

EGG Hypersensitivity as Measured by RAST and a Reverse Enzyme-Immunoassay

J. DOMÍNGUEZ, M. CUEVAS, I. MONEO, V. UREÑA, A. FERREIRA and A. BOOTELLO

Servicio de Inmunología, Centro Especial Ramón y Cajal, Madrid, Spain

The application of a reverse enzyme-immunoassay (REIA) to measure specific anti-egg IgE is described. The results obtained with the REIA and the RAST are shown. The REIA is economical, easy and fast to perform when the conjugate has been made, non-radioactive and offers a new alternative for the *in vitro* evaluation of egg white-specific IgE.

Key words: ELISA; enzyme-immunoassay; food hypersensitivity; RAST; specific IgE.

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CLINICAL ASPECTS

Egg white hypersensitivity is frequently found in atopic children. The method described in this paper measures the presence of the specific IgE simulating *in vitro* the binding to the mast cell of this specific IgE. Furthermore, being free of the competitive effect of blocking antibodies, this non-isotopic method offers a new perspective in the *in vitro* evaluation of the egg white-atopic patient.

Since its introduction by Wide et al. in 1967, the RAST has proven to be a valuable method for the *in vitro* diagnosis of immediate hypersensitivity reactions. The quantity of specific IgE determined by the RAST correlates with skin tests and histamine release (8, 15).

One of the fields in which the RAST shows lower resolution, however, is that of food allergy (6, 12). The inadequate representation of the different antigenic fractions on the disc (15), along with the frequent presence of allergen-specific non-IgE antibodies (blocking antibodies) (1, 2, 4) in these patients, are two important factors responsible for this loss of sensitivity.

We have previously described the application of a reverse enzyme-immunoassay for the determination of specific IgE against *Dermatophagoides pteronyssinus* and *Lolium perenne* (3, 10). Its design, using enzymatically labeled antigens, avoids the blocking antibodies problem as well as that derived from the linking of the allergenic fraction to the disc.

In this work, we describe the application of the above method to the allergens of the egg, one of the most frequent causes of food allergy.

MATERIAL AND METHODS

Sera

The sera of 96 patients, sent to our laboratory with suspected food sensitivity, have been studied. Aliquots of the sera were stored at -20°C until use.

Allergens

The allergenic solution was prepared by gently shaking 1 vol. of fresh egg white in 9 vol. 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.2 (PBS) for 1/2 h. Then, after 10 min of centrifugation at 1000 g, aliquots of the supernatant were either kept at -20°C or conjugated as described below. The total protein concentration

of this solution, measured by absorption at 280 nm, was 10 OD.

Conjugation of the allergen

The periodate oxidation method, described by Nakane (11) but with some slight modifications (3, 10), was used. Briefly, 5,000 U of Type VI peroxidase (Sigma Chemical Co., St. Louis, MO, USA) were diluted in 1 ml PBS and treated with 0.2 ml 1% 1-fluoro-2,4 dinitro-benzene (FDNB) (Eastman Organic Chemicals, Rochester, NY, USA) in absolute ethanol at room temperature. After 30 min, 0.2 ml of 0.3 M sodium metaperiodate was added. After 30 min in the dark, this mixture was chromatographed on a 20 ml Sephadex G-25 column (Pharmacia, Uppsala, Sweden) equilibrated in PBS and the brown-coloured fraction of the peroxidase was collected (approximately 1.8 ml). Of this activated peroxidase, 0.2 ml (equivalent to 1.6 mg) was then mixed with 0.2 ml of the antigenic solution together with 0.05 ml carbonate-bicarbonate buffer 1 M (pH 9.5) and kept at 4°C. After 18 h, 1 ml 0.5 M phosphate buffer (pH 7) containing 2 mg sodium borohydride was added. After 1 h, 10 mg D-lysine in 0.05 ml of PBS was added to block any aldehyde groups which could still possibly be active. After 1 hour, 1 ml of 1% bovine serum albumin (BSA) in PBS was also added. At this point, the conjugate solution was centrifuged at 1,000 g (4°C) in order to separate the large aggregates produced during the conjugation. After 20 min, this conjugate was resuspended in glycerin (50% v/v) and merthiolate was added to achieve a final concentration of 0.01 M. This "stock solution" was then kept at 4°C until use.

Coating of the plates

M-24-AR Dynatech U-shaped plates, treated with 0.1 ml per well of a 1/100 dilution of monospecific anti-human IgE (Tago Lab.) in PBS, were incubated at 4°C. After 18 h, the plates were washed 5 times with PBS 0.1% Tween 20 (PBS-Tw). The plates were then incubated at room temperature with 0.2 ml per well of a 2% BSA-PBS solution, to block any

residual binding sites on the plates. After 6 h, the plates were washed again (5 ×) in PBS-Tw. At this time the plates were either used immediately or stored at -20°C until use.

