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Capture assay for specific IgE

An improved quantitative method

V. Olivieri, I. Beccarini, G. Gallucci, T. Romano and F. Santoro

Radim S.p.A., R.&D. Laboratories, Via del Mare 131, 00040 Pomezia (Rome), Italy

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A capture assay for the measurement of specific IgE in the serum of allergic patients is described, using monoclonal anti-human IgE (coated to the wells of a microtiter plate) and biotinylated allergens in solution. In a single incubation, IgE is bound to the solid phase through the Fc fragment and biotinylated allergens react with their specific IgE Fab regions, if present. In a second step, streptavidin-HRP conjugate is added to reveal the amount of biotin fixed on the solid phase. Quantitative determinations are obtained by comparison with a standard curve of total IgE incubated with a biotinylated monoclonal anti-IgE, complementary to the one employed as capture antibody. The assay is unaffected by allergen-specific IgG, shows good intra- and interassay reproducibility and is linear over a wide range of specific IgE concentrations. The method could be used with a wide range of different allergens.

Key words: Specific IgE assay; Allergen

Introduction

The RAST technique, developed by Wide and co-workers (Wide et al., 1967), permitted for the first time the detection of allergen-specific IgE in human serum. This method, employing cellulose paper discs as the allergen-carrier solid phase (Ceska et al., 1972), has been widely used for in vitro diagnosis of IgE-mediated allergy, despite

some theoretical and practical limitations. Among the former is possible interference by allergen-specific IgG, which could result in an underestimation (Zeiss et al., 1981), and the use of different solid phases for standard and samples, with the resulting impossibility of obtaining a true quantitative assay of specific IgE. From the practical point of view RAST assays are difficult to automate and are time-consuming, although some modifications of the classical procedure have shortened the incubation times (Pecoud et al., 1986; Kemeny and Richards, 1988). Some years ago, Plebani et al. (1986) described a new assay for specific IgE, based on the capture of total IgE by a microtiter plate coated with anti-human IgE, followed by the addition of a biotinylated allergen. The binding of allergen to the specific IgE, if present, was revealed by incubating the mi-

Correspondence to: V. Olivieri, Radim S.p.A., R.&D. Laboratories, Via del Mare 131, 00040 Pomezia (Rome), Italy. Tel.: +39-6-91.08.364; Fax: +39-6-91.06.128.

Abbreviations: HRP, horseradish peroxidase; BSA, bovine serum albumin; OPD, *o*-phenylenediamine 2HCl; B-CAP-NHS, biotinyl- ϵ -aminocaproic acid *N*-hydroxysuccinimide ester; DMF, *N,N*-dimethylformamide; PBS, phosphate-buffered saline, pH 7.5.

croplate with avidin-HRP conjugate. This approach avoided the interference of allergen-specific IgG. Nevertheless the authors found a marked reduction in the level of specific IgE when high amounts of non-specific IgE were added to the sample suggesting an insufficient binding capacity of the anti-IgE coated solid phase.

In the assay described here we have been able to overcome this limitation by a careful selection of reagents and assay conditions. A quantitative estimation of the captured IgE was obtained by incubating dilutions of IgE standard in the first step of the assay with a biotinylated monoclonal anti-human IgE antibody complementary to the one employed for coating the solid phase. Streptavidin-HRP conjugate was used as the enzymatic tracer for both standard and samples.

Materials and methods

Reagents

Biotinyl- ϵ -aminocaproic acid *N*-hydroxysuccinimide ester (B-CAP-NHS) was purchased from Calbiochem, La Jolla, CA. BSA protease-free was from Interger Company, New York. Phospholipase A₂ (from bee venom), OPD tablets (15 mg), neomycin sulfate, streptomycin sulfate, amphotericin B and caprylic acid were purchased from Sigma Chemical Co., St. Louis, MO. Streptavidin and horseradish peroxidase were from Boehringer Mannheim, Germany. Purified human IgE (from myeloma serum) was purchased from Fitzgerald Industries, Chelmsford, MA. *Feldl*, purified according to Dandeu et al. (1990), was obtained from Immunopharm, France; all other allergen preparations were from Allergon, Engelholm, Sweden. Protein A-Sepharose CL-4B was obtained from Pharmacia, Uppsala. Microtiter plates (Maxisorp) were purchased from Nunc, Roskilde, Denmark. Ultrogel AcA44 was from I.B.F., France. All other chemicals were from Merck, Darmstadt, Germany.

