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A New Solid Support for Sandwich Enzyme Immunoassays of Human Immunoglobulin G *

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Two antibodies were prepared for use in a sandwich enzyme immunoassay of human IgG. Completely purified guinea pig anti-human IgG was labelled with β-D-galactosidase (EC 3.2.1.23), using a heterobifunctional cross-linker named GMBS. Partially purified anti-human IgG was immobilized on a new solid support: Amino-Dylark balls. Optimal conditions for immobilizing the antibody, using glutaraldehyde as the coupling reagent, were studied in detail. With the enzyme-labelled antibody and the solid-phase anti-human IgG, a sandwich enzyme immunoassay of human IgG with a lower limit of detection at 10.5 pM (0.3 ng/tube) was developed. A comparative study of the EIA method and a laser nephelometric method showed a good correlation. The specificity of the assay was excellent: all 4 types of IgG tested showed the maximum 0.0001%; human IgA, IgM and albumin possessed the maximum 0.54% in their cross-reactivity values with human IgG.

Key words: *enzyme immunoassay – human IgG – new solid support*

Introduction

Recently, solid-phase immunoassay has progressed rapidly owing to the easy separation of the bound label (Langone and Vunakis, 1981). Most of the solid matrices are based on the simple absorption of antigens or antibodies to plastic surfaces. In these cases it is not easy to prevent non-specific absorption of im-

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Abbreviations: Ig, immunoglobulin; anti-human IgG, guinea pig anti-human IgG antibody; EIA, enzyme immunoassay; GMBS, *N*-(gamma-maleimidobutyryloxy)succinimide; Gal, β-D-galactosidase; EDTA, ethylenediamine tetraacetate; BSA, bovine serum albumin.

munoreagents, or the desorption of antigen or antibody, from the solid immunosorbents. To diminish these problems the covalent bonding of antigens or antibodies has been studied in relation to the preparation of solid immunosorbents (Fuchs and Sela, 1978; Hendry and Herrmann, 1980) but satisfactory solutions have, to date, not been found.

Recently we developed enzyme immunoassays (EIAs) for rabbit IgG (Tanimori et al., 1983) and for the IgG specific to neocarzinostatin (Kitagawa et al., 1984) using an Amino-Dylark cylinder as the solid support. Applications of the solid-phase immunosorbents covalently bonded to amino groups of Amino-Dylark cylinders gave us the benefit of producing sensitive and accurate assays. The solid matrix could, however, still be improved. For example, there is the need for more convenience in handling and for low desorptions of the immunosorbent with wider measuring ranges without sacrificing accurate and reproducible assay results.

Ball-type Amino-Dylark, which may diminish the desorption problem in the solid-phase EIAs, was prepared for this purpose. We report here detailed comparisons of ball- and cylinder-type solid matrices and also an application of the ball type in a highly sensitive, accurate and specific sandwich EIA for human IgG.

Materials and Methods

Reagents

β-D-Galactosidase (Gal) from *Escherichia coli* (EC 3.2.1.23) was bought from Boehringer Mannheim, F.R.G.; human IgG from Miles Lab., Elkhart, IN; and DEAE-Sepharose CL-6B and cyanogen bromide-activated Sepharose 4B from Pharmacia Fine Chemicals, Uppsala. *N*-(gamma-maleimidobutyryloxy)succinimide (GMBS) (Tanimori et al., 1981) was the product of Dojin Chemicals, Kumamoto. The commercial Amino-Dylark cylinders (6 mm diameter, 4 mm height) and balls (6 mm diameter) were generous gifts from Sekisui Chem. Ind., Osaka. Other chemicals used were of reagent grade.

Purification of guinea pig antibody to human IgG

Guinea pig antiserum to human IgG (12 ml), which was elicited in the animal immunized with human IgG emulsified with Freund's complete adjuvant by the method of Vaitukaitis et al. (1971), was precipitated by 50% saturated ammonium sulphate. The precipitate was washed with a 33% saturated ammonium sulphate solution and dissolved in 0.02 M sodium phosphate buffer, pH 6.8 (7 ml). The solution was dialysed against the same phosphate buffer at 4°C overnight. The IgG solution was loaded onto a DEAE-Sepharose CL-6B column (1.5 × 20 cm) and eluted with the same buffer. The IgG fractions (nos. 4–12, 5 ml/tube) were pooled and dialysed against 0.02 M sodium phosphate-buffered saline, pH 7.0 (PBS). This partially purified IgG was used as the solid-phase antibody (see later). For enzyme labelling, the IgG fraction was further purified by affinity chromatography on a human IgG-coupled cyanogen bromide-activated Sepharose 4B column, prepared according to the manual of Pharmacia Fine Chemicals.

