classes of independent sites; n_j is the number of sites in class j ; K_j represents the association constant for the antibody clone for the j th class (Hunston, 1975). The discrete distribution of binding constants is replaced with a continuous function $N(K)$:

$$r = \int_{-\infty}^{+\infty} \frac{N(K) K [A]}{1 + K [A]} d/\log K_j \quad (3)$$

The probability density distribution function $N(K)$ for affinities can be estimated numerically from the experimental curve of r as a function of $\log A$ according to Hunston (1975):

$$N(K) = \frac{r(aA) - r(A/a)}{2 \log a} - \frac{a}{(a-1)^2} \times \frac{[r(a^2A) - r(A/a^2)] - 2[r(aA) - r(A/a)]}{2 \log A} \quad (4)$$

where $r(aA)$ is the value for the saturation level for point (aA) and (a) is an arbitrary constant less than 1. Values of $N(K)$ are obtained by Eq. (4) as a function of $\log K$ ($K = 1/A$). The function $N(K)$ is a probability density function, the fraction of surface under the curve corresponding to the interval ΔK represents the probability of finding a K in the interval of ΔK .

We should, at this point, stress once more that our contention that the distribution function reflects the probability density of intrinsic affinities, and not avidities, is based on the premises that: (a) in a reverse assay the antigen (ligand) titrates *all* antibody specificities and *all* affinities and the distribution is consequently both over different affinities to the same epitope and over all epitopes; (b) cross-linking both arms of an antibody molecule is unlikely because the most probable separation of 150 Å is far larger than the dimensions of Der p 1 or Amb a 1; further, Der p 1 has been shown to be highly asymmetric, and that there is no evidence that Amb a 1 has a repetitive structure; (c) the nearest neighbor probability gives an estimated average distance between captured serum IgE molecules of 1791 Å for the upper range of serum IgE concentrations tested (Chandrasekhar, 1943). The value of 1791 Å was arrived at by knowing the magnetic particle concentration of 1.865×10^9 beads/ml, bead diameter of 1 µm and using the upper range of serum IgE at a 1:10 dilution of 144 ng/ml.

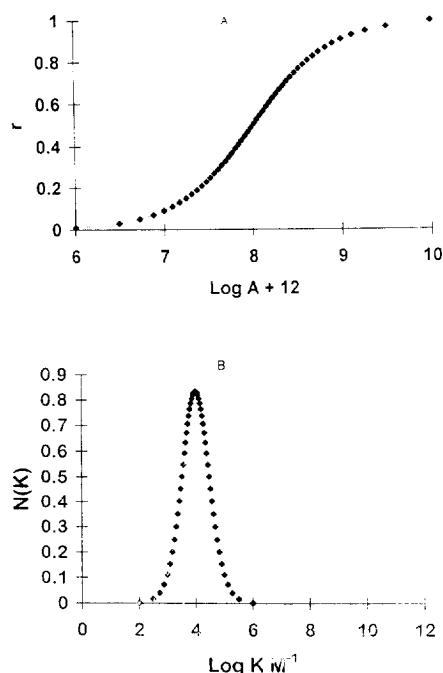


Fig. 5. (A) The theoretical binding isotherm for an arbitrary K_A of $1 \times 10^4 \text{ M}^{-1}$. (B) The affinity distribution function generated by Eq. (4) using the values from the binding isotherm.

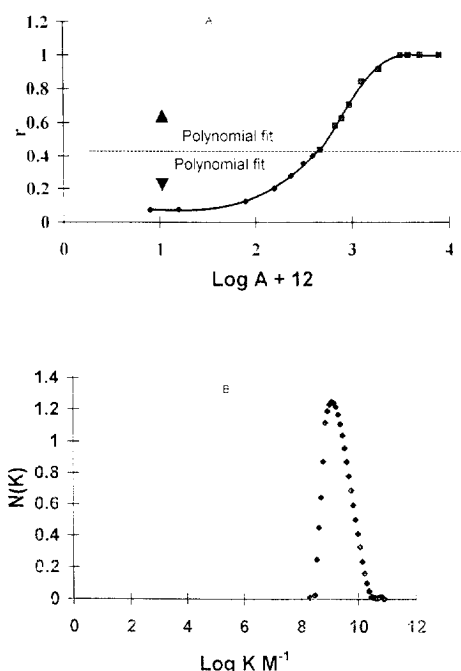


Fig. 6. (A) Binding isotherm for real data of Der p 1 binding to IgE in serum from an individual (#93834653). This data was fit by two multiple polynomials. Polynomial fit A: $y = 0.8509x^4 - 11.2x^3 + 54.383x^2 - 114.91x + 89.489$; Polynomial fit B: $y = 0.0733x^3 - 0.1966x^2 + 0.154x + 0.0421$. (B) The distribution function for this serum sample was calculated by applying Eq. (4) to data pairs, obtained at 0.1 intervals of log A, from the smoothed isotherm.

