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Magnetic enzyme immunoassay of anti-grass pollen specific IgE in human sera

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SUMMARY

This paper reports a magnetic solid-phase sandwich enzyme immunoassay for specific IgE antibodies in human sera. Crude extracts of grass pollen bound to magnetic polyacrylamide agarose beads were mixed with human serum to be tested. After washing in a magnetic rack, the beads were incubated with the glucose-oxidase-labelled sheep anti-IgE. The enzyme activity associated with the beads was measured by colorimetric assay. Results obtained from sixty-one human sera, as measured by the magnetic enzyme immunoassay, gave a linear correlation coefficient of 0·98 with the values as determined by radio-immunoassay.

This procedure, which allows the grass pollen specific IgE in human sera, to be measured, is easy to perform, reproducible and may avoid the use of radioactive compounds.

INTRODUCTION

The use of magnetic polyacrylamide agarose beads has recently been proposed as a way of insolubilizing antigen or antibody in order to determine the respective quantities of antibody and antigen in various sera (Guesdon & Avrameas, 1977).

In a previous study, we coupled complex antigens to magnetic polyacrylamide agarose beads and these were used in an enzyme immunoassay for quantification of specific antibodies to grass pollen and mites in rabbit sera (Guesdon, David & Lapeyre, 1977).

In the present paper, we report the same sandwich procedure using grass pollen as antigen, bound to magnetic polyacrylamide agarose beads and glucose-oxidase-labelled sheep anti-IgE, allowing the measurement of human specific IgE in allergic patient serum (David & Guesdon, 1977). The technique was optimized by studying the effect of the period incubation, the choice of glucose-oxidase-labelled antibody dilution and the different volumes of bead suspension and serum to be tested.

Sera from sixty-one grass pollen sensitive patients were tested and the results obtained with the magnetic enzyme immunoassay were compared to those obtained by radio-immunoassay

MATERIALS AND METHODS

Allergen extracts. Four crude grass pollen extracts of timothy (*Phleum pratense*), cocksfoot (*Dactylis glomerata*), rye (*Secalum cereale*) and perennial rye grass (*lolium perenne*) were obtained from the Service des Allergènes, Institut Pasteur, Paris. Each aqueous grass pollen extract contained approximately 20 mg protein/ml determined by Folin's technique.

Binding of the allergens to magnetic polyacrylamide agarose beads. Grass pollen extracts were bound to magnetic polyacrylamide agarose beads (Magnogel®, IBF, 92231 Gennevilliers, France) using glutaraldehyde as a coupling agent according to a procedure described previously (Guesdon, David & Lapeyre, 1977). Briefly, Magnogel was washed with distilled water using a magnet (Ticonam FC 57, Arelec, 64005 Pau, France) to maintain the beads in the vessel.

When the supernatant was completely clear, the gel was incubated overnight at 37°C in a 6% solution of glutaraldehyde in 0·1 M phosphate buffer, pH 7·4 and then extensively washed with water. An excess of grass pollen extracts (6 ml per 20 ml beads) in phosphate buffer pH 7·4 was incubated overnight at room temperature with the glutaraldehyde treated beads.

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Under these conditions about 3 mg of protein was bound per ml of beads. In order to block any remaining free aldehyde groups on the beads, the gel was suspended in 0·1 M phosphate buffer pH 7·4 containing 0·1 M lysine and left at room temperature overnight. After washing, the gel was diluted ten-fold in phosphate buffered saline (PBS) containing 0·02% Merthiolate and 0·1 % Tween 20.

IgE protein. Serum from patient Yu with IgE myeloma (Stefani & Mokeeva, 1972) was obtained through the courtesy of Dr Nezlin (Institute of Molecular Biology, Moscow). Immunoglobulin E was isolated from this serum by means of ion exchange chromatography on DEAE-cellulose (DE 32, Whatman) according to Nezlin *et al.* (1973).

Antiserum and antibodies. Antiserum to human IgE used in the present work was obtained by injecting a sheep several times with IgE myeloma protein Yu in Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan, USA). The antiserum was made monospecific by absorption on glutaraldehyde insolubilized human serum (Avrameas & Ternynck, 1969). Tested by immunelectrophoresis and single radial diffusion, it was found to reveal only IgE. The anti-IgE antibodies were isolated from this antiserum by using IgE myeloma protein Yu coupled to polyacrylamide agarose beads (Guesdon & Avrameas, 1976).

Coupling of antibodies with glucose oxydase. Isolated antibodies (5 mg) were coupled with 10 mg Aspergillus niger glucose oxidase (Grade I—Boehringer–Mannheim, Germany) using $1{\cdot}3\ 10^{-2}$ M glutaraldehyde (Avrameas, 1969).