Reverse enzyme-immunoassay technique (REIA)

This technique was performed as previously described (3, 10) but with slight modifications. Briefly, 0.05 ml of serum was mixed with an equal volume of PBS-Tw in each well and kept at 4°C for 18 h. After 5 washing steps, alternating PBS-Tw and tap water, 0.2 ml of a dilution of stock solution (equivalent to 1 µg peroxidase) in PBS-0.5% Tw-25% fetal calf serum was added to each well and left shaking at room temperature for 1 h. Then, the plates were washed 10 times, alternating PBS-Tw and tap water. When the plates were dry, 0.1 ml per well of a substrate containing 2 mg O-phenylene-diamine per ml and 0.03% H₂O₂ in 0.1 M citrate buffer (pH 5) was added. After 30 min at room temperature, 0.10 ml per well of 4N H₂SO₄ was added to arrest the reaction. Values that were 0.1 OD higher than the blanks were considered positive.

RAST

The RAST was performed according to the manufacturer's instructions (Pharmacia).

RESULTS

In order to decrease the background values and increase the positive results, several modifications were made with respect to previous methods (3, 10). First, the allergen incubation time was reduced to 1 h while shaking at room temperature. At the same time, the conjugate concentration was increased (14).

A series of RAST-positive sera were tested using 0.125, 0.25, 0.5 and 1 µg of peroxidase per well (Fig. 1). The maximum resolution between the different sera is obtained using 1 or 0.5 µg peroxidase per well. Both concentrations can be used without a significant rise in the blank values. For this reason we have used 1 µg/well for the rest of the assay.

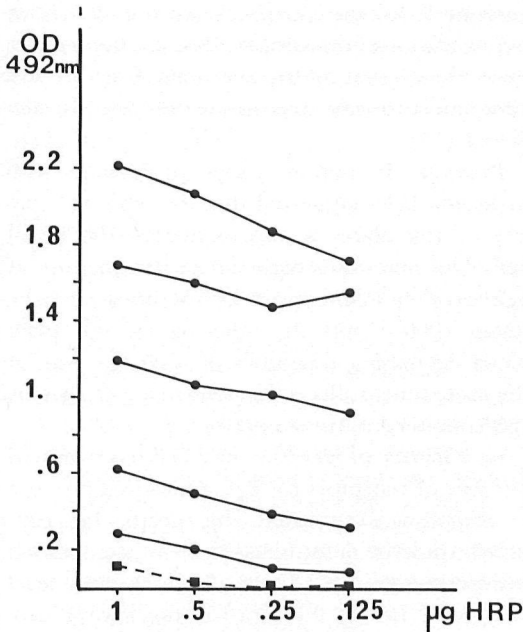


Fig. 1. Optical densities obtained from different sera (mean of duplicates) using 0.125, 0.25, 0.5 and 1 µg of peroxidase per well after 1 h of incubation (0.05 ml serum for 18 h at 4°C).

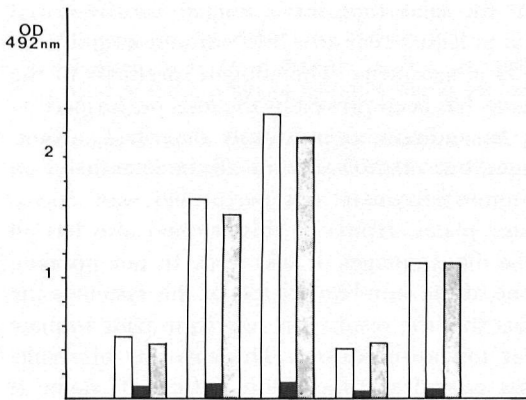


Fig. 2. Inhibition induced by adding 20 µg of unlabeled antigen (black bars) or 20 µg of unrelated antigen, β-lactoglobulin (shaded bars), to 0.2 ml labeled egg white, 1 µg peroxidase per well (open bars).