3.7. Steps of affinity distribution function determination using theoretical binding isotherm

3.7.1. Theoretical binding isotherm

A theoretical binding isotherm was calculated with an arbitrary affinity of $1 \times 10^4 \text{ M}^{-1}$ to validate the method of determining the affinity distribution function from a binding isotherm. Values of A were inserted into Eq. (5) to calculate the corresponding values of r from a transformation of the equilibrium equation.

$$\log K_A = \log \frac{r}{1-r} + \log \frac{1}{A} \quad (5)$$

The equation was used to generate a theoretical binding isotherm that was then used to calculate the affinity distribution function $N(K)$ using Eq. (4), (Fig. 5). The final result is an even distribution with

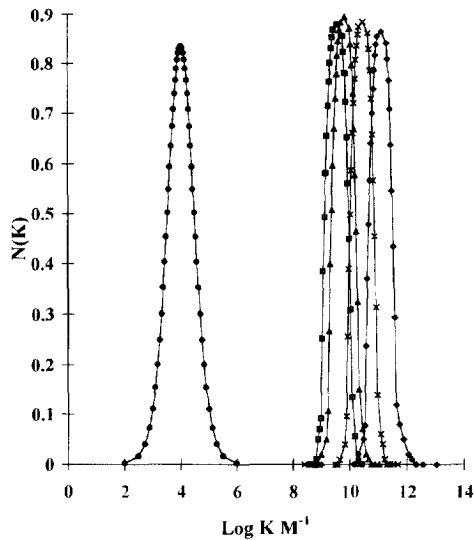


Fig. 7. Affinity distribution functions for four Der p 1 specific monoclonal antibodies and the theoretical distribution function for an arbitrary K_A of $1 \times 10^4 \text{ M}^{-1}$. Theoretical distribution function (●); C4.1 mAb (■); 4Cl mAb (▲); 5H8D8 mAb (*); 10B9 (◆).

$N(K)$ maximum for the theoretical affinity distribution at $1 \pm 0.04 \times 10^4 \text{ M}^{-1}$.

3.8. Steps of affinity distribution function determination using real data

The first step is to obtain binding data at increasing values of antigen using the Access[®] immunoassay. The data, from serum containing Der p 1 specific IgE, expressed as a fraction of saturation (r), are then plotted as a function of $\log A$ (Fig. 6A). Unlike the theoretical binding isotherm, a smoothing function is necessary, and is applied to the data by

computer using a multiple polynomial or spline function. The choice of a smoothing function is arbitrary and based on the best obtainable fit. A minimum of 10 points is used to most accurately reflect r vs. $\log A$. The smoothing function is then employed to estimate r at a series of antigen concentrations. The choice for $\log(a)$ is important for the sensitivity of $N(K)$. A value of $\log a = 0.2$ was used for our calculations. Values of r were taken, starting at the lowest value of $\log A$, at intervals of 0.05 covering the entire range of r to generate the affinity distribution function $N(K)$ using Eq. (4) (Fig. 6B). The reported results are used as an estimation of the range and probability of affinities. The affinity distribution function in Fig. 6B has a maximum value for the affinity distribution at $1.4 \times 10^9 \text{ M}^{-1}$, the most probable K_A .

3.9. Affinity distribution function of mAbs

The affinity distribution function was determined for four Der p 1 specific mAbs (Fig. 7). The affinity probability density function for a monoclonal shows the broadening of a theoretical line due to the approximations inherent in the numerical method. The data was also plotted in the form of Klotz plots, and the affinity was then compared to the values obtained by our method for determination of affinity distributions (Table 1). We find that major changes occur when the data is transformed into a Klotz reciprocal plot (Wilkinson, 1961). A weighting factor is derived from the variance and is used to correct for any random errors generated by the method as best as possible (Bevington, 1969). The values for ligand A were, in all cases, based on