Magnetic solid phase enzyme immunoassay. The procedure employed in this work was similar to that already described elsewhere (Guesdon & Avrameas, 1977). A given quantity (500 μl) of grass pollen extract bound beads was added to a series of disposable polystyrene test-tubes (13 × 75 mm). The beads were washed once. To each tube, 500 μl of serum adequately diluted in PBS containing 1% bovine serum albumin, 0·1% Tween 20 and 0·02% Merthiolate (medium 1) were added. When a small amount of specific IgE was expected the serum was tested undiluted. After 18 hr of rotation at room temperature, the beads were washed three times on a magnetic rack with 4 ml of PBS containing 0·1% Tween 20 (medium 2). Next, 500 μl of glucose-oxidase-labelled sheep anti-human IgE (2·5 μg), diluted in medium 1, were added to each tube. The tubes were allowed to rotate at room temperature for 4 hr and then excess conjugate was removed by washing three times with medium 2. The glucose-oxidase activity was then measured by adding 3 ml of fresh substrate solution to each tube. The substrate solution was prepared by dissolving 10 mg of D-glucose, 100 μg of horse radish peroxidase Grade II (RZ = 0·6, Boehringer–Mannheim, Germany) and 400 μg of o-phenylene diamine per ml of 10^{-2} M phosphate buffer pH 6·8. The enzyme reaction was performed by rotating the tubes horizontally for 30 min at room temperature in the dark. Then a magnet was used to attract the beads and keep them along the tube walls so as to allow the reaction mixture to be poured into a series of tubes containing 50 μl 4N hydrochloric acid, which stopped the reaction. The yellow colour of the product was read by measurement of the absorbance at 492 nm.

Estimation of anti-cocksfoot pollen specific IgE by radio-immunoallergosorbent test (RAST). Paper discs (Whatman No. 4) were activated with cyanogene bromide as described by Ceska, Erickson & Varga (1972) and used as a support for coupling the allergens. Twenty ml of crude extract cocksfoot pollen was stirred at 4°C overnight with 500 mg of activated paper discs according to the method described by Topping *et al.* (1977). The following procedure was performed according to Wide, Bennich & Johansson (1967). Each paper disc was incubated at room temperature for 3 hr with 50 μl of test serum. All liquid was removed and after washing three times with saline, 50 μl of ^{125}I-anti IgE solution (Pharmacia, Uppsala) were added to each disc. The tubes were left overnight and then washed three times. The radioactivity on the disc was measured using a gamma-counter. The uptake of added radioactivity was measured in allergic, non-allergic sera and in pooled umbilical cord sera, as a non-allergic control.

Allergic and non-allergic sera. Sera from allergic and non-allergic patients were kindly supplied by Dr N. Donat (Hôpital Boucicaut, Paris). Hypersensitivity was determined by case history and skin testing.

RESULTS AND DISCUSSION

Choice of the enzyme

In a recent publication (Guesdon & Avrameas, 1977), it was shown that alkaline phosphatase seemed to be a more effective label than glucose oxidase in a magnetic enzyme immunoassay. But the low effectiveness of glucose oxidase for the measurement of antibody by this enzyme immunoassay was due to the procedure employed for quantifying this enzyme. In this experiment, glucose oxidase was quantified by measuring the chromogenic derivative which appeared after oxidation of o-dianisidine. This chromogenic derivative was found to be only partially soluble and to be bound irreversibly with the beads. In this paper we describe how o-dianisidine was replaced by a more suitable chromogen, i.e. o-phenylene-diamine. The coloured reaction product was stable and did not precipitate even at a high concentration. On the other hand, the phosphatase activity found in the grass pollen extracts did not allow the use of this enzyme as a label.

Effect of incubation time

In a previous experiment, the first incubation period was tested. This varied from 30 min to 18 hr

(30 min, 1 hr, 2 hr, 4 hr and 18 hr) while the second incubation period was kept constant (2 hr). This experiment was performed with 1/8 dilution of an allergic patient serum (500 μl), 500 μl of perennial rye grass pollen bound beads and 2·5 μg of glucose-oxidase labelled anti-IgE. Under these conditions, binding of specific IgE to insolubilized allergen gradually increased from 30 min to 18 hr (Table 1).