Serum samples

Serum samples were collected from non-atopic subjects and allergic patients and stored at -20°C until assayed.

Monoclonal anti-IgE production and purification

BALB/c mice were immunized by repeated intraperitoneal injections with 50 μg of purified human IgE. Fusion of spleen cells with SP2/O-Ag 14 myeloma cells was performed according to Köhler and Milstein (1975). From the hybridoma selection, two monoclonals were chosen, both of IgG1 class, directed against two distinct, non-overlapping epitopes on the Fc fragment. The affinity constants of these reagents were 3×10^9 l/m (clone D015) and 1.5×10^9 l/m (clone D016). Antibodies were purified from ascitic fluid according to McKinney and Parkinson (1987). Briefly, ascites were diluted 1/5 in 0.06 M acetate buffer pH 4.0, before albumin and other non-IgG proteins were precipitated with caprylic acid. After centrifugation, the supernatant was dialyzed against PBS and the antibodies concentrated by ammonium sulfate precipitation.

Biotinylation

Allergens and antibodies were dissolved in 0.1 M NaHCO₃ and biotinylated by adding appropriate amounts of 25 mM B-CAP-NHS in DMF. The concentrations of protein and the added volumes of 25 mM B-CAP-NHS were as follows: for monoclonal anti-IgE D016, 1.3 mg/ml and 14 μl /ml; for *Feldl* and phospholipase A₂, 1 mg/ml and 10 μl /ml; *Lolium perenne* pollen was extracted at 2 mg/ml in 0.1 M NaHCO₃, the extract filtered (0.22 μm pore size) and added with 10 μl /ml of 25 mM B-CAP-NHS. After incubation for 3 h at room temperature, the mixtures were dialyzed against PBS overnight at 4°C . To the biotinylated products 1% BSA, 0.05% Tween 20 and 0.5 mg/ml NaN₃ were added and they were stored in aliquots at -20°C until used.

Preparation of streptavidin-HRP conjugate

4 mg of streptavidin were conjugated with 5 mg of HRP according to the NaIO₄ method of Nakane and Kawaoi (1974). The conjugate was gel filtered on Ultrogel AcA 44 column (1.6 $\times$ 85 cm) eluted in PBS. The peak corresponding to the monomeric conjugate was collected and 1% BSA, 0.1 mg/ml neomycin, 0.2 mg/ml streptomycin and 5 μg /ml amphotericin B were added before storage at 4°C . The conjugate was used for six months without detectable loss of activity.

Preparation of anti-IgE coated microplates

Purified anti-IgE (clone D015) was partially denatured before coating by short time exposure to low pH, in order to increase binding to the solid phase (Ishikawa et al., 1980). Antibodies were first diluted at 300 $\mu\text{g/ml}$ in 0.1 M citrate buffer pH 2.6 and kept for 5–10 min at room temperature, then further diluted at 10 $\mu\text{g/ml}$ in 1 M potassium phosphate buffer pH 8.5. The resulting coating solution was distributed in Nunc Maxisorp microplates (300 $\mu\text{l/well}$) and incubated overnight at room temperature. The plates were aspirated and washed four times with 0.1 M citrate buffer pH 5.0 containing 0.1% BSA, dried under vacuum and stored at 4°C.