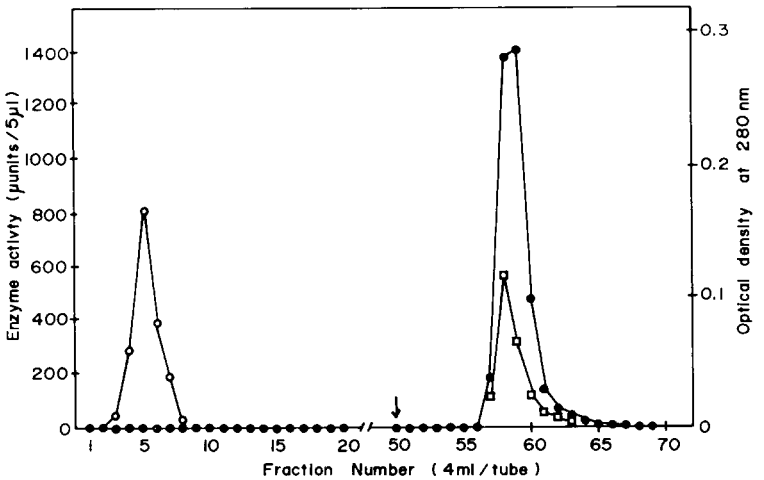

Fig. 1. Elution profiles of anti-human IgG-Gal from a DEAE-Sepharose CL-6B column. The arrow indicates the change in NaCl concentration (0.1–0.4 M) in 0.05 M sodium phosphate buffer (pH 7.0). ○, OD at 280 nm; ●, enzyme activity of the conjugate measured in 5 μl of each fraction at 30°C for 5 min; □, immunoreactive enzyme activity determined by the EIA described in Materials and Methods, except that 5 μl of each fraction and human IgG-coated Amino-Dylark were used.

Enzyme labelling of anti-human IgG antibody

A tetrahydrofuran solution of GMBS (0.9 μg, 3.2 nmol/90 μl) was added dropwise to a 1 ml solution of anti-human IgG (0.5 mg, 3.2 nmol) in 0.02 M phosphate-buffered saline (pH 7.0) (PBS), with vortex mixing, and was incubated at 30°C for 30 min. One ml of the reaction mixture was then added dropwise to a 1 ml PBS solution of β-galactosidase (Gal) (340 μg, 630 pmol) over 10 min with gentle mixing, and the solution was then incubated at 30°C for 2 h. The reaction mixture was directly chromatographed on a DEAE-Sepharose CL-6B (1.5 × 23 cm), previously swollen with 0.05 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl. Stepwise elution was then performed with the same buffer containing 0.1 and 0.4 M NaCl. The enzyme and the immunoreactive enzyme activities in 5 μl of each fraction (5 ml/tube) were then measured (see legend to Fig. 1) and the immunoreactive enzyme activity was found to be almost parallel to the pure enzyme activity.

Measurement of β-D-galactosidase (Gal) activity

Five μl of diluted enzyme solution were incubated with 0.15 ml of 0.1 mM 7-β-D-galactopyranosyloxy-4-methylcoumarin in a 0.05 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% BSA and 0.1% NaN_3 (buffer A) at 30°C for 30 min. The reaction was stopped by the addition of 2 ml of 0.2 M glycine-NaOH buffer, pH 10.3, and the 7-hydroxy-4-methylcoumarin liberated was measured spectrofluorometrically. The Gal-labelled anti-human IgG was expressed in units of Gal activity; and 1 unit of the enzyme activity was defined as the amount able to hydrolyze 1 μmol of the substrate/min.

