

Amplification of the enzyme-linked immunosorbent assay for measuring allergen-specific IgE and IgG antibody

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Summary

The amplified enzyme-linked immunosorbent assay (a-ELISA) has been successfully applied to the measurement of allergen-specific IgE and IgG antibodies. Data presented indicate a high positive correlation among measurements of IgE-specific antibodies to ragweed antigen E using the a-ELISA, RAST and radiometric assay of Zeiss. Similarly, a high correlation between allergen-specific IgG antibodies measured by the a-ELISA and Farr assay was also observed. Data presented indicate that the measurement of serum IgE antibodies in the linear region of the ELISA titration plot is unaffected by competition with IgG antibodies. The assay is sensitive, reliable, and without risk to laboratory personnel. It is capable of measuring both IgE and IgG antibodies in patients using the same assay system.

Introduction

In recent years, the immunology of allergic diseases has been explored with great success using radioimmunoassays to measure IgE and IgG antibodies (Wide, Bennich & Johansson, 1967; Zeiss *et al.*, 1973; Gleich, Adolphson & Yunginger, 1980). Assays which employ radioactive materials are associated with attendant risks and problems; the materials are relatively unstable, costly, and potentially hazardous. The advantages of enzyme-linked immunoassays for measuring allergen specific antibody are numerous. These include the safety of using very stable enzyme-antibody complexes without the risk associated with radioactive materials, the ease and quickness of performing the test, and the use of relatively inexpensive materials. The enzyme-linked immunosorbent assay (ELISA) first described by Engvall & Perlmann (1972) has been modified to improve its sensitivity and to eliminate the need for specifically purified antiglobulins which are needed to prepare antibody-enzyme conjugates (Butler, McGivern & Swanson, 1978). The latter feature is especially important when antibody

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with subclass or of minor immunoglobulin classes, such as IgE, is measured. This modification, termed the amplified ELISA (a-ELISA), has been successfully used to measure serum and secretory antibodies of different classes and subclasses in rats, rabbits and cattle (Butler *et al.*, 1978; Butler, Cantarero & McGivern, 1980a; Butler *et al.*, 1980b; Butler, 1981). Amplification is achieved through the use of soluble anti-enzyme immune complexes bound to the antiglobulin (anti-IgG, anti-IgE, etc.) through the use of a 'bridging' antibody. The use of anti-enzyme immune complexes saves preparatory time, increases sensitivity, and detects antibody of various classes by changing only the 'primary' antiglobulin without having to conjugate a new antibody to an enzyme. Antibody-enzyme complexes eliminate the potential for the loss of antibody or enzyme activity through modification by covalent bonds.

We report here the application of the a-ELISA to measure allergen-specific IgE and IgG antibodies in humans. This methodology is compared to standard radiometric methods of measuring serum allergen-specific antibody.

Materials and methods

Preparation and source of antigens

A partially purified ragweed extract was prepared after the method of Patterson *et al.* (1978). Equal parts of short ragweed (*Ambrosia elator*) and giant ragweed (*Ambrosia trifida*) pollens (Hollister-Stier Laboratories, Spokane, WA) were ether defatted and extracted in isotonic PBS at 4°C for 24 hr. The extract was precipitated in 90% saturated ammonium sulphate and solubilized with PBS. Following dialysis with a Spectrapore No. 3 membrane (Spectrum Industries, Los Angeles, CA), the extract was chromatographed over a Bio-Gel p-2 column (2.5 × 30 cm) (Bio-Rad Laboratories, Richmond, CA). The protein content of the excluded portion of the column was determined by the microbiuret method. This preparation was called ragweed extract (RGW). A portion of the RGW was radiolabelled for binding studies with ¹³¹I using the chloramine-T method (McConahey & Dixon, 1966). Other antigens studied included a commercial lyophilized extract of *Alternaria* species (Center Laboratories, Port Washington, NY) and a purified extract of ragweed Antigen E kindly supplied to us by Dr Sylvia Cunningham of the NIH.

Immunochemical reagents

Rabbit antiserum to human IgE was obtained commercially (Behring Diagnostics, Sommerville, NJ). This antiserum was absorbed twice prior to use in the a-ELISA on an affinity column containing human IgG (Cautrecassas & Anfinsen, 1971) to remove cross-reacting contaminants. The human IgG was isolated from the 33% saturated ammonium sulphate insoluble fraction of whole human serum by ion-exchange chromatography on DEAE-Cellulose (DE-52, Whatman). The protein eluted in the 'fall-through' fraction from a column equilibrated with 0.01 M phosphate buffer, pH 8.0 was determined to contain only IgG.