Assay procedure

IgE standard diluted in bovine serum and serum samples were pipetted into the microplate (100 $\mu\text{l/well}$). Biotinylated anti-IgE clone D016, diluted 1/1500 in PBS containing 1% BSA, 0.05% Tween 20 and 0.5 mg/ml NaN_3 , was added to the standard wells (100 $\mu\text{l/well}$). To the samples wells, 100 μl of biotinylated allergens diluted in the same buffer were added. The working dilution of each allergen was determined by titration experiments (see results section). The microplate

was incubated for 1 h on an orbital shaker (1200 rpm) at room temperature, then washed four times with PBS-0.05% Tween 20. Streptavidin-HRP conjugate was diluted 1/3500 in 20 mM Tris-HCl buffer pH 7.5 containing 0.1 M NaCl, 1% BSA, 0.1 mg/ml neomycin, 0.2 mg/ml streptomycin and 5 $\mu\text{g/ml}$ amphotericin B and added to all wells (200 $\mu\text{l/well}$). The plate was incubated for 30 min at room temperature on an orbital shaker and then washed as above. To each well 200 μl chromogen solution (3 mg/ml OPD and 0.03% H_2O_2 in 0.15 M phosphate-citrate buffer pH 5.0) were added and the plate was incubated for 15 min at room temperature. The enzyme reaction was stopped by adding 100 μl of 2 N H_2SO_4 and the absorbance measured at 492 nm with an Anthos 2001 Reader. The concentration of specific IgE in the samples was then obtained from the standard curve.

Assay calibration

The IgE standard employed in the present method was calibrated against the 2nd I.R.P. for IgE (WHO 75/502), which contains 5000 IU/ampoule. However, since this reference preparation is intended only for calibration of total IgE assays, the specific IgE levels measured

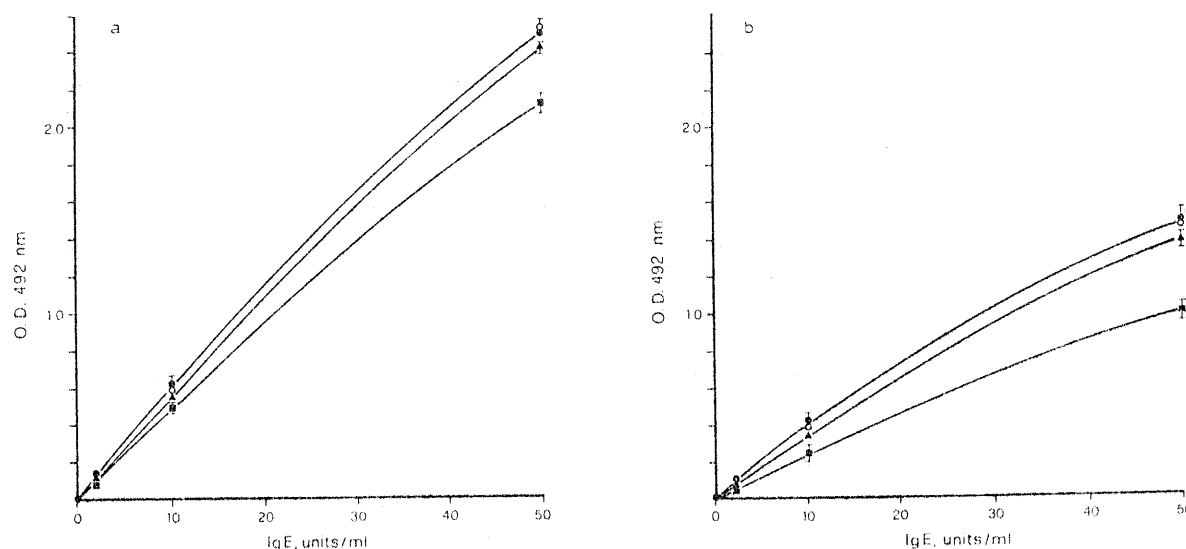


Fig. 1. Dose-response curve for IgE obtained with microplates coated with different concentrations of anti-IgE D015, with (a) or without (b) previous exposure of the antibodies to 0.1 M citrate buffer pH 2.6 for 5 min. Coating buffer was (in both cases) 1 M potassium phosphate buffer pH 8.5. Concentrations of anti-IgE in the coating solution were 20 $\mu\text{g/ml}$ (●), 10 $\mu\text{g/ml}$ (○), 5 $\mu\text{g/ml}$ (▲) and 2.5 $\mu\text{g/ml}$ (◻). Biotinylated anti-IgE D016 was diluted 1/1500. Bar = $\pm$ SD.

in the samples by the present method were expressed in U/ml.

