

Assay of IgE antibodies against June and Timothy grasses by an immunoperoxidase technique

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(Received 11 April 1979; accepted for publication 3 May 1979)

Summary

The results of eighty assays for serum IgE antibodies against June and Timothy grass antigens with a solid-phase immunoperoxidase technique are correlated with those obtained with the RAST procedure. The allergens were insolubilized in polystyrene microtitre plates. After allowing the allergen-specific IgE antibodies in the test serum to bind the allergen, the plates were incubated with horseradish peroxidase-conjugated rabbit antihuman IgE. The enzyme bound to the plates was assayed spectrophotometrically using *o*-phenylene diamine as the substrate.

The coefficients of variation for within-batch reproducibility ranged from 4.57 to 11.97%, and those for between-batch variance from 8.25 to 13.7%. The slope of absorbance values of the immunoperoxidase assay is shallower than that of the bound radioactivity in the RAST technique. Notwithstanding, the immunoperoxidase method allows separation of non-atopic controls from the sera containing specific IgE antibodies and categorization of positive sera into weakly, moderately, and strongly positive results. Free of the disadvantages inherent in the use of radioactive material, this assay offers an alternative to the RAST test for the assay of allergen-specific IgE antibodies.

Introductions

The radioallergosorbent test (RAST) for the assay of allergen-specific IgE antibodies (Wide, Bennich & Johansson, 1967) in the *in-vitro* diagnosis of atopic disorders has gained widespread acceptance in recent years. It has been shown to correlate well with clinical history of allergy (Strenius *et al.*, 1971) *in-vivo* diagnostic tests (skin tests, Aas & Johansson, 1971; Norman, Lichtenstein & Ishizaka, 1973; provocation tests, Engvall & Perlmann, 1971), and Prausnitz-Kustner titres (Evans *et al.*, 1975). The requirements of the radioimmunoassay techniques, however, are such that the search for an

enzymatic immunoassay replacing the isotope label with an enzymatic marker continues. The applications of the enzyme-linked immunosorbent assay (ELISA) (Hoffman, 1973) using alkaline phosphatase (Eriksson & Ahlstedt, 1977) and galactosidase (Weltman *et al.*, 1976) have been reported. Largely because of the method of enzyme conjugation chosen, and also due to unwieldy molecular size of the enzyme in the case of galactosidase (mol wt 598,000), these assays have failed to match the sensitivity and specificity of the RAST test.

Horseradish peroxidase (mol wt 40,000) has served well as the enzymatic marker in several applications of the ELISA principle (Alpert & Coston, 1975; Bosch *et al.*, 1975; Pesce *et al.*, 1974). We have described the use of this enzyme in immunoenzymatic techniques for the localization of IgE in tissues (Ali *et al.*, 1979a), and in an assay for ragweed-specific IgE antibodies in serum (Ali *et al.* 1979b). In this paper, we are reporting correlation of data for IgE antibodies against June and Timothy grasses assayed by our version of the ELISA principle and the RAST technique. For conjugation with rabbit antihuman IgE, we employed a previously described modification (Mesa-Tejada *et al.*, 1978) of the method of Nakane & Kawaoi (1974), which minimizes the undesirable side reactions and allows a high yield of functionally useful enzyme-antibody conjugates with the major species containing two to three molecules of enzyme per molecule of antibody.

Materials and methods

Antibody-enzyme conjugate

The IgG fraction of rabbit antihuman IgE (Behring Diagnostics) was purified by sequential sodium sulphate fractionation, ion-exchange chromatography on DEAE cellulose, and repeat sodium sulphate fractionation. Prior to conjugating the purified IgG fraction to horseradish peroxidase (Sigma, type VI, RZ 3.4), the free amino groups on the peroxidase molecule were blocked with phenyl isothiocyanate. The carbohydrate moiety was then oxidized with periodate to yield peroxidase aldehyde which, following dialysis, was coupled to the amino group of the IgG molecule by formation of Schiff's base. Sodium borohydride was employed to stabilize the Schiff's base. Finally, ammonium sulphate precipitation was used to purify the conjugate. The RZ values of the conjugate so prepared were 0.5–0.6.

Buffers

Incubation buffer. Dulbecco's PBS was passed once through 0.45 µ Nalgene filter (Nalge Co.), pH 7.4, containing 5% chicken serum (Grand Island Biological Co.), 5% Trasylol (Mobay Chemical Co.), 0.1% tween 20, and 0.1 mM merthiolate.

Enzyme buffer. McIlvain's buffer, pH 6.0 (36.85 ml 0.1 M citric acid and 63.15 ml 0.2 M Na₂HPO₄), freshly prepared.