Rabbit antiserum to human IgG was raised in rabbits by toepad injection of 3 mg of purified IgG emulsified in Freund's complete adjuvant. Rabbits were boosted by i.v. injection of 0.5 mg of IgG 30 days later and the serum collected 1 week after the booster.

The preparation of guinea-pig anti-rabbit IgG and rabbit antisera to alkaline phosphatase (calf intestine, Type IV, Sigma Chemical Co., St. Louis, MO) have been

described previously (Butler *et al.*, 1978). The soluble anti-alkaline phosphatase immune complex was prepared by solubilization of the equivalence immune precipitate with excess enzyme as described elsewhere (Butler *et al.*, 1978; Butler, in press).

Measurement of antibody using the a-ELISA

RGW and other antigen were adsorbed to the wall of polystyrene tubes (12×55 mm) (Sarstedt Laboratories, West Germany) by incubation in a roller drum at 37°C for 3 hr in the presence of 0.1 M sodium carbonate buffer, pH 9.6 (Engvall & Perlmann, 1972; Butler *et al.*, 1978) (Fig. 1). The adsorptive characteristics of RGW were measured using radiolabelled RGW as described by Cantarero, Butler & Osborne (1980). Antigen-coated tubes were washed twice with 0.05% PBS-Tween® 20 (PBS-T) buffer, and serial dilutions of patient sera were added and incubated overnight in a roller drum at room temperature. These tubes, which contained antigen-bound specific antibody, were washed twice in PBS-T buffer and a 1/500 or 1/1000 dilution of rabbit anti-human IgE or IgG respectively was added.

Following the addition of secondary antibody, the tubes were again washed and guinea pig anti-rabbit IgG-bridging antibody was added. The reaction was incubated for 2 hr at room temperature. The tubes were washed and a 1/2000 dilution of the anti-enzyme soluble complex added. The reaction was again incubated for 2 hr at room temperature and the substrate, p-nitrophenyl phosphate (1 mg/ml) (Sigma Chemical Co., St Louis, MO), was added at timed intervals in 0.05 M sodium carbonate buffer, pH 9.8 at room temperature. The reaction was stopped with 0.1 ml, 0.1 N NaOH, and the optical density (OD) was read spectrophotometrically at 400 nm on a Beckman 24 spectrophotometer (Beckman Instruments, Creve Coeur, MO) equipped with an automatic 'sipper' device.

Test sera

Sera used for the radiometric assays and a-ELISA were obtained from volunteer

ELISA for Ragweed Specific Antibody

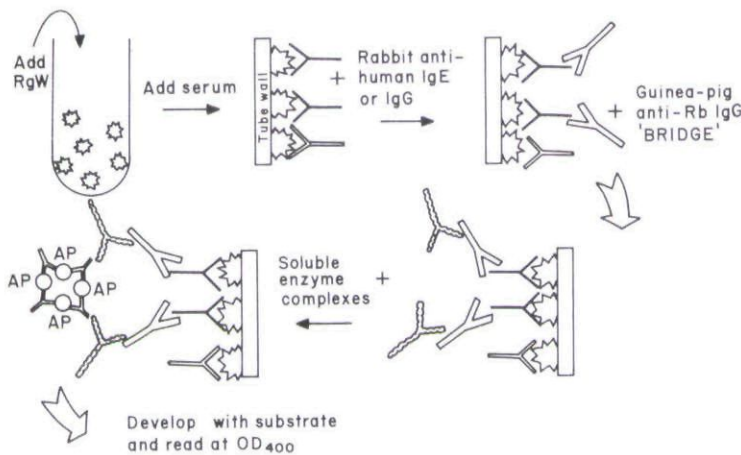


Fig. 1. Schematic flow diagram of the multiple steps required to measure ragweed-specific antibody of the IgE or IgG class. Addition steps move from adsorption of antigen to polystyrene tube (left upper corner) to addition of soluble complexes (lower left corner). After each step, tubes are washed twice with PBS-T to remove unbound reactants.

donors who had ragweed sensitive pollenosis. Sera was also obtained from normal, non-atopic donors, and all samples were stored at -20°C until examined. The five serum samples and the values for allergen-specific antibody shown in Table 3 were kindly provided by Dr C. R. Zeiss, Northwestern University Medical School. The values for antigen-E specific IgE were determined by the polystyrene tube radio-immunoassay (PTRIA) of Zeiss (Zeiss *et al.*, 1973). Antigen E-specific IgG antibody was determined by a modification of the Farr technique (Lidd & Farr, 1962). These sera were obtained from subjects who had been treated with high dose allergy immunotherapy according to a specific experimental protocol using an antigen extract prepared in a manner similar to RGW (Becal *et al.*, 1978).