Results

Optimal assay conditions

As shown in Fig. 1a, pre-treatment of anti-IgE at pH 2.6 before coating resulted in a marked increase in binding capacity to the microplate, as judged by the dose-response curve obtained with total IgE standard and biotinylated anti-IgE diluted 1/1500 compared to microplates coated with the same concentrations of antibody but without pre-treatment (Fig. 1b). This effect is probably due to the increased exposure of hydrophobic areas of the Fc region of the IgG molecule after partial denaturation at low pH, and was observed by Conradie et al. (1983) who also employed other denaturing procedures such as 3 M urea or high temperature.

In order to verify assay sensitivity as a function of biotinylated anti-IgE concentration, serial dilutions of this reagent were tested. The highest sensitivity was obtained with a dilution of 1/1000–1/2000 (Fig. 2), corresponding to a concentration of 1.2–0.6 $\mu\text{g/ml}$. When higher con-

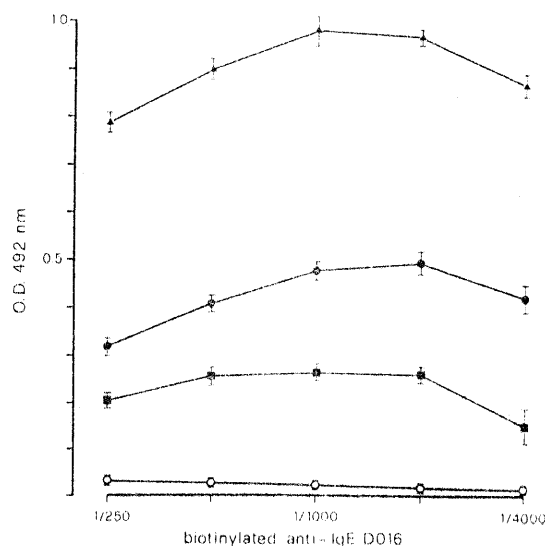


Fig. 2. Variation of assay sensitivity as a function of biotinylated anti-IgE D016 concentration. IgE standard at 20 (▲), 10 (●), 5 (■) and 0 U/ml (○) was assayed with several dilutions of biotinylated anti-IgE. Bar = $\pm$ SD.

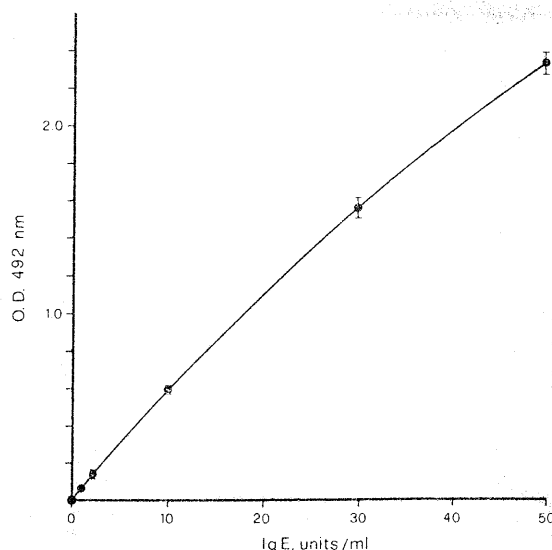


Fig. 3. Standard curve of total IgE obtained with optimized assay conditions (mean of six consecutive assays). Sensitivity limit (mean zero standard OD + 3 SD) was 0.15 U/ml. Bar = $\pm$ SD.

centrations of biotinylated anti-IgE were employed the optical density decreased, possibly because of an excessive binding of biotin to the solid phase resulting in an impaired binding of streptavidin-HRP conjugate during the second step of the assay. Indeed under conditions of reagent excess not all the biotin molecules would be accessible for binding to the streptavidin, despite the use of a spacer arm (ϵ -aminocaproic acid) between the biotin residue and the antibody.