Antigen coating buffer. Na₂CO₃, 3.18 gms; NaHCO₃ 5.86 gms; dissolved in water up to 100 ml (stored at 4°C) and diluted 1:20 in distilled water immediately before use, pH 9.6.

Antigens

The antigen extracts were obtained from Pharmacia Diagnostics, Piscataway, N.J. and were used in the following concentrations: Timothy grass, 79,000 PNU/ml; June grass, 86,000 PNU/ml; and birch tree 80,000 PNU/ml. Polystyrene microtitre plates (Cook

Microtitre™, 81 × 124 mm) were used to insolubilize antigens by incubating in each well 2 µl of extract in 200 µl of coating buffer overnight at 4°C. The plates were subsequently washed and stored at -20°C.

IgE antiserum specificity

The specificity of the anti IgE antiserum conjugated with peroxidase was assured by an absorption study. Equal volumes of the sera containing allergen-specific IgE antibodies against birch tree and June grass antigens were incubated with equal volumes of appropriate antigen extracts overnight at 4°C. After centrifugation at 8000 × *g* for 0.5 h, the supernatant was assayed for specific IgE antibodies against birch tree and June grass antigens. To demonstrate the independence of the results of this assay from raised levels of total IgE, sera of two patients, who were highly allergic to both Timothy and June grasses, and had markedly elevated serum levels of total IgE but were RAST negative for birch tree, were tested with birch tree antigens.

Test and control sera

The test sera were obtained from patients who underwent an investigation for symptoms of inhalant allergies and had raised serum levels of total and allergen-specific IgE as determined by the PRIST (Pharmacia Diagnostic) and the RAST tests respectively.

For negative controls, sera from cords of six newborns and four laboratory technicians without any history of allergic symptoms and with negative RAST test results were used.

Immunoperoxidase assay

All assays were performed in duplicate. The procedural steps for the assay were as follows: (1) incubation of 100 µl of test serum with 100 µl of incubation buffer overnight at 4°C in a polystyrene microtitre plate well with previously adsorbed allergen; (2) four washes with PBS containing 0.1% tween 20 by immersing the plate in the buffer with agitation and manually forcing out the contents of the wells followed by blotting; (3) application of peroxidase-conjugated rabbit antihuman IgE (10 µg/ml) for 2 h at room temperature incubation buffer; (4) repeat washes times 4; (5) addition of 250 µl of the substrate (0-phenylene diamine (Eastman Kodak), 0.5 mg/ml) in enzyme buffer containing 0.006% of hydrogen peroxide at room temperature for 1 hr; (6) and arrest of colour reaction by the use of 5N H₂SO₄.

The colour product was transferred from the microtitre plate wells into the cuvettes of a double-beam spectrophotometer, diluted 1:1 with 0.5 N H₂SO₄, and the absorbance was measured at 492 nm against the assay reagent blank (prepared by omitting the step of incubation with serum). The O.D. of the blank was subtracted from that of the assay results automatically by the spectrophotometer.

The RAST

The RAST test (Pharmacia Diagnostics) was performed according to the manufacturer's directions.

June and Timothy grass antigens

The correlative data for eighty analyses for IgE antibodies against June and Timothy grasses performed with the immunoperoxidase and the RAST techniques is displayed in Fig. 1. The mean absorbance value of six cord sera was 0.028. No sera of non-atopic

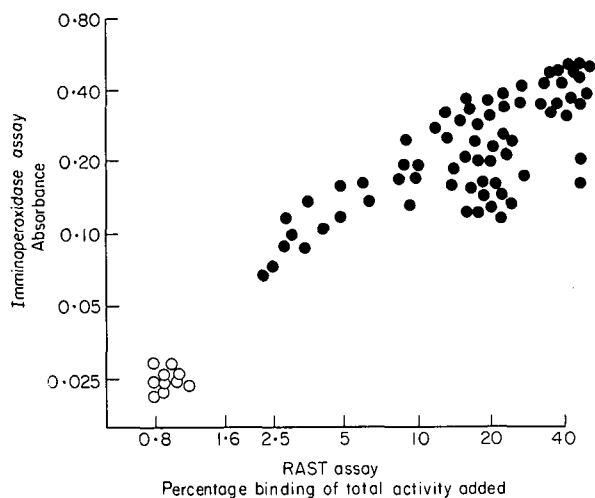


Fig. 1. Correlation of net absorbance values obtained with the Immunoperoxidase assay with bound radioactivity as determined by the RAST technique. ●, Atopic patients; ○, controls.

control individuals produced absorbance of more than one and one-half times the mean absorbance value for the cord sera. The range of absorbance values between the non-atopic controls and the strongly positive sera in the immunoperoxidase assay was not as wide as that of bound radioactivity values in the RAST technique. However, except for a few outlying values, there was a correlation between these two methods as regards their quantitative function.