TABLE 1. Effect of variations in first incubation period, the second period being constant (2 hr)

First incubation period	Absorbance
30 min	0·35
1 hr	0·38
2 hr	0·42
4 hr	0·46
18 hr	0·51

TABLE 2. Effect of variations in second incubation period, the first period being constant (18 hr)

Second incubation period	Absorbance
30 min	0·31
1 hr	0·37
2 hr	0·51
4 hr	0·68
18 hr	0·65

In a second experiment, the first incubation period was maintained constant (18 hr) and the length of the second incubation was varied from 30 min to 18 hr (Table 2). The enzymatic activity on the beads increased during the first 4 hr and then reached a peak. In view of these results and in order to be able to perform the assay in one working day, the incubation times chosen for all the other tests were 18 hr for the first incubation and 4 hr for the second incubation.

Effect of glucose-oxidase labelled antibody concentration

A given quantity (500 μl) of perennial rye grass pollen bound beads, 500 μl of three patient serum dilutions (1/2, 1/8, 1/32) and a constant volume (500 μl) of labelled antibody solution containing various concentrations of antibody were used. Fig. 1 shows a rapid increase up to 5 μg/ml and a slower increase thereafter. The absorbance increased 100% between 2·5 and 5 μg/ml but only 25% between 5 and 10 μg/ml. For that reason, a concentration of 5 μg/ml was deemed most suitable for the subsequent experiments.

Choice of the volume of perennial rye grass pollen bound bead suspension

Four volumes (150 μl, 500 μl, 1 ml and 2 ml) of the ten-fold diluted bead suspension (stock suspension) were tested. All the other parameters were maintained constant. The experiment showed that enzymatic activity increased with bead volume (Fig. 2). However, this increase was not pronounced enough to justify, from a practical point of view, the use of higher volumes exceeding 500 μl of stock suspension. This volume provided a sufficient excess of allergen.

Effect of the volume of test serum

Specific IgE concentration was estimated in different volumes of diluted allergic patient serum.

It was found (Fig. 3) that 500 μl of serum gave sufficient absorbance even with the highest dilution (1/32) and the values obtained with the three dilutions showed a more significant difference.

Magnetic enzyme immunoassay of anti-grass pollen specific IgE in human sera and correlation between the values obtained by this enzyme immunoassay and by radio-allergosorbent test

An allergic human serum was tested against the four different species of grass pollen extract: timothy, cocksfoot, perennial rye grass and rye. The curves obtained are shown in Fig. 4. In the same serum we tested the reproducibility of this technique measuring the absorbance in ten samples by dilution. The results showed a maximal variability of 15% for each dilution. When normal human sera were used, the

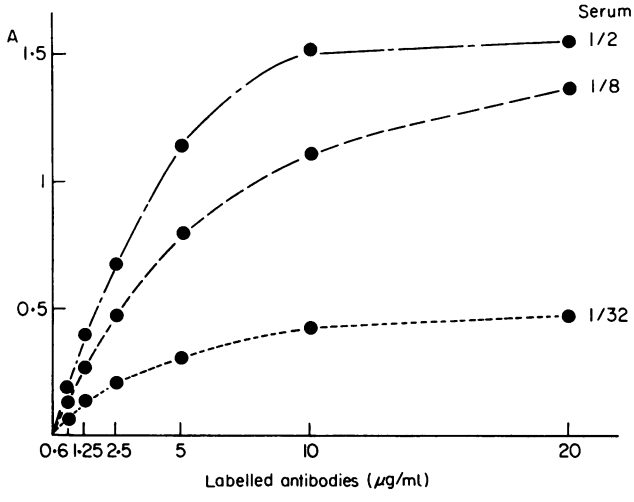

FIG. 1. Determination of the optimal labelled antibody concentration.

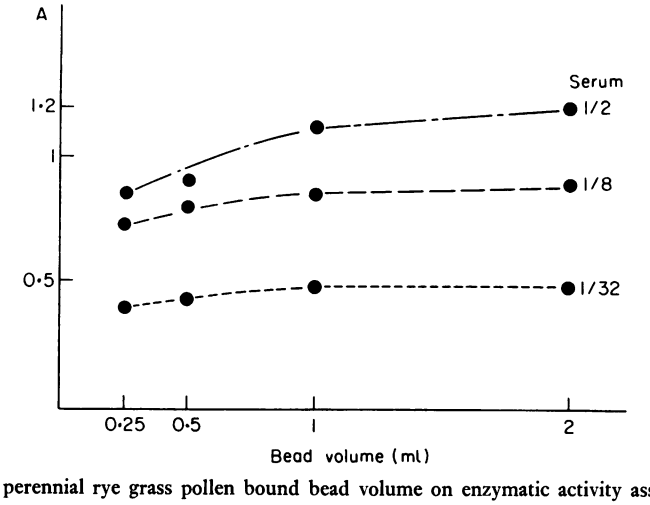

FIG. 2. Effect of perennial rye grass pollen bound bead volume on enzymatic activity associated with the solidphase