Using the above described conditions we obtained the standard curve shown in Fig. 3. The assay sensitivity, calculated as the dose corresponding to the mean + 3 standard deviations of the zero standard optical density (determined on 20 replicates) was 0.15 U/ml.

The optimal working dilution of each biotinylated allergen was chosen on the basis of titration experiments performed with sera from allergic and non-allergic subjects. For each allergen the highest concentration was selected which gave optical densities lower than the sensitivity limit of the assay using the sera of non-allergic individuals. This corresponded to 4 $\mu\text{g/ml}$ for *Feld1*, 3.3 $\mu\text{g/ml}$ for phospholipase A_2 and 5 $\mu\text{g/ml}$ for *Lolium perenne* pollen.

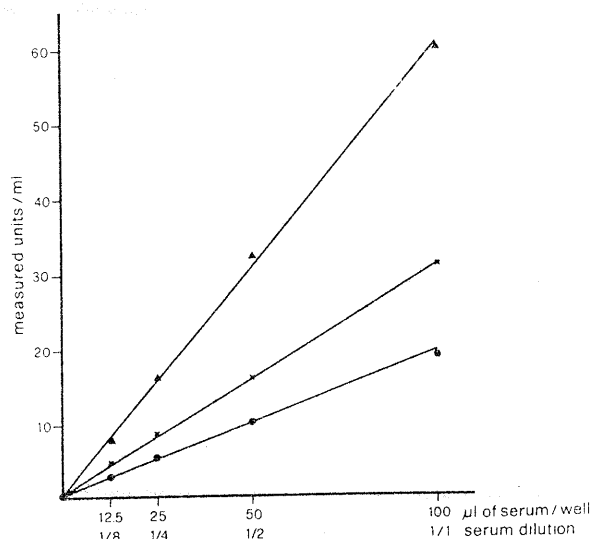


Fig. 4. Linearity test. Sera from three patients allergic to, respectively, *Lolium perenne* (▲), bee venom (×) and cat epithelium (●) when assayed at several dilutions against the standard curve showed a linear relationship between measured specific IgE and sample dilution.

Linearity test

Sera from three patients known to be allergic to, respectively, *Lolium perenne*, bee venom and cat epithelium were assayed undiluted and at different dilutions in bovine serum, in parallel with the standard curve. Fig. 4 shows that a linear relationship was found between the measured specific IgE levels and sample dilution (expressed as sample volume added to the well) on a linear-linear scale.

Assay reproducibility

Intra- and interassay variability was assessed employing three different allergens and positive sera containing high, medium and low concentrations of specific IgE. These results are shown in Table I.

Interference by high total levels of IgE

Two sera from allergic patients, one positive for cat epithelium and the other one positive for bee venom, were each mixed with different volumes of a serum containing 2200 IU/ml of total IgE, but RAST negative for both allergens. Each mixture contained 50% of allergic serum, variable amounts of total IgE and was made up to a

TABLE I

INTRA- AND INTERASSAY COEFFICIENT OF VARIATION (CV) OBSERVED ON THREE SERUM SAMPLES OF PATIENTS ALLERGIC TO DIFFERENT ALLERGENS (n = number of assays)

Sample	Allergen	Specific IgE U/ml $\pm$ SD	CV	n
<i>Intra-assay variability</i>				
P421	<i>D. pteronyssinus</i>	1.24 ± 0.06	4.8%	10
P373	Cat epithelium	18.70 ± 0.58	3.1%	10
P279	<i>Lolium perenne</i>	49.30 ± 2.29	4.6%	10
<i>Interassay variability</i>				
P420	<i>D. pteronyssinus</i>	2.42 ± 0.22	9.1%	10
P350	Cat epithelium	16.16 ± 1.60	9.9%	10
P279	<i>Lolium perenne</i>	50.12 ± 4.10	8.2%	10

constant volume by adding bovine serum. These mixtures were assayed with the present method for specific IgE against, respectively *FeldI* and phospholipase A_2 , and the result expressed in U/ml. The total IgE concentrations of each mixture was also assayed with an immunoradiometric assay (total IgE IRMA kit, Radim, Italy) and they were found to agree closely with the expected values. Fig. 5 shows the variations of measured specific IgE as a function of total IgE. For total

