

A new immunoperoxidase assay for *Lolium perenne*-specific IgE in serum based on the biotin/avidin system (BAS)

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(Received 4 October 1982; revised text accepted 13 July 1983)

Summary

A new solid-phase immunoassay based on the biotin/avidin system (BAS) for measuring serum *Lolium perenne* (LP)-specific IgE antibody is described. LP-specific IgE was assayed by the BAS assay and RAST for comparison in the sera of thirty-two normal asymptomatic subjects RAST-negative for LP and of twenty-six subjects with hay fever and RAST-positive for LP. The specificity of the BAS assay for LP-specific IgE was demonstrated by absorption experiments. An overall agreement of 91% (53/58) was observed between the BAS and RAST and a high correlation ($r=0.87$, $P<0.001$) was found between the LP-specific IgE determined by the two methods. The advantages of the BAS assay as compared to both the RAST and classical ELISA are discussed.

Introduction

Since introduction of the radioallergosorbent test (RAST) in 1967 for assay of allergen-specific IgE antibodies in serum, the results observed with this test have been shown to correlate with clinical history (Stenius *et al.*, 1971), diagnostic skin tests (Aas & Johansson, 1971; Norman, Lichtenstein & Ishizaka, 1973) and provocation tests (Berg, Bennich & Johansson, 1971) in subjects with atopic diseases. In this paper we describe the use of a solid-phase immunoassay based on the biotin/avidin system (BAS) in which specific IgE antibodies against *Lolium perenne* (LP) are detected using an antihuman IgE-biotinylated antiserum and a horseradish peroxidase-conjugated avidin.

Materials and methods

Antigens

Extracts in 50% glycerine of *Lolium perenne* (Lot 8036192) containing 10.000 PNU/ml and *Dermatophagoides pteronyssinus* (DPP) (Lot 804399) containing 5000 PNU/ml

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purchased from Bayropharm (Milan, Italy) were used as antigens either for coating to Sepharose 4B, for sensitizing microtitre plates (M129A Dynatech Laboratories) or for coating to paper discs in the RAST.

Reagents

The IgG fraction of a goat antihuman-IgE antiserum (Lot IgE-IgG-018-1) was purchased from Atlantic Antibodies (Maine, U.S.A.). Horseradish peroxidase-avidin D (Lot 01118) was purchased from Vector Laboratories Inc. (California, U.S.A.). Activated biotin (Enzotin) was purchased from Enzo Biochem Inc. (New York, U.S.A.).

Sera

Sera obtained from thirty-two normal subjects with a negative history for symptoms of hay fever and RAST-negative for circulating IgE antibodies against LP, and from twenty-six subjects with symptoms of hay fever and RAST-positive for circulating IgE antibodies against LP were evaluated for serum LP-specific IgE by both RAST and BAS assay.

Buffers

Antigen coating buffer: carbonate buffer, pH 9.6, was prepared by dissolving 3.18 g Na_2CO_3 and 5.86 g NaHCO_3 in distilled water up to 100 ml and diluted 1:20 in distilled water before use. Incubation buffer for the sample to be tested and the biotinylated antihuman IgE: phosphate-buffered saline (PBS), 0.15 M, pH 7.2 containing 2% bovine serum albumin (BSA, Sigma Chemical Co., Missouri, U.S.A.).

Incubation buffer for horseradish peroxidase-avidin D: PBS containing 15% fetal calf serum (FCS, Labtek, Milan, Italy). Washing solution: normal saline containing 0.1% Tween 20. Enzyme buffer: 0.1 M KH_2PO_4 and 0.1 M K_2HPO_4 in proportions to give a final pH of 6.0.

Biotinylation of immunoglobulins

The IgG fraction of goat antihuman-IgE antiserum was conjugated to activated biotin following the manufacturer's directions. Briefly: 100 μl of 0.3 M Na borate buffer, pH 9.1 was mixed with 200 μl of dimethylsulfoxide and the mixture allowed to equilibrate at 4°C. Then 100 μl of the protein (precooled at 4°C) to be biotinylated was added followed by addition of activated biotin in a ratio of 1:10 activated biotin/protein. The mixture was incubated at 4°C for 2 hr. After incubation 200 μl of 1 M cacodylate buffer, pH 6.0 were added. Volume was brought to 1 ml with cold distilled water and dialysed against PBS overnight at 4°C.

RAST procedure

The RAST was performed according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden) with the exception that the discs coated with LP extract were prepared in our laboratory according to the method of Ceska, Erickson & Varga (1972). Results were scored as positive when the percent counts bound was greater than the mean value + 2 s.d. calculated in the negative subjects.