enzymatic activity was not significantly different from that of the background (about 0·02) and this probably corresponds to the non-specific absorption of the enzyme-antibody conjugate on the beads. One allergic patient serum diluted ten times was incubated with increasing amounts of soluble perennial rye grass pollen extract for 8 hr at laboratory temperature. The specific IgE was measured by the magnetic enzyme immunoassay using perennial rye grass pollen bound beads. The inhibition curve (Fig. 5) shows the 100% inhibition that was obtained with pollen extract diluted ninety times. The specificity is demonstrated by the low absorbance of non allergic serum and by the inhibition test with the grass pollen sensitive patient serum.

In subsequent experiments, insolubilized cocksfoot pollen extract was used to test sixty-one human sera by the present magnetic enzyme immunoassay. These sera were also submitted to the radio-allergosorbent test. The two series of values obtained by the two different techniques are reported in Table 3. In all sera tested there was agreement between enzymatic activity associated with the cocksfoot allergen bound beads and ^{125}I bound to insolubilized cocksfoot allergen. A high degree of radioactivity corresponded to intense absorbance and a low degree of radioactivity to weak absorbance. The statistical

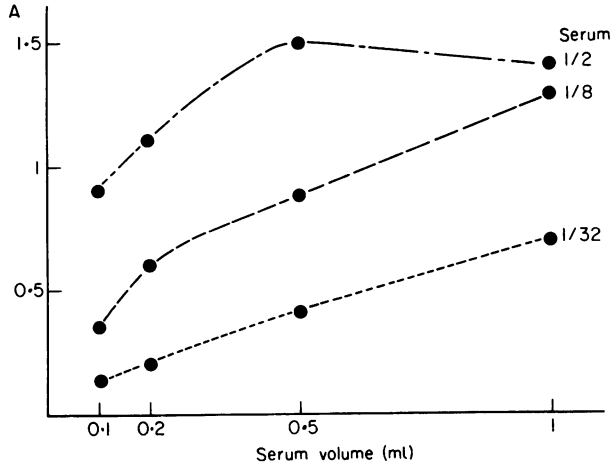

FIG. 3. Effect of patient serum volume on enzymatic activity associated with the beads.

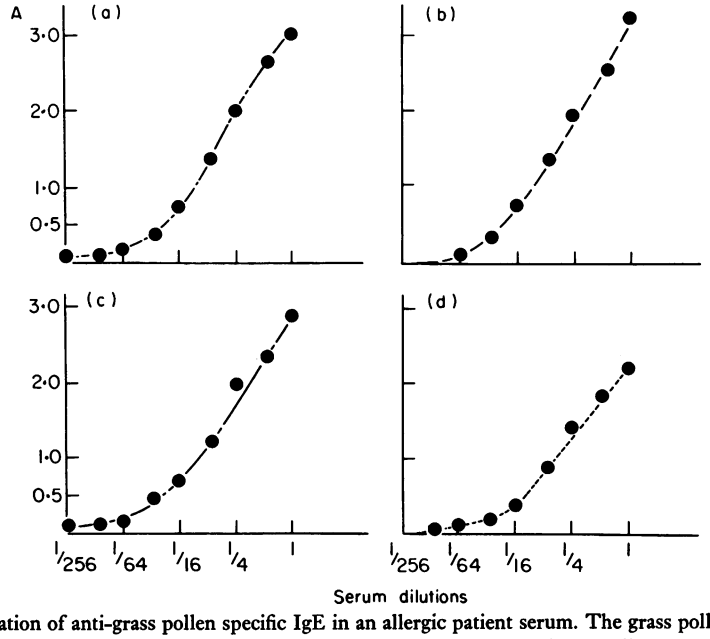

FIG. 4. Estimation of anti-grass pollen specific IgE in an allergic patient serum. The grass pollens used were (a) cocksfoot pollen; (b) timothy pollen; (c) perennial rye grass pollen and (d) rye pollen.

parameters were obtained from the comparison of the two different methods. The correlation between them is demonstrated by the correlation coefficient (r = 0·98) and illustrated by the regression line shown in Fig. 6, where the Logit of percentage of total counts bound were plotted against $\log_{10}$ of absorbance. With the number of sera tested (n = 61) the slope of the least squares line is 2·01 and the y intercept of the least squares line is −0·39.