Preparation of anti-human IgG-coated Amino-Dylark cylinders and balls

The amino-Dylark cylinders and balls were washed with a detergent (Scat 20-X; Nakarai Chemicals, Kyoto) and immersed in 1% glutaraldehyde for 1 h while being shaken. This was followed by washing with 0.02 M phosphate-buffered saline, pH 7.0 (PBS). The solid supports were immersed in 0.01% anti-human IgG in PBS at room temperature for 20 min and then at 4°C for 2 h. After washing with PBS and buffer A, successively, the Amino-Dylark cylinders and balls coated with anti-human IgG were stored in buffer A at 4°C until use.

Immunoassay procedure by the sandwich method

Amino-Dylark cylinders and balls coated with anti-human IgG were incubated at 30°C for 2 h while being mechanically shaken, with standard human IgG or sample antigens dissolved in a final 0.2 ml volume of buffer B (0.06 M sodium phosphate, pH 7.4, containing 0.01 M EDTA and 0.1%BSA). After 2 washes with 1 ml of buffer A, the bound human IgG on the solid supports was reacted with the Gal-labelled guinea pig anti-human IgG (200 μU) dissolved in 0.2 ml of buffer A, by incubation at 30°C for 2 h with shaking. Each Amino-Dylark cylinder or ball was washed twice with 1 ml of buffer A and was transferred to a fresh tube to eliminate non-specific enzyme activity bound to the wall of the test tube. The enzyme activity bound to the solid supports was assayed.

Results

Preparation of solid-phase antibody

The optimal concentration of glutaraldehyde for immobilization of guinea pig anti-human IgG to the amino groups of Amino-Dylark balls was first tested using various concentrations of glutaraldehyde. Thus, binding of the solid-phase immunosorbents to 0, 1 and 100 ng of human IgG was compared using Gal-labelled anti-human IgG as the indicator (Table I). The maximum a/c value was obtained for the 1% glutaraldehyde solution. Although the 5% solution gave a similar a/c value, a concentration of 1% was chosen, based on the a/b values shown in Table I.

The optimal concentration of anti-human antibody used for the preparation of the solid adsorbant was then examined. Table II shows that the concentration of the antibody is also an important factor for the assay, and the 10 μg/ml solution which gave the lowest assay variation yet with sufficient binding to human IgG was chosen, on the basis of CV and bound values (Table II).

Table III summarizes the experiment to determine the optimal incubation time for immobilizing the anti-human IgG. A time of 1 h was chosen, based on the CV and binding values.

The optimal incubation times for the two-step immunoreactions in the EIA were then studied by similar methods to those reported in our previous paper (Tanimori et al., 1983). Almost the same experimental results were obtained (data not shown). Thus, 2 h was chosen for both incubation times of the two-step immunoreaction (the first reaction between antibody-coated balls and human IgG samples (1, 10 and 100

TABLE I

COMPARISON OF THE CONCENTRATIONS OF GLUTARALDEHYDE (GA) USED TO IMMOBILIZE ANTI-HUMAN IgG ON AMINO-DYLARK BALLS

Bound enzyme activities of the sandwich EIA of human IgG (0, 1 and 100 ng) were measured using the glutaraldehyde-treated Amino-Dylark balls.

GA concentration (%)	Bound enzyme activity			a/b	b/c	a/c
	With antigen (ng/tube)		Without antigen 0 (c)			
	100 ng (a)	1 ng (b)				
0.2	389.8	67.4	24.2	5.8	2.8	16.1
1	423.3	85.5	11.4	5.0	7.5	37.2
5	440.8	99.0	12.5	4.5	7.9	35.2
25	348.1	54.8	17.6	6.4	3.1	19.8

TABLE II

EFFECT OF THE CONCENTRATION OF ANTI-HUMAN IgG ANTIBODY USED FOR IMMOBILIZATION

Amino-Dylark balls pretreated with 1% gultaraldehyde were incubated for 2 h with the antibody and the amounts of the immobilized antibody were determined by the sandwich EIA for human IgG using either 100 ng human IgG or a zero antigen (Ag) control.