Results

Antigen coupling

Figure 2 shows the binding behaviour of ^{131}I -RGW antigen and indicates that the maximal percentage binding of antigen to the tube wall occurred when between 500 and 600 ng RGW were added. More RGW adhered to the wall of the tube when large amounts of antigen were added, although a smaller percentage of the added antigen was bound. Therefore, 500 ng of added RGW was chosen as the amount to add to each polystyrene tube in order to conserve antigen and guard against the possibility that

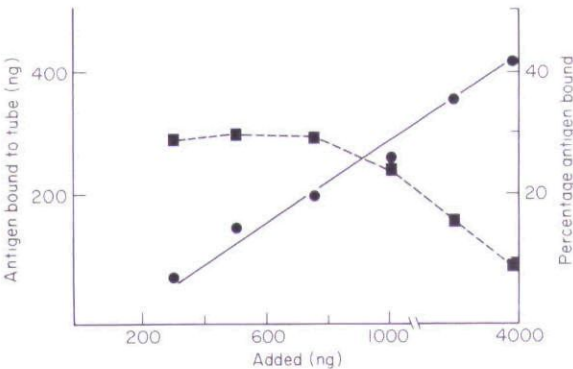


Fig. 2. Characteristics of binding of partially purified ragweed antigen to polystyrene tubes. Dashed line (■---■) represents percentage antigen bound. Solid line (—) represents nanograms of antigen bound to tube after incubation.

Table 1. Effect of temperature on antigen binding in polystyrene tubes

RGW antigen added (ng)	Conditions	Percentage bound	Antigen bound (ng)
860±2	1 hr, RT*	20·6	177
	3 hr, RT	21·1	183
	1 hr, 37 °C	22·1	191
	3 hr, 37 °C	26·3	226

* RT=room temperature.

larger amounts of bound antigen might be less stable during the assay (Cantarero *et al.*, 1980). Incubation of the tube for 3 hr at 37°C resulted in a slightly higher percentage of bound antigen compared to a 1 hr incubation at room temperature (Table 1).

Calculation of antibody activity

Figure 3 shows examples of typical sigmoidal curves obtained from an a-ELISA titration where the log of OD₄₀₀ is plotted against the log of the serum dilution.

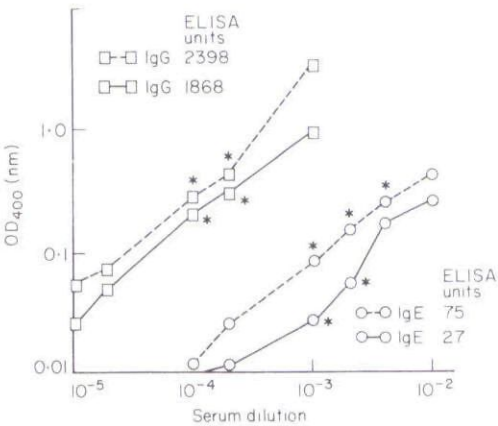


Fig. 3. Demonstration of typical titration curves obtained by plotting mean optical density of triplicate samples corrected for normal serum background against serum dilution on full logarithmic graph paper. (---), Sample a, Ragweed specific IgG (□) and IgE (○), (—), sample b, Ragweed specific IgG (□) and IgE (○). *Only those points indicated by asterisks were used to calculate ELISA units.