Table 1
Comparison of affinity determinations for four Der p 1 specific monoclonals by Klotz analysis and use of affinity distribution function $N(K)$ for sera containing Amb a 1 specific IgE and Der p 1 specific IgE

mAb	From Klotz analysis, $K_A \text{ (M}^{-1}\text{)}$	From affinity distribution function, 37°, pH 7.4, $\langle K_A \rangle^a \text{ (M}^{-1}\text{)}$
C4.1/102	$3.0 \pm 0.32 \times 10^9$	3.5×10^9
4Cl	$1.1 \pm 1.12 \times 10^{10}$	1.0×10^{10}
5H8D8	$1.3 \pm 1.12 \times 10^{10}$	2.8×10^{10}
10B9	$6.0 \pm 0.17 \times 10^{11}$	1.2×10^{11}
Patient #93915542		7.8×10^8 and 8.7×10^9
Patient #93923463		7.0×10^6 and 3.6×10^7

^aDetermined as maximum value for $N(K)$.

Coomassie® Plus (Pierce) protein assay with BSA as a standard.

Comparing, in Fig. 7, the distribution function calculated from the theoretical data, and the distribution function for the four monoclonal antibodies, we notice that the function for a mAb is compressed at the base. This is the consequence of the random error of about 4% present in actual data. We cannot define the isotherm below 5% saturation and for the last 95–100%. We consequently reach apparent 0% and 100% saturation lines earlier. A calculation shows that, in such a case, the slope of the isotherm (Fig. 7) increases and the truncated distribution function narrows at the base. Using only a section around 50% saturation would produce a delta-like function.

Monoclonal antibodies were attached to separate magnetic particles and mixed together or attached simultaneously to the same magnetic particle to investigate the validity of the method in terms of being able to separate two antibodies with different affinities. This experiment also investigates the possible effects of avidity. The monoclonal antibodies used were C4.1/102, 10B9 and 5H8. These mAbs are noncompeting antibody molecules with different epitope specificities (Lomardero et al., 1990). The first experiment was performed with a mixed solution of magnetic particles coated with monoclonal antibodies 10B9 and C4.1. Table 2 compares the K_A values for the distribution functions for 10B9 and C4.1 monoclonal antibodies determined individually, and the affinity distribution function for 10B9 and C4.1 antibodies determined simultaneously. The method is clearly able to separate the two different antibody molecules with differing affinities. The next experiment was to compare the affinities obtained when two monoclonal antibodies were coated onto the same magnetic particle (Table 2). The resulting affinities are comparable to the affinities of the

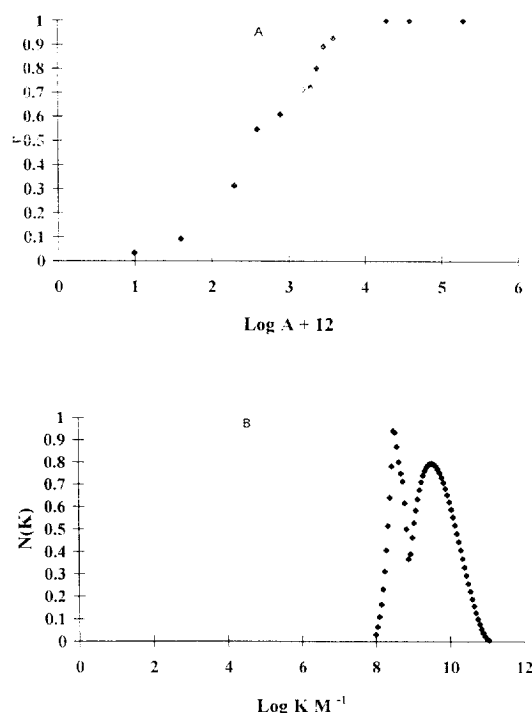


Fig. 8. Binding isotherm for serum from an individual (#93923463) containing Der p 1 specific IgE that was fit and used to generate the affinity distribution function in part B.

antibodies that were determined individually. In addition, these results seem to indicate that there are minimal effects due to avidity when using the new two-step sandwich immunoassay. In these experiments, if the two different antibodies bound to the same antigen molecule on two different epitopes, the resulting affinity distribution functions values would vary from the results obtained from the affinity distribution functions of the two antibodies determined separately.