Concentration (μg/ml)	Bound enzyme activity				
	With 100 ng human IgG	CV (%)	Without Ag	CV (%)	Bound (%)
2	343.0 ± 20.5	6.0	3.7 ± 0.37	10.0	21.9
4	341.7 ± 6.8	2.0	4.0 ± 0.50	12.5	21.8
10	674.6 ± 23.4	3.5	4.1 ± 0.05	1.2	43.1
20	771.9 ± 29.6	3.8	4.1 ± 0.36	8.8	49.3
40	685.8 ± 13.1	1.9	4.2 ± 0.15	3.6	43.8

TABLE III

EFFECT OF INCUBATION TIME OF ANTI-HUMAN IgG DURING IMMOBILIZATION TO AMINO-DYLARK BALLS PRETREATED WITH 1% GLUTARALDEHYDE

The sandwich EIA for 100 ng human IgG was used as the test method and the total enzyme activity that became bound was measured.

Incubation (h)	Bound enzyme activity	Binding (%)	CV (%)	Blank (without IgG)
1/6	737.2 ± 20.6	47.4	2.8	4.1
1/2	760.1 ± 12.6	48.9	1.7	4.6
1	784.7 ± 10.3	50.5	1.3	4.0
2	800.1 ± 15.0	51.5	1.9	4.2
4	768.1 ± 44.6	49.4	5.8	4.6
8	754.6 ± 20.8	48.5	2.8	4.7
16	636.6 ± 25.8	41.0	3.9	5.8

TABLE IV

DESORPTION OF IMMOBILIZED ANTIBODY AGAINST HUMAN IMMUNOGLOBULIN G FROM AMINO-DYLARK BALLS AND CYLINDERS WITH OR WITHOUT PRETREATMENT OF GLUTARALDEHYDE (GA) AS CROSS-LINKER USING β-D-GALACTOSIDASE-LABELLED HUMAN IgG AS THE TRACER

Incubation (h)	Bound enzyme activity (%)			
	Cylinders		Balls	
	With Ga	Without GA	With GA	Without GA
0	100	100	100	100
4	94.2	60.0	98.8	68.2
12	89.3	36.2	97.4	33.3
24	78.2	34.2	96.6	35.5

ng); and the 2nd reaction between the human IgG bound on the solid support, and Gal-labelled anti-human IgG).

Comparison of cylinder- and ball-type Amino-Dylark as solid supports for EIA of human IgG

The stability of Amino-Dylark balls and cylinders for sandwich EIA of human IgG was compared as follows: anti-human antibody bound to these solid supports under the conditions described above was periodically incubated in buffer B and was then reacted with an aliquot of human IgG. The bound human IgG was followed by the bound enzyme activities of the Gal-labelled anti-human IgG, which should reflect desorptions of the immobilized antibody to the solid supports. Results of the effect of glutaraldehyde treatment in these experiments are presented in Table IV.

The ball-type Amino-Dylark showed only 3.4% desorption of bound enzyme activity, while 21.8% reduction was observed for that of the cylinder type after 24 h incubation in buffer B.

In contrast, judging from the binding percentages of the bound enzyme activity, little difference was observed for the desorption of antibody adsorbed to both solid matrices without using glutaraldehyde.

Dose-response curves for sandwich EIAs of human IgG using Amino-Dylark balls and cylinders are shown in Fig. 2.

The assay range for the EIA measured using the balls was wider than that observed using the cylinders.

Accuracy of sandwich EIA for human IgG using Amino-Dylark balls as solid support

Precision tests for the method are summarized in Table V. Good recoveries (98.8–103.3%) were obtained for 5 IgG samples, with coefficients of variation of less than 7.5 and 17.1% for intra- and inter-assays respectively.

Specificity of the EIA

The specificity of the EIA was examined using rabbit, horse, mouse and pig IgG.