Results

Reproducibility

For within-batch variation, the absorbance of two sera with positive RAST test results for June and Timothy grasses was measured six times in the same batch. Coefficients of variation were 4.57 and 11.97% respectively (Table 1). For between-batch variation, duplicate assays were performed with the same serum on six different days. The coefficients of variation were 8.25 and 13.71% respectively (Table 2).

Table 1. Within-batch reproducibility of the immunoperoxidase assay for IgE antibodies against June and Timothy grasses

Allergen	Serum no.	Absorbance		Coefficient of variation (%)
		Mean*	Range	
June grass	1	0.153	0.150-0.160	4.57
	2	0.123	0.118-0.130	6.42
Timothy grass	1	0.096	0.090-0.107	11.97
	2	0.089	0.084-0.091	11.68

*Mean for six determinations

Table 2. Between-batch variability of the Immuno-peroxidase assay for IgE antibodies against June and Timothy grasses

Allergen	Absorbance		Coefficient of variation (%)
	Mean*	Range	
June grass	0.218	0.208–0.226	8.5
Timothy grass	0.070	0.061–0.074	13.71

* Mean for duplicate assays performed on six different days.

Assay specificity

In the absorption studies, designed to assure the specificity of the IgE antiserum, the assays performed before and after absorption of the test sera with appropriate antigens showed precipitate drops in the absorbance values (from 0.192 to 0.022 for birch tree, and from 0.31 to 0.043 for June grass antigens). In the studies performed to assure the independence of this assay from the elevated levels of total IgE, the absorbance values of two sera with class 4 RAST results for antibodies against June and Timothy grass when assayed for antibirch antibodies were 0.048 and 0.012 respectively.

Discussion

In solid-phase immunoenzymatic techniques, the sensitivity of the assay is principally a function of the quality of the enzyme-antibody conjugate; the assay specificity is essentially a measure of the specificity of the antibody. The accuracy and precision of the assay results is adversely affected by the presence in the conjugate of undesirable by-products of the reaction of conjugation such as enzyme-enzyme conjugates, IgG-IgG conjugates, and large aggregates. Thus, both the choice of the enzyme and the nature of the conjugation reaction are critically important to the quality of the conjugate.

In previously reported enzyme immunoassays for allergen-specific IgE antibodies in serum employing alkaline phosphatase (Eriksson & Ahlstedt, 1977) and galactosidase (Weltman *et al.*, 1976), the enzymes were conjugated to the antibodies with the glutaraldehyde method. In both instances, the information concerning the quality of the conjugate was lacking.

In our experience, horseradish peroxidase conjugate prepared by the formation of Schiff's base, the precise nature of which is known (Nakane & Kawasi, 1974) has proven to be superior to those of alkaline phosphatase and galactosidase made with the glutaraldehyde procedure. Among the advantages of the peroxidase conjugate are: (1) less cumbersome and more successful separation of functionally useful conjugates from the unwanted by-products of reactions of conjugation; (2) higher final yield of the conjugate, and (3) stability of the purified fraction for a longer period of time (up to 9 months at -20°). By sedimentation analysis, the main species in the peroxidase conjugate used in this assay was found to be composed of two to three molecules of the enzyme per molecule of IgG.

Among the factors influencing the assay specificity are the animal species of the antiserum and the serum used for maintaining the optimal concentration of the

proteins in the incubation buffer. The occurrence of heterophil antibodies in the test sera may introduce problems of cross-reactivity in the system. After our own initial experience with goat, and in view of that of others with sheep (Eriksson & Ahlstedt, 1977), we selected rabbit as the source of the antibody. For the incubation buffer, chicken serum proved quite satisfactory. Trasylol was added to the incubation buffer to inhibit any protease activity that may exist in the system.

For the allergen sources investigated with this immunoperoxidase technique, the sensitivity and specificity of this assay compare favorably with the RAST test. With few exceptions, the values of absorbance with the immunoperoxidase procedure correlated well with percentage binding of the radioactivity in the RAST test. For IgE antibodies against June and Timothy grasses, the slope of the immunoperoxidase assay is not as deep as that of the RAST technique. Notwithstanding, the immunoperoxidase method allows separation of sera into categories of weakly positive (absorbance 0.6 to 0.1), moderately positive (absorbance between 0.1 and 0.2), and strongly positive (absorbance > 0.2). In clinical applications of enzymatic and radiometric assays, the distinction between the negative controls and low positives is most critical. In our data, there is a clear separation between the absorbance values of our negative controls (cord sera and non-atopic individuals) and the sera containing allergen-specific IgE antibodies as determined by the RAST test.

We believe the elimination of the need for isotopic material, and the ease of performance of the assay allowed by the microtitre plate (ninety-six wells per plate, for up to forty-eight individual allergen assays in duplicate) are significant advantages of this immunoperoxidase method over the RAST test.