Table 2

Comparison of K_A values derived from affinity distribution functions for Der p 1 specific monoclonal antibodies with different epitope specificities

Monoclonal Antibody	5H8D8	10B9	C4.1/102	C4.1 + 10B9 coated on different MPs ^a	5H8D8 + 10B9 coated on same MPs ^a
K_A (M^{-1})	2.8×10^{10}	1.2×10^{11}	3.5×10^9	3.9×10^9 (C4.1) and 2.2×10^{11} (10B9)	3.0×10^{10} (5H8) and 2.9×10^{11} (10B9)

^aMagnetic particles.

3.10. Affinity distribution function for serum IgE

The affinity distribution functions were determined for sera of allergic patients with measurable concentrations of Der p 1 and Amb a 1 specific IgE. The binding isotherms and affinity distribution functions are shown for two serum, each with two major clones of Der p 1 specific IgE (Fig. 8) and Amb a 1 specific IgE (Fig. 9). Multiple polynomial smoothing functions were applied to the binding isotherms as in Fig. 6 (data not shown), and the affinity distribution functions calculated for each serum sample.

In order to ascertain that the form of the distribution was not unique to Der p 1, and the method is general, we biotinylated purified Amb a 1 and carried out a series of studies. The form of the IgE distribution was obtained with equal ease (Fig. 9). The most probable K_A values for the Amb a 1 IgE affinity distributions were, as a rule, similar to the most probable K_A values obtained for the Der p 1 IgE affinity distributions.

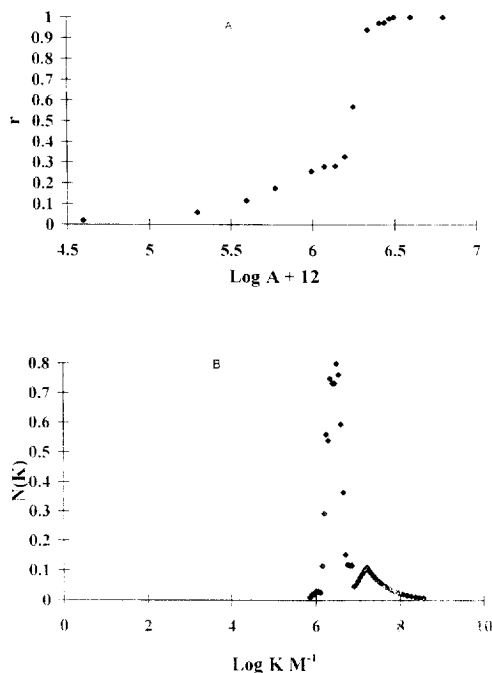


Fig. 9. Binding isotherm for serum from an individual (#93915542) containing Amb a 1 specific IgE that was fit and used to generate the affinity distribution function in part B.

4. Discussion

In order to be able to calculate the affinity distribution function for a polyclonal antibody response, we need a complete binding isotherm with a well-defined state of ligand saturation. We also need to find conditions where ligand crosslinking of the two binding sites of an immunoglobulin is minimal to allow us to use affinity constants instead of the less well-defined avidity (Berzofsky et al., 1993). Many of the previously used methods suffer from this and other drawbacks.

Most methods use 'forward' solid-phase strategies in which allergen is absorbed to a solid surface prior to binding with an unknown quantity of antibody. However, there are well-known inherent problems with these methods that question the validity of these methods in determining the affinity of antigen-specific antibodies. Proteins tend to undergo denaturation, and allergenic determinants may be lost when adsorbed to a solid phase (Soderquist and Walton, 1980). Also, chemical manipulations of antibody during the labeling process may influence antibody binding capacity, thereby lowering the value of the association constant, K_A . Finally, competition between antigen-specific IgG and IgE may result in false affinity determinations (Haba and Nisonoff, 1985).

An indirect ELISA avoids many of the pitfalls associated with other enzyme-linked immunoassays as reviewed by Pesce and Michael (1992), yet it only permits the determination of affinity up to $1 \times 10^9 \text{ M}^{-1}$. One attempt made so far to establish the affinity of IgE present in very low levels used murine monoclonals and an ELISA-based assay using fluorescence in which the affinity was determined under a multitude of assumptions including using only a fraction of the binding curve (Pathak et al., 1989). A PRIST method, where the antibody instead of the antigen was immobilized, has been used to determine the affinity of human cow hair and dander-specific IgE from the classical Scatchard analysis, but again only a fraction of the binding curve was used (Prah and Nexø, 1982). A Scatchard or Klotz analysis is only applicable for nonheterogeneous populations or populations of limited heterogeneity of antibody.