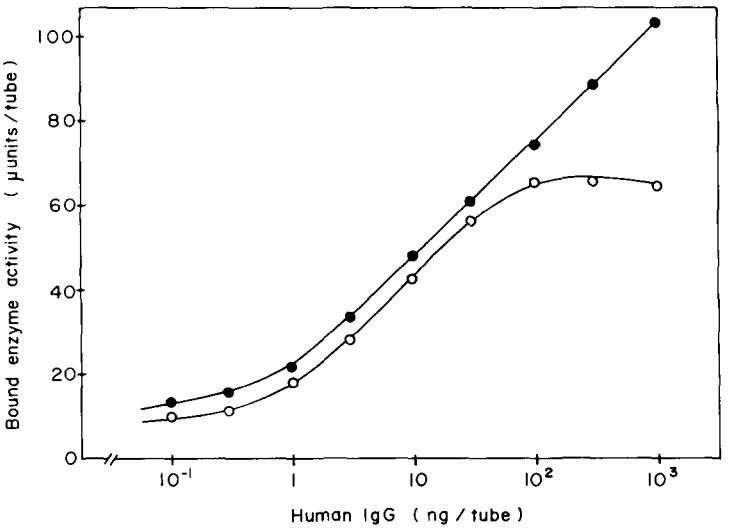

Fig. 2. Typical calibration curves for sandwich EIA of human IgG with anti-human IgG-loaded Amino-Dylark balls (●) or cylinders (○). Bound enzyme activity (μU) plotted against log dose of human IgG (100 pg to 300 ng).

TABLE V

PRECISION OF ENZYME IMMUNOASSAY OF HUMAN IgG

Sample	IgG added (ng/tube)	Estimated [a] (ng/tube)	Recovery (%)	CV [b] (%)	n [c]
Intra-assay	0.3	0.307 ± 0.023	102.3	7.5	5
	1.0	0.988 ± 0.019	98.8	1.9	5
	3.0	3.026 ± 0.043	100.9	1.4	5
	10.0	10.06 ± 0.33	100.6	3.3	5
	30.0	30.26 ± 2.11	100.9	7.0	5
Inter-assay	0.3	0.310 ± 0.053	103.3	17.1	5
	1.0	1.008 ± 0.090	100.8	8.9	5
	3.0	3.001 ± 0.137	100.0	4.6	5
	10.0	10.01 ± 0.566	100.1	5.7	5
	30.0	30.73 ± 4.149	102.4	13.5	5

[a] Mean ± SD.
[b] Per cent coefficient of variation.
[c] Number of assays.

The lowest detectable amounts were tested. All showed extremely low cross-reactivities, with rabbit IgG being the highest (0.0001%), as shown in Table VI.

The cross-reactivities of human IgM, IgA and albumin were also low by the EIA (Table VII). Human IgM showed the largest value of 0.54%.

TABLE VI

CROSS-REACTIVITIES OF RABBIT, HORSE, MOUSE AND PIG IgGs IN THE SANDWICH EIA OF HUMAN IgG

Amount of human IgG corresponding to the bound enzyme activities of the test sample was obtained from the standard curve of human IgG.

Amount of IgG added (μg/tube)	Cross-reactivity (%)			
	Rabbit IgG	Horse IgG	Mouse IgG	Pig IgG
1	–	–	–	–
10	–	–	–	–
100	0.0001	–	–	–
1000	0.0002	0.00006	–	0.000036

TABLE VII

CROSS-REACTIVITIES OF HUMAN IgA, IgM AND ALBUMIN IN THE SANDWICH EIA OF HUMAN IgG

Amount of human IgG corresponding to the bound enzyme activities of the test sample was obtained from the standard curve of human IgG.

Amount of protein added (μg/tube)	Cross-reactivity (%)		
	Human IgA	Human IgM	Human albumin
1	0.42	0.54	–
10	0.065	0.086	0.017
100	–	–	0.0075

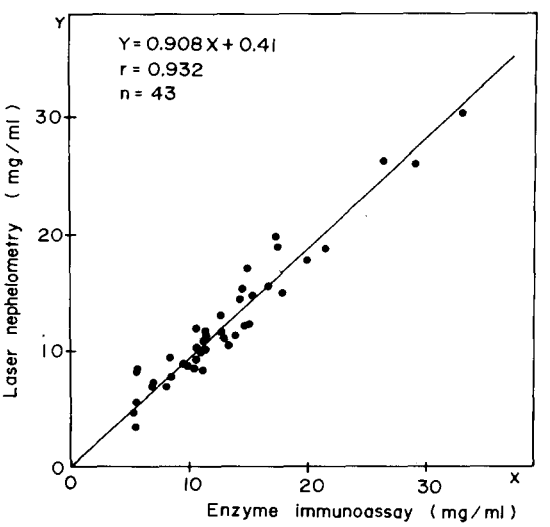

Fig. 3. Correlation of values for human IgG concentrations in 43 serum samples determined by the sandwich EIA and a laser nephelometric method respectively. Coefficient of correlation $r = 0.932$; $y = 0.908x + 0.41$.