BAS assay

All assays were performed in duplicate. The steps were as follows: polystyrol

microplates (M129A Dynatech Laboratories) were sensitized by incubating 200 μ l of a 1:50 dilution of LP extract in coating buffer for 3 hr at 37°C, then overnight at 4°C. After three washings with the washing solution, each well was filled with 200 μ l of a 1:5 dilution of the sample to be tested in PBS BSA 2%. After 2 hr at 37°C followed by three washings, the wells were filled with 200 μ l of a 1:5000 dilution of antihuman-IgE biotinylated antiserum in PBS BSA 2%. After 2 hr at 37°C followed by three washings, the wells were filled with 200 μ l of a 1:1000 dilution of horseradish peroxidase-avidin D in PBS FCS 15%. After 2 hr at 37°C followed by three washings, the substrate 200 μ l of *O*-phenyldiamine (2 mg/ml) (Sigma Chemical Co., Missouri, U.S.A.), dissolved in the enzyme buffer containing 0.015% hydrogen peroxide, was added to each well and the reaction was allowed to develop at room temperature for 20 min. The colour reaction was stopped by adding 50 μ l of H₂SO₄. The plate was read at 492 nm in a Titertek Multiskan photometer (Flow Laboratories).

The results were expressed in arbitrary units per ml (AU) with reference to pooled sera (reference pool) from four subjects with a positive RAST for LP and symptoms of hay fever; 1 AU was defined as the absorbance observed with a 1/5 dilution of the reference pool. For each sample AU were calculated as follows:

$$\text{AU} = \frac{\text{absorbance of sample}}{\text{absorbance of reference pool}}$$

The results were scored as positive when the AU were greater than the mean value + 2 s.d. calculated in the negative controls. Because of the considerable variability in background binding of the various RAST discs using non-IgE-containing control specimens, we chose to set the threshold for positivity of the RAST and BAS as the mean + 2 s.d. of percent counts bound, or of AU calculated in the negative subjects.

Inhibition test

To assure antibody specificity an inhibition test was designed as follows: serial dilutions of the reference pool were absorbed with 0.5 mg of Sepharose 4B coated to LP, DPP and BSA extracts by rotating the tubes on a rotator at room temperature for 6 hr. The tubes were then centrifuged for 20 min at 500 g and the supernatant was tested for the presence of LP-specific IgE antibodies using the BAS assay. The BAS assay was also done on serial dilutions of the same reference pool unadsorbed.

Results

Quality control

The standard deviations and coefficients of variation for the BAS assay obtained with one cord serum, one negative serum and sera from two RAST-positive patients with symptoms of hay fever are given in Table 1.

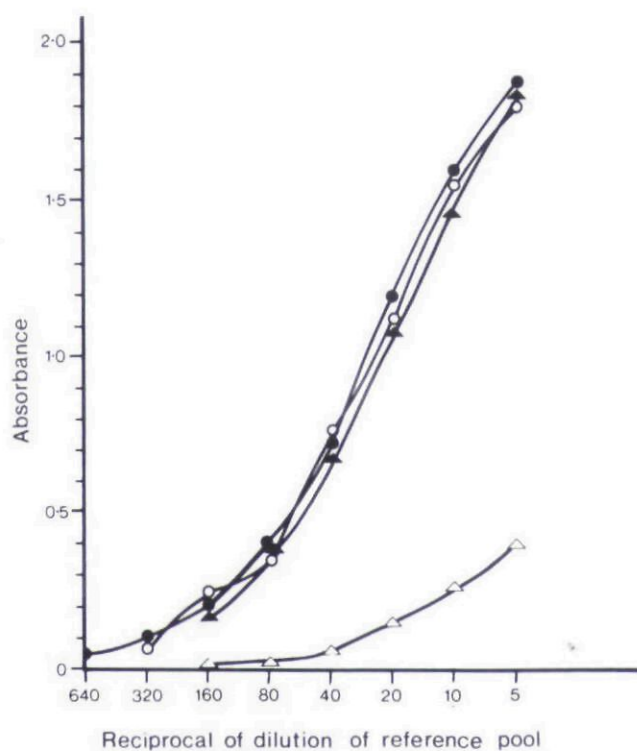
Specificity

As shown in Fig. 1 there was no difference in the absorbance values of the unadsorbed reference pool and the same adsorbed with DPP or BSA coated to Sepharose 4B, while the absorbance values greatly decreased after adsorption with LP coated to Sepharose 4B.

Table 1. Quality-control data for the BAS assay of LP-specific IgE antibodies

Serum	Absorbance		Coefficient of variation (%)
	Mean*	$\pm$ s.d.	
Cord serum	0.075	0.023	7.3
Positive serum	0.785	0.054	17.0
Positive serum	0.746	0.038	12.0
Negative serum	0.110	0.018	5.7

* Mean of ten determinations.

**Fig. 1.** Comparison of the dose-response curves of the reference pool unadsorbed (●---●), adsorbed with BSA (○---○), DPP (▲---▲), LP (△---△) coated to the Sepharose 4B.*Correlation between RAST and BAS assay*

In the thirty-two RAST-negative subjects the mean values $\pm$ 1 s.d. were 2.9% counts bound $\pm$ 1.0 in the RAST and 0.04 AU $\pm$ 0.02 in the BAS assay, while in the twenty-six RAST-positive subjects they were, respectively 23.0% counts bound $\pm$ 11.0 and 1.17 AU $\pm$ 0.90. In fifty-three of the fifty-eight subjects the results were in agreement (Table

Table 2. Comparison of the results of the BAS assay and RAST for LP-specific IgE antibodies: overall agreement 91% (53/58 assays)

		RAST		
		Positive	Negative	Total
BAS assay	Positive	23	2	25
	Negative	3	30	33
	Total	26	32	58

Statistical analysis $\chi^2=0.0032$ not significant.