Attempts have been made to accurately describe the distribution of antibody affinities (reviewed by

Karush, 1962). Hunston (1975) developed a theoretical approximation method to treat ligand binding data. This method was further refined and shown theoretically to be able to describe the distribution of affinities from ligand binding data (Thakur et al., 1980). This type of affinity distribution function has not been applied to experimental data, to the best of our knowledge.

Heterogeneity has mostly been characterized by an index of heterogeneity, described by Sips (1948), which includes the doubtful assumption that the affinities fit a distribution similar in shape to a normal distribution. Attempts to use the Shannon coefficient as an index of a polyclonal response have not been very successful showing low resolving power between a monoclonal and polyclonal response (Rath et al., 1988). The affinity distribution function gives far better resolving power and gives more information than heterogeneity coefficients and histograms.

The best previous method for affinity determination is surface plasmon resonance, which can detect affinities up to $1 \times 10^{11} \text{ M}^{-1}$ (Malmquist, 1993). This method needs 0.07 nM of specific antibody on a solid state and solutions required for coupling are in the μM range. This is a kinetic method, and in the case of heterogeneity of binding constants, we have to resolve exponentials both for on and off rates, which is a notoriously difficult problem. Even if rates could be determined for a heterogeneous response, the problem of matching on and off rates remains.

Our approach avoids previous problems with allergen immobilization and labeling methods that lead to inconsistencies as reviewed in Azimzadeh et al. (1992). In our method, serum IgE is captured before incubation with labeled allergen avoiding the competition between IgG and IgE (Haba and Nisonoff, 1985), allowing a sampling of all IgE antibody populations (low and high affinity) present in the serum sample. A further advantage is that there is no restrictions on the binding between the antibody and the epitopes presented by the allergen in solution, resulting in binding at a variety of different sites. Considering the distance between the two antibody binding arms of the antibody is at least 150 Å (Posner et al., 1991), and that the structure of the Der p 1 molecule is clearly asymmetric (Topham et

al., 1994), the crosslinking of immobile IgE is very unlikely in contrast to assays with immobilized antigen.

Once more, we summarize here the experimental arguments in support of our contention that we are measuring affinity distribution and not avidity. (1) Inverse plot of monoclonals give very fine straight lines, although we are dealing as in the case of IgE with bivalent antibody. (2) Mixtures of monoclonals recognizing different epitopes give binding curves resolvable into its constituents. (3) Raw binding isotherms show clear stable saturation curves.

No work is ever free of the possibility of having a hidden variable present. At our level of precision, we see no sign of such a variable. Although it remains a possibility that avidity may play a role when measuring the binding of much larger theoretical antigens. In the concentration range of the captured antibody, it remains highly improbable.

The biotinylation of the antigen did not appear to affect the binding between the antigen and the antibody based on the results from the competition between labeled and unlabeled antigen. Although, it is recommended to perform an inhibition assay using 50% competition between labeled and unlabeled antigen when using a new system to check for the effects of the biotinylation procedure.

Our method exhibits a 4% error in replicability of duplicates as shown in Section 2. The high reproducibility is due to the precision of the automated Access[®] system (Patterson et al., 1994). The use of chemiluminescent detection gives a better signal to noise ratio than fluorescent detection system due to decrease in background. This allows us to determine complete binding curves at extremely low levels of specific antibody. Using serum with Der p 1 specific IgE level of 0.193 ng/ml we have, after a 1:10 dilution, 19.3 pg specific IgE per ml. One hundred microliters is added to each reaction vessel. This leaves 1.93 pg attached to the solid state. If 25% of the binding sites are saturated with Der p 1 (0.25 ng/ml), this corresponds to the presence of 0.6 pg of antibody complexes. We observe for this, a signal 3 times above the background. We believe that 150 pg/ml of specific IgE in serum represents at present the limit for obtaining a full binding isotherm. The limits of affinity we can measure are given for high affinity by difficulty in determining true free ligand

concentration for $K_A > 1 \times 10^{11} \text{ M}^{-1}$. The solid state IgE concentration has to be diminished radically with loss in signal-to-noise ratio. For low-affinity limit, we recognize that $k_{\text{off}} \geq 10^{-3} \text{ s}^{-1}$ leads to appreciable loss of bound ligand during the steps following equilibration. The method is less suitable for $K_A < 1 \times 10^6 \text{ M}^{-1}$ affinities, for which the off rates have been shown to approach $1 \times 10^{-3} \text{ s}^{-1}$. The optimal affinity range for our method is $1 \times 10^6 < K_A < 1 \times 10^{11} \text{ M}^{-1}$.