Table 2. Demonstration of the calculation of ELISA units (EU) for IgG and IgE antibody activity

Sample 1*	Serum dilution	OD ₄₀₀ §	OD ₄₀₀ (Corrected)†	Dilution factor	Result	EU
IgG antibody activity	1/1000	2.500	1.995	1000	1995	2434‡
	1/3000	1.176	0.819	3000	[2457]	
	1/10000	0.523	0.241	10000	[2410]	
	1/30000	0.569	0.282	30000	8460	
IgE antibody activity	1/50	0.832	0.431	50	21.6	70.1‡
	1/100	0.651	0.383	100	38.3	
	1/250	0.529	0.248	250	[67.0]	
	1/500	0.401	0.149	500	[74.5]	

* Serum sample no. 1 is also shown on Table 3. The EU values represent those of a patient treated with high dose allergy immunotherapy.
† Corrected indicates OD sample—OD of a serum pool of ten normal, non-allergic donors.
‡ Mean of values which are in agreement and follow the straight line portion of a plot of log₁₀ of the corrected OD₄₀₀ and log₁₀ serum dilution factor.
§ OD₄₀₀= optical density at 400 nm.
¶ Brackets indicate those values which were averaged to obtain EU because they fell in the linear portion of the log-log plot.

Normally, patient serum dilutions in the range of 1/50–1/500 were optimal for measurement of IgE antibodies and a dilution range of 1/1000–1/30 000 was appropriate for IgG titration. Only the near-linear portion of the curve was used to calculate ELISA units (EU) of antibody activity by multiplying the OD₄₀₀ (corrected for normal human serum) × the reciprocal of the appropriate serum dilution (Table 2). The slope of the straight line portion of the log-log plot was approximately 45°, making it possible to obtain the approximate same value in EU when OD measurements fell in the linear region of the titration (Table 2). EU values differing by a coefficient of variation (c.v.) of less than 15% were considered to be in the linear portion of the curve (Butler *et al.*, 1980a). The mean of values falling into this range were averaged to express the EU activity of the particular serum (Table 2).

Serum samples were assayed in triplicate at each dilution and averaged to obtain a mean OD₄₀₀ value. Standard deviations ranged from 0.001 to 0.07. The largest standard deviations occurred when large OD₄₀₀ values were reached near the curvilinear portion of the log-log plot. These values usually did not enter into calculations since they did not fall in the linear portion of the log-log curve. Five samples were analysed for IgE and IgG on two or three occasions over a three month period. The variation in values obtained during this period had an average coefficient of variation of 12.6%.

Comparison to other radiometric assays

Allergen-specific IgE measurements obtained using the a-ELISA were compared with those obtained by the RAST (radioallergosorbent test) (materials kindly donated by Pharmacia, Inc., Piscataway, NJ) and to those obtained using the radiometric method of Zeiss *et al.* (1973). Figure 4 shows the highly significant correlation between the data for the IgE-ragweed-specific-antibody measured by RAST and the a-ELISA ($\tau=0.7$, $P<0.001$) in sera obtained from a group of normals and atopic subjects who were untreated.

Table 3 shows the comparison between the values for serum specific IgE measured by the a-ELISA and by the radiometric method of Zeiss. Also shown are comparative data obtained with the a-ELISA and modified Farr assay for the measurement of specific IgG antibodies. The relative ranking determined by the a-ELISA and the Zeiss assay using Antigen E adsorbed to the tube was similar. When RGW was used as the antigen, more antibody activity was detected, and in one case the ranking of the relative antibody activity was reversed (Table 3, patients 4 and 5). Using the values for

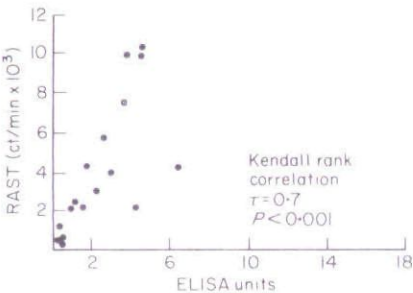


Fig. 4. Correlation for ragweed-specific IgE determined by RAST and a-ELISA. ● = Individual serum sample, atopic, untreated subject or normal.

Table 3. Comparison of antigen specific IgE and IgG antibody using the Zeiss, Farr, and a-ELISA methods.

Subject Rank	Antigen-specific IgE antibody		
	Zeiss-AgE* (ng/ml)	a-ELISA (EU)† AgE§	a-ELISA (EU)-RGW‡§
1	828	21.1	70.1
2	513	15.9	64.3
3	245	9.0	47.7
4	335	4.8	20.0
5	122	2.0	12.5

Subject Rank	Antigen-specific IgG antibody		
	Farr Assay-AgE(ng/ml)	a-ELISA (EU)¶ AgE	a-ELISA (EU)§-RGW
1	9600	656	3608
2	8400	549	2501
3	2880	218	2434
4	1600	64	394
5	920	55	977

* AgE = Antigen E, adsorbed to polystyrene tubes.