TABLE VIII

RECOVERY TEST OF HUMAN IgG ADDED TO A HUMAN SERUM SAMPLE CONTAINING 4.02 ng OF HUMAN IgG

The sample solution (50 μl) was prepared as a 200,000-fold dilution of normal human serum in buffer B.

IgG added (ng/tube)	Estimated IgG level [a] (ng/tube)	Recovery of added IgG		n [b]
		(ng/tube)	(%)	
0	4.02 ± 0.16			3
3	7.14 ± 0.35	3.12	104.0	3
10	14.00 ± 1.50	9.98	99.8	3
30	33.60 ± 8.10	29.58	98.6	3

[a] Mean ± SD.
[b] Number of assays.

Fig. 3 compares the assay results for human IgG in sera obtained from Nagasaki University Hospital, determined by the present method and by laser nephelometry (Nohtomi and Inoue, 1979). The values between the limits of detection gave the regression: $y = 0.908x + 0.41$, $r = 0.932$.

The IgG content of the samples determined by both methods agreed well, with a few exceptions. The sample which showed the largest discrepancy by the 2 methods was further studied in a recovery test with 3 known amounts of added human IgG (3, 10 and 30 ng). The IgG content in the sample was confirmed by the EIA as being 4.02 ng (16.08 mg/ml in the original serum), with good recoveries of the added IgG samples (Table VIII).

Discussion

The rapid enzyme labelling method (Tanimori et al., 1983) has also been successfully used for Gal labelling of guinea pig anti-human IgG with the aid of a heterobifunctional cross-linker, GMBS (Kitagawa and Aikawa, 1976; Tanimori et al., 1981). The Gal-labelled antibody was isolated from the reaction mixture by chromatography of the DEAE-Sepharose CL-6B column (Fig. 1) and the immune specificity of the enzyme conjugate in the fractions estimated by EIA was closely parallel to its enzyme activity. Fractions 57–58 of the peak enzyme activity were thus chosen as the labelled antibody for EIA of human IgG.

The optimal conditions for immobilizing anti-human IgG antibody with the amino groups of Amino-Dylark balls using glutaraldehyde as the cross-linker were established by 3 experiments concerning the concentrations of glutaraldehyde (Table I) and anti-human IgG (Table II), as well as the incubation time for immobilizing the immunosorbent (Table III).

In addition, the optimal times for the first and second incubations in a sandwich EIA of human IgG were also established, using the solid-phase immunosorbent and

β-Gal-labelled anti-human IgG as the tracer (data not presented).

Using the optimal conditions established for the balls, anti-human IgG antibody was also coupled to the cylinders of Amino-Dylark. A comparison of their stability as the solid support for EIA of human IgG was made (Table IV). It is concluded that ball-type Amino-Dylark is superior to the cylindrical form in the following respects: convenience of handling, especially in the washing step; low desorption of the immobilized antibody; and wide measuring ranges for the assay (Fig. 2). Above all, the low desorption of the immobilized antibody is the most important characteristic of the Amino-Dylark balls.

The accuracy of the assay for human IgG was excellent, with over 98–104% recoveries, and with CV values of less than 17.1% over 5 point determinations (Table VI) for both intra- and inter-assay experiments.

The specificity of the EIA of human IgG presented here was also excellent, as shown by the cross-reactivity experiments for IgGs of rabbit, pig, etc. (Table VI), as well as for human IgA, IgM and albumin (Table VII). The sandwich EIA was compared with the laser nephelometric method, and a good correlation was obtained.

Although the reason for the difference between the cylinder and the ball immunosorbents in their desorption behaviour is not clear, especially the low desorption of the bound antibody from the Amino-Dylark balls may be responsible for the consistently low enzyme-linked immunoassay backgrounds of the control samples with consequent greater sensitivity and assay accuracy.

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