Previous studies report the affinity of murine IgE monoclonal antibodies to be 1×10^8 to $1 \times 10^9 \text{ M}^{-1}$ (Pathak et al., 1989). Another study determined the affinity of cow hair and dander-specific human IgE to be in the range of 1 to $150 \times 10^9 \text{ M}^{-1}$ (Prah and Nexø, 1982). A more recent study has determined the range of Amb a 5 specific IgE in allergic patients to be 2.6×10^{11} to $9.0 \times 10^9 \text{ M}^{-1}$ (Kim et al., 1996). The distribution functions for our serum Der p 1 specific IgE (Table 1) fits well into this range.

The method is fully automated from the production of the binding curve to the computer-generated affinity distribution function. In addition, our method does not require high cost-specific instrumentation. The Access[®] instrument, available in many clinical laboratories, can be used for affinity determinations without any modifications to the currently available system.

This method is a promising tool for future studies, where there is a question concerning the role that the affinity of the immune response plays in the outcome of a disease process. It can be easily adapted to study the effects of affinity on a variety of biological responses.

References

- Azimzadeh, A., Pellequer, J.L., Van Regenmortel, M.H.V., 1992. Operational aspects of antibody affinity constants measured by liquid-phase and solid-phase assays. *J. Mol. Recogn.* 5, 9–18.
- Berzofsky, J.A., Berkower, I.J., Epstein, L., 1993. Antigen-antibody interactions and monoclonal antibodies. In: Paul, W.E. (Ed.), *Fundamental Immunology*. Raven Press, New York, pp. 421–465.
- Bevington, P.R., 1969. *Data Reduction and Error Analysis for the Physical Sciences*. McGraw-Hill, New York.
- Ceska, M., Lundkvist, U., 1972. A new and simple radioimmunoassay method for the determination of IgE. *Immunochemistry* 9, 1021–1030.
- Chandrasekhar, S., 1943. Stochastic problems in physics and astronomy. *Rev. Mod. Phys.* 15, 1–89.
- Chapman, M.D., Platts-Mills, T.A.E., 1980. Purification and characterization of the major allergen from *Dermatophagoides pteronyssinus*-antigen p₁. *J. Immunol.* 125, 587–592.
- Chapman, M.D., Rowntree, S., Mitchell, E.B., Di Prisco de Fuenmajor, M.C., Platts-Mills, T.A.E., 1983. Quantitative assessments of IgG and IgE antibodies to inhalant allergens in patients with atopic dermatitis. *J. Allergy Clin. Immunol.* 72, 27–33.
- Cooper, L.J.N., Robertson, D., Granzow, R., Greenspan, N.S., 1994. Variable domain-identical antibodies exhibit IgG subclass-related differences in affinity and kinetic constants as determined by surface plasmon resonance. *Mol. Immunol.* 31, 577–584.
- Eisen, H.N., 1980. *Immunology*, 2nd edn. Harper and Row, MD.
- Gretch, D.R., Suter, M., Stinski, M.F., 1987. The use of biotinylated monoclonal antibodies and streptavidin-affinity chromatography to isolate herpes virus hydrophobic proteins or glycoproteins. *Anal. Biochem.* 169, 270–277.
- Haahtela, T., Jaakonmaki, I., 1981. Relationship of allergen-specific IgE antibodies, skin prick tests and allergic disorders in unselected adolescents. *Allergy* 36, 251–256.
- Haba, S., Nisonoff, A., 1985. Quantitation of immunoglobulin E antibodies by radioimmunoassay in the presence of high concentrations of non-immunoglobulin E antibodies of the same specificity. *J. Immunol. Methods* 85, 39–52.
- Hunston, D.L., 1975. Two techniques for evaluating small molecule-macromolecule binding in complex systems. *Anal. Biochem.* 63, 99–109.
- Karush, F., 1962. Immunologic specificity and molecular structure. *Adv. Immunol.* 2, 1–40.
- Kim, K.-E., Rosenberg, A., Roberts, S., Blumenthal, M.N., 1996. The affinity of allergen specific IgE and the competition between IgE and IgG for the allergen in Amb a V-sensitive individuals. *Mol. Immunol.* 33, 873–880.
- King, T.P., Norman, P.S., Connell, J.T., 1963. Isolation and characterization of allergen from ragweed pollen II. *Biochemistry* 3, 458–469.
- Klotz, I.M., Darnall, D.W., Langerman, N.R., 1953. In: Neurath, H., Hill, R.L. (Eds.), *Quaternary Structure of Proteins in the Proteins*, Vol. 1, Chap. 8. Academic Press, New York.
- Lomardero, M., Heymann, P.W., Platts-Mills, T.A.E., Fox, J.W., Chapman, M.D., 1990. Conformational stability of B cell epitopes on group I and group II *Dermatophagoides* spp. allergens. Effect of thermal and chemical denaturation on the binding of murine IgG and human IgE antibodies. *J. Immunol.* 144, 1353–1360.
- Malmquist, M., 1993. Surface plasmon resonance for detection and measurement of antibody-antigen affinity and kinetics. *Curr. Opin. Immunol.* 5, 282–286.
- Neter, J., Wasserman, W., 1974. *Applied Linear Statistical Models*. Richard D. Irwin, Homewood, IL, pp. 113–121, 509.
- Pathak, S.S., Vos, Q., Savelkoul, H.F.J., 1989. Terasaki-ELISA