Finally, a good correlation was observed, but it must be noticed that the MEIA is less sensitive than RAST since MEIA allowed the determination of specific IgE in 500 μl of serum diluted from 1/1 to 1/128, while RAST was able to measure specific IgE in 50 μl of the same serum diluted from 1/1 to 1/256. Moreover the incubation times were not the same for the both techniques. The first incubation period was 18 hr in the MEIA and 3 hr in the RAST, while the second incubation period was 4 hr in the

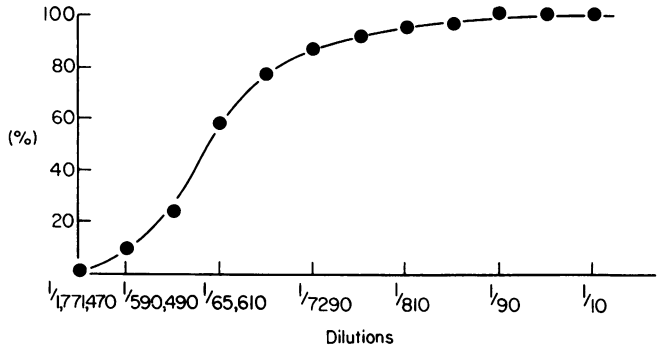

FIG. 5. Inhibition curve. The percentage of inhibition is plotted against the dilution of soluble perennial rye grass pollen extract.

TABLE 3. Estimation of anti-cocksfoot specific IgE in human sera by radio-allergosorbent test (RAST) and by magnetic enzyme immunoassay (MEIA)

Sample number	Percentage of radioactivity* bound to the solid phase (RAST)	Absorbance read* (MEIA)	Sample number	Percentage of radioactivity* bound to the solid phase (RAST)	Absorbance read* (MEIA)
1	63	2·354	31	29	0·687
2	55	2·052	32	27	0·740
3	53	2·020	33	27	0·560
4	51	1·735	34	23	0·244
5	50	1·753	35	23	0·340
6	50	2·040	36	23	0·425
7	47	1·820	37	22	0·360
8	47	1·484	38	22	0·331
9	46	1·562	39	22	0·183
10	46	1·424	40	18	0·115
11	45	0·975	41	18	0·256
12	41	1·595	42	15·6	0·150
13	41	0·963	43	15	0·140
14	40	1·130	44	14	0·146
15	40	0·757	45	13	0·144
16	40	1·049	46	12	0·096
17	39	1·050	47	12	0·145
18	39	0·672	48	12	0·113
19	39	0·883	49	12	0·095
20	39	0·910	50	9	0·055
21	38	1·146	51	6·5	0·064
22	37	0·860	52	6·5	0·095
23	35	0·510	53	6·5	0·068
24	34	0·614	54	5	0·050
25	33	0·530	55	4·5	0·051
26	33	0·626	56	2·6	0·025
27	33	0·614	57	2·1	0·051
28	31	0·600	58	2·0	0·027
29	31	0·600	59	2·0	0·025
30	30	0·442	60	1·7	0·035
			61	1·6	0·030

* The background values were subtracted from the results obtained.

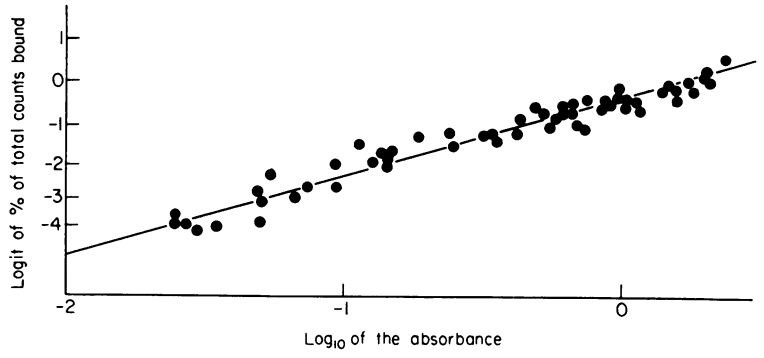

FIG. 6. Comparison between the results obtained by the radio-allergosorbent test (RAST) and those obtained by magnetic enzyme immunoassay (MEIA). The logit-log representation is used.

MEIA and lasted overnight in the RAST. It must be also mentioned that MEIA required agitation during the incubations.

In conclusion, the allergens present in crude grass pollen extracts can be covalently bound to magnetic polyacrylamide agarose beads using glutaraldehyde as a coupling agent. The immunoadsorbents obtained thus can be used for measuring anti-grass, pollen specific IgE in sera from allergic patients. The good correlation observed with RAST justifies the use of magnetic enzyme-immunoassay to measure anti-grass pollen specific IgE. It offers several advantages because it does not require multiple centrifugation, is relatively easy to handle, regularly reproducible and avoids the use of radioactive compounds.

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