Specificity. Having previously proven that the REIA is highly IgE specific, we set out to verify the specificity of the method for this particular antigen. To do this, a series of positive sera were

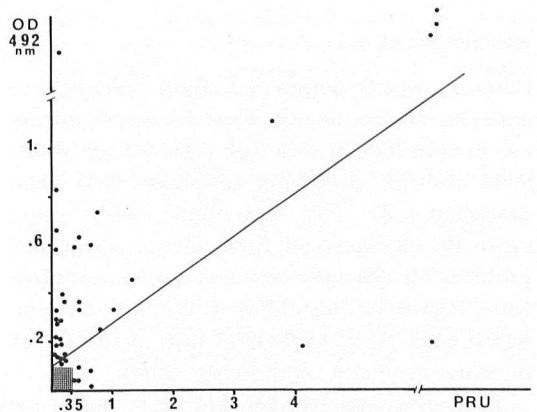


Fig. 3. Lineal regression between REIA, expressed in corrected OD, and RAST. The bar in the left lower corner represents 54 RAST and REIA negative sera. ($r = 0.86$).

incubated for 18 h at 4°C. Thereafter, 1 µg of the peroxidase conjugate per well was mixed with 20 µg of unlabeled antigen or 20 µg of unrelated antigen (β-lactoglobulin) and processed in the usual way (Fig. 2).

Reproducibility. The coefficient of variation (CV) obtained with five sera, with OD between 0.637 and 0.201, assayed 8 times in a single run varied between 2.9% and 12.6%.

Because of the high interassay variability with CV in 4 different runs which oscillated between 10% and 43%, ($\bar{x} = 30.16$, $SD = 10.87$, $n = 7$), we tried to reduce the CV by means of a standard curve obtained with a serial two-fold dilution, in fetal calf serum, of a pool of six positive sera. The OD of the first assay were considered OD reference units. In the successive days, the standard curve OD were plotted against the reference units on a log-log graphic paper and the OD values obtained with patient sera were corrected. The CV obtained after this correction oscillated between 2% and 24.3% ($\bar{x} = 12.04$, $SD = 7.01$, $n = 7$; $P < 0.01$).

Fig. 3 shows the lineal regression between the results obtained after testing 96 sera using both the RAST, expressed as Phadebas Rast Units, and the REIA, expressed in corrected OD.

DISCUSSION

Clinical manifestations of food allergy are generally the result of a Type I hypersensitivity mechanism mediated by IgE (7) although short-term non-IgE sensitizing antibodies have been described (13). This and other aspects often make the diagnosis of food allergy a complex problem for allergists. For this reason, a controversy regarding the validity of skin tests as compared to *in vitro* alternatives such as the RAST immunoenzymatic methods has arisen.

The advocates of the skin tests claim that there exists no relation between the levels of serum IgE and the IgE bound to the mast cells (5, 12). They also claim the importance of the above mentioned IgG₄ antibodies (16) and the limited scope of the RAST in the case of some foods (6) probably due to difficulties in linking some allergens to the disc (15).

On the other hand, the skin test is limited by the high frequency of false-positives and negatives, the danger of systemic reactions (5), as well as the insufficient maturity of the skin in very young children to provide reliable results. Furthermore, young children are especially prone to this kind of sensitivity. For all of the above mentioned reasons, the search continues for the ideal *in vitro* method.

The REIA is based on a principle contrary to the RAST: the first step is the isolation of the serum IgE and then the specific IgE is detected through the use of allergens labeled with enzymes. This method has previously proven to be specific for IgE and antigen, at least for those allergens tested so far (3, 10).

The REIA, like the method of Zeiss (17), avoids the interference of the blocking antibodies which are eliminated by the washing steps that follow the serum incubation stage.

We do not know if all the allergenic fractions have been optimally labeled, if any kind of steric hindrance exists with a given allergenic fraction, nor have we tried to separate the unlabeled fractions which are a source of competition for the labeled ones. Because the heterogeneous character of the proteins in the sample (9) makes purification nearly impossible, and as the results of the 96 sera studied were

acceptable for our purposes, we found further manipulations unnecessary. Besides, there exists no evidence that all the allergenic fractions are adequately represented on the discs used for the RAST (15).

Previous inhibition assays performed with myeloma IgE, suggested that the binding capacity of the plates is approximately 100 ng of IgE. This amount is higher than the quantity of IgE existing in the majority of the sera to be tested (900 U/ml). In other cases, the plate binds decreasing quantities of total IgE but, at the same time, reflects the percentage of specific IgE contained in the sera (10).

As a matter of fact, the mast cell has a limited number of receptors for IgE. Once said number of receptors is saturated, the specific IgE captured would be determined by its representation within the total IgE. Thus, a high absolute level of specific IgE in the sera will not always have the same clinical relevance.

All these facts can explain the discordances found between the RAST and the REIA.

The labeling procedure is inexpensive, easy to perform, risk-free, and the conjugated allergen can be employed for several thousands of tests. At the same time this conjugate can be stored for at least a year at -70°C without a significant loss in sensitivity. The antigen specificity of the assay has been proved by the tests performed.