† EU = ELISA units, antibody activity.

‡ RGW = ragweed antigen, adsorbed to polystyrene tubes.

§ Kendall rank correlation, $\tau = 0.8$.¶ Kendall rank correlation, $\tau = 1.0$.

antigen-specific IgE determined by Zeiss, we calculated that the a-ELISA could detect approximately 500 pg of IgE antibody activity. Table 4 also shows a very high correlation for specific IgG levels and ranking when data determined by the a-ELISA and Farr assay are compared.

Discussion

The ELISA avoids some of the disadvantages of the radiometric assays while retaining many of their important features. The use of the ELISA for the detection of antibody is attractive because: (1) there is no immediate risk to those handling the materials and there is little potential for any long-term risk, (2) normally the cost of the components and equipment is low, (3) antibody-enzyme conjugates are stable and are more easily stored for extended periods of time than radiolabelled reagents, (4) the sensitivity of the assay is the same as for radiometric assays, and (5) the ELISA is highly adaptable to commercial use, or use 'in the field' with minimal need for equipment since the results can be determined visually.

The a-ELISA is an improvement over the standard ELISA for the measurement of antibodies from minor classes because it is more sensitive and it alleviates the need for specifically purified antiglobulin from which to prepare antibody-enzyme conjugates. The anti-enzyme soluble complexes of the a-ELISA have less potential for reduced

antibody capacity or enzyme activity which may result from conventional conjugation by glutaraldehyde (Engvall *et al.*, 1972).

These data demonstrate that the a-ELISA can be effectively used to measure allergen-specific antibodies of the IgE or IgG class. The high positive correlation among specific IgE data obtained using the a-ELISA, RAST and the Zeiss method are very reassuring and somewhat surprising. The surprising observation is that the radiometric method of Zeiss is designed to eliminate competition between blocking IgG and allergen-specific IgE in the assay procedure (Zeiss *et al.*, 1973). The high correlation with the data of Zeiss suggests that competition between specific IgG and IgE is not a factor in the a-ELISA if EU values are calculated from the linear log-log titration. The close correlation between specific IgG data obtained with the a-ELISA and the Farr assay means that antibodies of both classes (IgG and IgE) can be measured using the same method. This is always a desired experimental situation when comparative data is the aim of the detection assay. We have used the a-ELISA to measure ragweed specific IgG and IgE in a group of 100 patients treated with allergy immunotherapy (Metzger *et al.*, 1981; Metzger *et al.*, 1980). Its usefulness in that situation was demonstrated by its ability to detect the expected dramatic rise in blocking antibody. Confirmation by RAST for allergen-specific IgE in serum was also obtained.

The a-ELISA is highly adaptable to measuring many different antigen-specificities (Voller, Bartlett & Bidwell, 1978). It is limited only by the binding characteristics of the antigen to the solid phase support and perhaps its purity. In this regard, we have also measured changes in *Alternaria*-specific antibodies following repeated bronchial challenge in asthmatic subjects (Metzger *et al.*, 1981).

The greater sensitivity of the a-ELISA may be due to the addition of a bridging antibody or the use of enzyme-antibody complexes rather than conjugated enzyme. The latter eliminates the effect of attaching a large molecular weight substance to the antibody which may influence the reactivity of the molecule (Voller *et al.*, 1978).

Currently, the EU provides only an estimate of relative antibody activity, but efforts are in progress to prepare standard curves for absolute antibody quantitation similar to those in use for other species (Butler *et al.*, 1980a, b; Butler, 1981). The a-ELISA currently requires time allotments from a 48-hr period to complete an assay. However, current work using microtitre wells indicates the capability of contracting the entire assay into just one work-day requiring only 3 hr of actual technical time with additional conservation of reagents and buffers.

In summary, we believe that the data presented indicate that the a-ELISA is an improved method of measuring allergen-specific antibody of the IgE and IgG classes which has certain advantages over standard radiometric assays. Its reliability and sensitivity combined with automation may allow it to be commercialized, therefore, making it more accessible than current radiometric assays for detection of allergen-specific antibodies.

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