Acknowledgments

The authors acknowledge the technical assistance of Roger Maldini and Carol Frka and the help of Gertrude Martin in preparing the manuscript.

References

- AAS, K. & JOHANSSON, S.G.O. (1971) The radioallergosorbent test in the *in-vitro* diagnosis of reaginic allergy. A comparison of diagnostic approaches. *Journal of Allergy and Clinical Immunology*, **48**, 134.
- ALI, M., MESA-TEJADA, R., FAYEMI, A.O., NALEBUFF, D.J. & CONNELL, J.T. (1979a) The localization of IgE in tissues by an immunoperoxidase technique. *Archives of Pathology and Laboratory Medicine*, **103**, 274.
- ALI, M., RAMANARAYANAN, M., CONNELL, J.T., NALEBUFF, D.J., FAYEMI, A.O. & MESA-TEJADA, R. (1979b) An immunoperoxidase assay for serum ragweed-specific IgE. *Annals of Allergy*, **42**, 231.
- ALPERT, E. & COSTON, R.L. (1975) Serum alpha-fetoprotein (AFP) in benign and malignant gastrointestinal diseases. Evaluation of an immunoenzymatic assay. *Clinica Chimica Acta*, **58**, 77.
- BERG, T., BENNICH, H. & JOHANSSON, S.G.O. (1971) *In vitro* diagnosis of atopic allergy. I. A comparison between provocation tests and the radioallergosorbent test. *International Archives of Allergy and Applied Immunology*, **40**, 770.
- BOSCH, A., VAN HELI, H. & BRANDS, J. *et al* (1975) Methods for the determination of total estrogens (TE) and human placental lactogen (HPL) in plasma of pregnant women by enzyme-immunoassay (EIA). *Clinical Chemistry*, **21**, 1009. (Abstract).
- ENGVAL, E. & PERLMANN, P. (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry*, **8**, 871.
- ERIKSSON, N.E. & AHLSTEDT, S. (1977) Diagnosis of reaginic allergy with house dust, animal dander and pollen allergens in adult patients. V. A comparison between the enzyme-linked immunosorbent assay (ELISA), provocation tests, skin tests and RAST. *International Archives of Allergy and Applied Immunology*, **54**, 88.
- EVANS, R., REISMAN, R.E., WYPYCH, J.I. & ARBESMAN, C.E. (1972) An immunologic evaluation of ragweed-sensitive patients by newer techniques. *Journal of Allergy*, **49**, 285.

- GUESDON, J.-L., THIERRY, R. & AVRAMEAS, S. (1978) Magnetic enzyme immunoassay for measuring human IgE. *Journal of Allergy and Clinical Immunology*, **61**, 23.
- HOFFMAN, D.R. (1973) Estimation of serum IgE by an enzyme-linked immunosorbent assay (ELISA). *Journal of Allergy and Clinical Immunology*, **51**, 303.
- MESA-TEJADA, R., KEYDAR, I., RAMANARAYANAN, K.M., OHNO, T., FENOGLIO, C. & SPIEGELMAN, S. (1978) Immunohistochemical detection of a cross-reacting virus antigen in mouse mammary tumors and human breast carcinomas. *Journal of Histochemistry and Cytochemistry* **26**, 532.
- NAKANE, P.K. & KAWAOI, A. (1974) Peroxidase-labelled antibody. A new method of conjugation. *Journal of Histochemistry and Cytochemistry*, **22**, 1084.
- NORMAN, P.S., LICHTENSTEIN, L.M. & ISHIZAKA, K. (1973) Diagnostic tests in ragweed hay fever: a comparison of direct skin tests, IgE antibody measurements, and basophil histamine release. *Journal of Allergy and Clinical Immunology*, **52**, 210.
- PESCE, A., MENDOZA, BOREISH, I. *et al.* (1974) Use of enzyme-linked antibodies to measure serum anti-DNA antibody in systemic lupus erythematosus. *Clinical Chemistry*, **20**, 353.
- STRENIUS, B., WIDE, L., SEYMOUR, W.M., HOLFORD-STREVS, V. & PEPYS, J. (1971) Clinical significance of specific IgE to common allergens. *Clinical Allergy*, **1**, 37.
- WELTMAN, J.K., FRACKELTON, A.R., JR., SZARO, R.B. & ROTMAN, B. (1976) A galactosidase immunosorbent test for human immunoglobulin E. *Journal of Allergy and Clinical Immunology*, **58**, 426.
- WIDE, L., BENNICH, H. & JOHANSSON, S.G.O. (1967) Diagnosis of allergy by an *in-vitro* test for allergen antibodies. *Lancet*, **ii**, 1005.

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