In addition to previously described advantages, our method also has all the benefits of an immunoenzymatic test performed with microtiter plates. However, this method also has all the disadvantages of said tests. In our opinion, one of the stumbling-blocks of this system is the fact that the results can vary from plate to plate for unknown reasons. This diversity of results has caused us to establish the cut-off point at values 0.1 OD higher than the blanks, which is greater than the mean plus 2 SD. At the same time, the interassay CV can be reduced using references with known values.

The RAST, on the other hand, does not seem to have this problem and this method is currently being used due to its high specificity and repeatability.

Finally, the different results obtained using both methods have lead us to begin an in-depth

study of the clinical significance of both tests. Nonetheless, at this stage, it is our opinion that these two *in vitro* alternatives represent a real help to the allergist in the field of food hypersensitivity.

REFERENCES

- Adkinson, N. F.: The radioallergosorbent test in 1981. Limitations and refinements. *J. Allergy Clin. Immunol.* 67, 87–89, 1981.
- Alvarez, E., Cuevas M., Moneo, I. & Bootello, A.: Falta de correlación entre el RAST y el ELISA en alergia a alimentos. Interferencia por anticuerpos específicos de otras clases. *Allergol. Immunopathol.* 9, 417–426, 1981.
- Cuevas, M., Moneo, I., Ureña, V., Domínguez, J. & Bootello, A.: Reverse enzyme immunoassay for the determination of *Lolium perenne* IgE antibodies. *Int. Arch. Allergy Appl. Immunol.* 72, 184–187, 1983.
- Dannaeus, A. & Inganas, M.: A follow-up study of children with food allergy. Clinical course in relation to serum IgE- and IgG-antibody levels to milk, egg and fish. *Clin. Allergy* 11, 533–540, 1981.
- Dockhorn, R. J.: Using the RAST and PRIST with overview of clinical significance. *Ann. Allergy* 49, 1–8, 1982.
- Fries, J. H.: Food allergy: current concerns. *Ann. Allergy* 46, 260–263, 1981.
- Golbert, M. T.: Food allergy and immunologic diseases of the gastrointestinal tract. In Patterson, R. (ed.): *Allergy disease: Diagnosis and management*, pp. 409–439. J. B. Lippincott Co., Philadelphia, Toronto, 1980.
- Johansson, S. G. O. & Bennich, H. H.: The clinical impact of the discovery of IgE. *Ann. Allergy* 48, 325–330, 1982.
- Langeland, T.: A clinical and immunological study of allergy to hen's egg white. *Allergy* 37, 323–333, 1982.
- Moneo, I., Cuevas, M., Ureña, V., Alcover, R. & Bootello, A.: Reverse enzyme immunoassay for the determination of *Dermatophagoides pteronyssinus* IgE antibodies. *Int. Arch. Allergy Appl. Immunol.* 71, 285–287, 1983.
- Nakane, P.: Preparation and standardization of enzyme-labeled conjugates. In Nakamura, R. M., Dito, W. R. & Tucker, S. E. (eds.): *Immunoassays in the clinical laboratory*, pp. 81–87. Liss, New York, 1979.
- Oehling, A., Martín-Gil, D., Antepara, I., Domínguez, M. A. & Subirá, M. L.: The reliability of the RAST in food allergy. *Allergol. Immunopathol.* 9, 217–222, 1981.
- Parish, W. E.: Detection of reaginic and short-term sensitizing anaphylactic or anaphylactoid antibodies to milk, in sera of allergic and normal person. *Clin. Allergy* 1, 369, 1971.
- Saunders, G. C.: The art of solid-phase enzyme immunoassay including selected protocols. In Nakamura, R. M., Dito, W. R. & Tucker, S. E. (eds.): *Immunoassays in the clinical laboratory*, pp. 99–118. Liss, New York, 1979.
- Sweeney, M. J., Kay, C., Klotz, L. R. & Klotz, S. D.: An IgE inhibition assay for the detection of allergen specific IgE. *Ann. Allergy* 46, 295–300, 1981.
- Vijay, H. M., Perelmutter, L. & Bernstein, I. L.: Possible role of IgG₄ in discordant correlations between intracutaneous skin tests and RAST. *Int. Arch. Allergy Appl. Immunol.* 56, 517–522, 1978.
- Zeiss, C. R., Levitz, D. & Suszko, I. M.: Quantitation of IgE antibody specific for ragweed and grass allergens; binding of radiolabeled allergens by solid-phase bound IgE. *J. Allergy Clin. Immunol.* 62, 83–89, 1978.

Address:

Javier Domínguez Juncal
Servicio de Inmunología
Centro Especial Ramón y Cajal
Carretera de Colmenar Km 9.1
Madrid 34
Spain

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