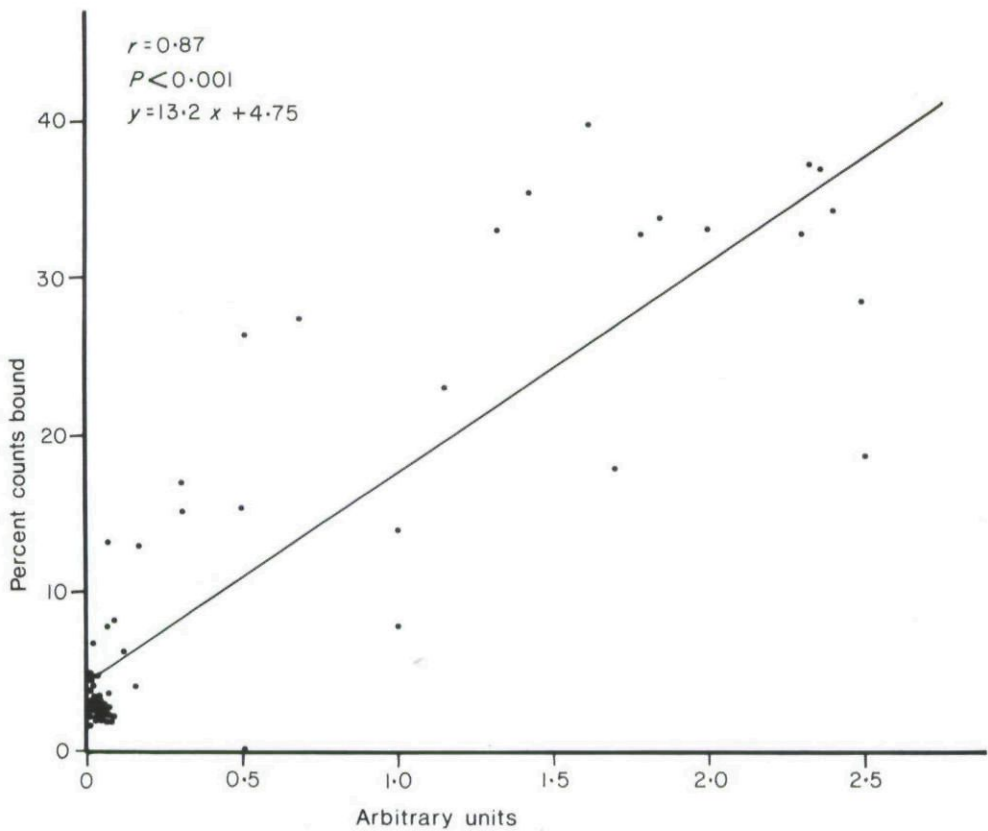


Fig. 2. Correlation between LP-specific IgE assayed by the BAS assay (AU) and RAST (% counts bound).

2). In addition the correlation between AU in the BAS assay and percent counts bound in the RAST (Fig. 2) was statistically significant ($r = 0.87$, $P < 0.001$).

Discussion

The BAS assay described in this paper is based on the extremely high affinity of avidin for biotin. Many molecules of biotin can be coupled to a protein and the biotinylated

protein then binds an avidin for each biotin; after coupling of peroxidase to the avidin, many molecules of peroxidase are bound to each avidin. As shown by the adsorption experiments, the antibodies detected by the BAS assay were specific for LP. The BAS assay correlated well with the RAST; the overall agreement was 91%.

The BAS assay offers various advantages over both the RAST and the classical ELISA technique which employs antibody conjugated to peroxidase. The advantages with respect to the former are: (i) no radioactivity is required; (ii) supplies and equipment are less expensive; (iii) it is very simple and rapid to perform; (iv) the microtitre plates are easily handled: all ninety-six wells can easily be rinsed with a squeeze bottle and repeatedly washed between steps, and (v) it is possible to use a photometer which reads one plate in a minute for the BAS assay.

With respect to the latter the advantages are: (i) in the classical ELISA, antibody is coated to the enzyme either with glutaraldehyde (Avrameas, 1969) or per-iodic acid (Nakane & Kawaoi, 1974) which partially alter the biological activity of proteins, while biotinylation has a minimal effect on their biological properties; (ii) the number of enzyme molecules coated to antibody by the glutaraldehyde or per-iodic-acid methods is much smaller than with the BAS assay; (iii) only a single labelled conjugate, namely avidin, need be kept on hand for use with a variety of biotinylated antibodies; and (iv) in our hands this BAS assay overcame the problem of background colour sometimes encountered with the classical ELISA.

A disadvantage of the BAS assay over the classical ELISA is that it requires an additional step.

Although we have described the BAS assay for only one antigen, we are currently using this technique with several other antigens with equally good results.

Acknowledgments

We thank Mr A. Ascione for expert technical assistance and Ms M.G. Zambon for her secretarial assistance. This work was partly supported by CNR, Rome.

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