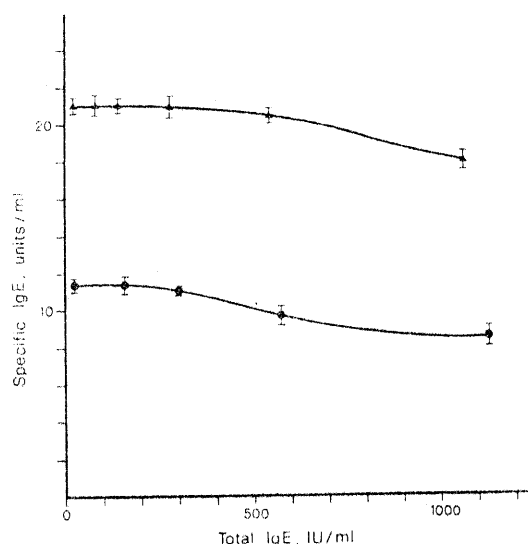


Fig. 5. Interference of non-specific IgE on the specific IgE assay. Serum from two patients allergic to bee venom (▲) or cat epithelium (●) were spiked with different amounts of non-specific IgE and assayed. Bar = $\pm$ SD.

IgE levels increasing from 25 to approximately 1100 IU/ml, the apparent reduction of specific IgE was 25% for *FeldI* positive serum (8.6/11.5 U/ml) and 15% for phospholipase A₂ positive serum (18.0/21.0 U/ml).

Interference by allergen-specific IgG

Sera from nine beekeepers receiving immunotherapy with bee venom for 3 or more years were treated with Protein A-Sepharose CL-4B in order to remove IgG. Packed gel and serum, 0.5 ml each, were incubated for 3 h at room temperature on an end-over-end rotator and the serum recovered by centrifugation. As a control, the sera were also submitted to the same treatment, using uncoupled Sepharose CL-4B. Both control and treated sera were tested for bee venom-specific IgE using both the RAST assay and the present method. The results are illustrated in Table II. No difference was seen in the specific IgE concentration between control and treated sera when tested with the capture-assay method. With the RAST assay, 2 out of 9 sera showed a two-fold increase in specific IgE levels after removal of IgG blocking antibodies.

Specific IgE assay on allergic sera

Fig. 6 illustrates the results obtained on a group of 95 sera from allergic patients tested for eight different allergens using both the present method and the RAST procedure. For samples ranging from 0.35 to 15.0 RAST units/ml, specific IgE measured with the present method fell

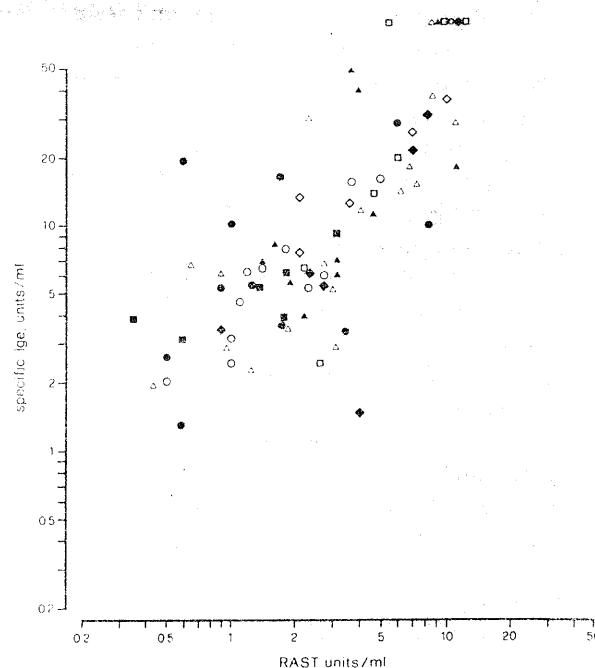


Fig. 6. Comparison between specific IgE concentrations obtained with the RAST procedure (abscissa) and the present method (ordinate) on the same samples. The following allergens were tested: cat epithelium (▲), bee venom (○), *D. pteronyssinus* (□), α -lactalbumin (◇), β -lactoglobulin (◆), birch pollen (●), *Cladosporium erbarum* (■) and *Lolium perenne* (△).