- for murine IgE: III. Determination of concentration and functional affinity by sequential equilibrium binding analysis. *J. Immunol. Methods* 123, 71–81.
- Patterson, W., Werness, P., Payne, W.J., Matsson, P., Leflar, C., Melander, T., Quast, S., Stejskal, J., Carlson, A., Macera, M., Shubert, F.W., 1994. Random and continuous-access immunoassays with chemiluminescent detection by Access[®] Automated Analyzer. *Clin. Chem.* 40, 2042–2045.
- Pesce, A.J., Michael, J.G., 1992. Artifacts and limitations of enzyme immunoassays. *J. Immunol. Methods* 150, 111–119.
- Posner, R.G., Erickson, J.W., Holowka, D., Baird, B., Goldstein, B., 1991. Dissociation kinetics of bivalent ligand immunoglobulin E aggregates in solution. *Biochemistry* 30, 2348–2356.
- Prahl, P., Nexø, E., 1982. Human serum IgE against two major allergens from cow hair and dander: Determination of affinity and quantity of antigen-specific IgE. *Allergy* 37, 49–54.
- Rath, S., Stanely, C.M., Steward, M.W., 1988. An inhibition enzyme immunoassay for estimating relative antibody affinity and affinity heterogeneity. *J. Immunol. Methods* 106, 245–249.
- Sips, R., 1948. On the structure of a catalyst surface. *J. Chem. Phys.* 16, 490–495.
- Soderquist, M.E., Walton, A.G., 1980. Structural changes in proteins adsorbed on polymer surfaces. *J. Colloid Interface Sci.* 75, 386–397.
- Sorensen, K., 1996. Biotinylation of a small amount of protein using a single dialysis cassette. *Am. Biotechnol. Lab.* 14, 30–31.
- Steward, M.W., 1981. The biological significance of antibody affinity. *Immunol. Today* (July) 134–140.
- Thakur, A.K., Munson, P.J., Hunston, D.L., Rodbard, D., 1980. Characterization of ligand-binding systems by continuous affinity distributions of arbitrary shape. *Anal. Biochem.* 103, 240–254.
- Topham, C.M., Srinivasan, N., Thorpe, C.J., Overington, J.P., Kalsheker, N.A., 1994. Comparative modelling of major house dust mite allergen Der p 1: Structure validation using an extended environmental amino acid propensity label. *Protein Eng.* 7, 869–894.
- Van der Zee, J.S., van Swieten, P., Jansen, H.M., Aalberse, R.C., 1988. Skin tests and histamine release with P₁-depleted *Der-matophagoides pteronyssinus* body extracts and purified P₁. *J. Allergy Clin. Immunol.* 81, 884–896.
- Wilkinson, G.N., 1961. Statistical estimations in enzyme kinetics. *Biochem. J.* 80, 324–332.
- Zeder-Lutz, G., Altschuh, D., Geysen, H.M., Trifilieff, E., Sommermeyer, G., Van Regenmortel, M.H.V., 1993. Monoclonal antipeptide antibodies: Affinity and kinetic rate constants measured for the peptide and the cognate protein using a biosensor technology. *Mol. Immunol.* 30, 145–155.