within the range 1.4 to > 50 U/ml. All the samples with > 17.5 RAST U/ml gave values of > 50 U/ml in our assay; 11 of them were diluted 1/10 in bovine serum and were reassayed using both methods. The lowest value obtained (1.4 U/ml) with the RAST-positive sera was about ten fold higher than the sensitivity limit of the assay.

TABLE II

IgG INTERFERENCE IN SPECIFIC IgE ASSAY

RAST assay (RAST U/ml)		Present method (U/ml)	
Control sera	Treated sera	Control sera	Treated sera
1.6	1.7	4.9	4.8
2.1	2.3	6.4	6.5
11.0	15.0	40.0	38.8
8.0	17.0	45.0	44.7
7.1	6.9	14.4	14.6
10.5	11.0	36.4	35.9
1.3	2.6	8.0	8.2
3.1	3.0	11.1	11.4
1.8	1.9	7.7	7.5

Discussion

The capture-assay method described here represents an improvement, from both the theoretical and practical point of view, over conventional assays for the determination of specific IgE in allergic patients. Our findings confirm that interference by allergen-specific IgG is completely avoided by this approach, as already demonstrated by Plebani et al. (1986). The very low concentrations of specific IgE detected, however,

and the variable ratio between specific and total IgE demand a very efficient anti-IgE coated solid phase. The coating procedure adopted proved to be adequate for our method, both in terms of sensitivity (minimal detectable level of IgE was 0.15 U/ml) and for the absence of significant interference by high levels of non-specific IgE in the assay. Indeed, an increase of total IgE in the sample of more than 40 times resulted in a reduction of only 15–25% of the measured specific IgE, which is of little clinical relevance.

The pre-treatment at low pH of the coating antibodies was, in our experience, very effective in reducing the so called 'hook effect', i.e., the reduced binding of tracer antibody at high levels of analyte (Olivieri et al., 1988), and should be considered the method of choice for all immunoassays requiring a very high binding capacity of solid phase.

A common feature of RAST procedures and the present method is that both produce chemical modification of the amino groups of the allergen molecule, since these are involved in the covalent binding with CNBr-activated cellulose or in the biotinylation procedure, respectively. In both cases, care must be taken in order to avoid an excessive modification of the antigenic structure, since this can, in our experience, result in a marked increase in the nonspecific binding of biotinylated allergen to the microplate.

The assay design, being based on the capture of IgE molecules through their Fc fragment, permits a correct quantitative estimation of all specific IgE against a total IgE standard, provided that all the biotinylated components of the assay are carefully optimized as described in the results section, i.e., titrated in order to obtain the highest sensitivity without a significant background signal. Furthermore, the use of a common solid phase for standard and samples and the use of allergens in the liquid phase greatly simplifies the whole assay procedure, which can be easily performed by computer-assisted automatic liquid handling equipment.

Dilution tests performed on the sera of allergic patients yielded linear relationships between the measured level of specific IgE and sample dilution, thus indicating the absence of saturation effects even at high levels of analyte. Low intra-

and interassay variabilities were obtained for all the allergens tested.

In addition to those described, the method has been applied in our laboratory to several other allergens, and can be employed also to detect IgE directed against small molecules, by using a suitable hapten derivative. As an example, good results were obtained with penicillin G and V, by conjugation with poly-L-lysine (MW 26500) through 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, followed by biotinylation of residual amino groups of the polylysine. This indicated the potential value of the present method for in vitro diagnosis of IgE mediated allergy.

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