# ENZYME-LINKED SANDWICH IMMUNOASSAY OF MACROMOLECULAR ANTIGENS USING THE RABBIT ANTIBODY-LOADED SILICONE PIECE AS A SOLID PHASE

Yoshitaka HAMAGUCHI, Kanefusa KATO, Eiji ISHIKAWA, Keiko KOBAYASHI* and Nobuhiko KATUNUMA*

*Department of Biochemistry, Medical College of Miyazaki, Kiyotake, Miyazaki 889-16, and *Department of Enzyme Chemistry, Institute for Enzyme Research, School of Medicine, Tokushima University, Tokushima 770, Japan*

Received 22 July 1976

## 1. Introduction

Several procedures have been developed for the enzyme-linked solid phase sandwich immunoassay of macromolecular antigens [1–5]. We have recently developed a novel method for the conjugation of Fab′ fragments of rabbit immunoglobulin G (IgG) with β-D-galactosidase (EC 3.2.1.23) from *Escherichia coli* using *N,N′-o*-phenylenedimaleimide and have demonstrated that 0.3 fmol of a macromolecular antigen, human IgG, can be determined by the sandwich procedure using the purified rabbit antibody (Fab′)-β-D-galactosidase complex and the rabbit antibody (IgG)-coupled Sepharose 4B as a solid phase [6,7]. (Fab′: monovalent fragments derived from pepsin-treated IgG.) We describe a highly sensitive sandwich immunoassay of macromolecular antigens using the rabbit antibody (Fab′)-β-D-galactosidase complex and the rabbit antibody (IgG)-loaded silicone piece as a solid phase.

## 2. Materials and methods

Human IgG was obtained from Miles Laboratories, Inc. (Kankakee) and its amounts were determined from the absorbance at 280 nm [8]. 2,4-Dinitrophenyl(DNP)-human IgG was prepared using 2,4-dinitrobenzene sulfonic acid (sodium salt) (Tokyo Kasei Kogyo Co., Ltd., Tokyo) by the method of Eisen et al. [9]. The number of DNP residues coupled to human IgG and the amount of DNP-human IgG was calculated from the absorbance at 280 and 360 nm [8,9]. Rabbit anti-sera against human IgG and DNP-bovine serum albumin were obtained from Medical and Biological Laboratories Ltd. (Nagoya) and from Miles Laboratories, Inc. (Kankakee), respectively. IgG fractions from the anti-sera and non-specific rabbit serum were prepared by fractionation with $Na_2SO_4$ [10] followed by passage through a column of DEAE-cellulose (DE 52, Whatman Biochemicals Ltd., Kent) [11]. Crystalline ornithine δ-aminotransferase (EC 2.6.1.13) from rat liver and rabbit anti-sera against the crystalline enzyme were prepared as described previously [12]. The preparation of the crystalline enzyme was homogeneous on ultracentrifugation and electrophoresis and could be stored in 0.1 M sodium phosphate buffer, pH 7.5 without loss of its activity at −20°C for at least 6 months. Amounts in μg of the crystalline enzyme were determined by the method of Lowry et al. [13] using bovine serum albumin (Fraction V, Armour Pharmaceutical Co., Chicago) as a standard and its amounts in fmoles were calculated using its molecular weight of 170 000 [12]. IgG fraction from the anti-serum against the enzyme was obtained using $(NH_4)_2SO_4$ [14] and DEAE-cellulose [11].

### 2.1. *The rabbit antibody (Fab′)-β-D-galactosidase complexes*

The rabbit antibody (Fab′)-β-D-galactosidase complexes were prepared as described previously [7]. Their amounts are expressed as units of the enzyme activity, which are defined as described previously [7]

2.2. *The rabbit antibody (IgG)-loaded silicone pieces*

Silicone tubes (2.5 mm in inner diameter and 4 mm in outer diameter; Silicone No.3, SH type, Fuji Kobunshi Kogyo Co., Ltd., Tokyo) were cut into pieces of 3 mm in length followed by longitudinal cut into half. The silicone pieces thus obtained were washed with a detergent (Scat 20-X, Nakarai Chemicals Ltd., Kyoto) followed by washing with water. They were then immersed in 0.25 M sodium phosphate buffer, pH 7.5, containing rabbit IgG fraction (20 to 2000 $\mu$g/ml) for 30 min and stored at 4°C overnight. They were then washed successively with 0.25 M sodium phosphate buffer, pH 7.5 and with 0.01 M sodium phosphate buffer, pH 7.0 containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% $NaN_3$ and 0.1% bovine serum albumin (Buffer A) and stored in Buffer A at 4°C until use.

2.3. *Sandwich procedure*

The rabbit antibody IgG-loaded silicone pieces were incubated with various amounts of antigens in a final volume of 0.15 ml of Buffer A with shaking at 37°C for 4 h, and the incubation mixtures were allowed to stand at 4°C overnight. The silicone pieces were then washed twice with 1 ml of Buffer A and incubated with the rabbit antibody (Fab')-$\beta$-D-galactosidase complex in a volume of 0.15 ml of Buffer A with shaking at 37°C for 6 h. The silicone pieces were then washed twice with 1 ml of Buffer A and transfered into another test tube to eliminate the enzyme activity due to the non-specific binding of the complex to the wall of test tubes, and the enzyme activities bound to the silicone pieces were determined. The silicone pieces were pre-incubated in 0.1 ml of Buffer A at 30°C for 5 min and the enzyme reaction was started by adding 50 $\mu$l of $3 \times 10^{-4}$ M 4-methylumbelliferyl-$\beta$-D-galactoside (Sigma Chemical Co., St. Louis). After 5 to 20 min of incubation at 30°C with shaking, the amounts of 4-methyl-umbelliferone formed were determined by fluorometry as described previously [7]. The amounts of the complexes used were 2050, 2000 and 1650 units for human IgG, DNP-human IgG and ornithine $\delta$-aminotransferase from rat liver, respectively. These amounts were adjusted to the minimum that gave the maximal ratios of the enzyme activity bound in the presence of antigens to that in their absence.

The sandwich immunoassay using the rabbit antibody (IgG)-coupled Sepharose 4B was performed as described previously [7]. The amount of the complex used was equal to that in the assay with the silicone pieces.

2.4. *Temperature of experiments*

Experiments were carried out at room temperature (20 to 25°C) throughout, unless otherwise specified.

## 3. Results and discussion

Typical results of the sandwich immunoassays are shown in fig.1. In all the assays, the complexes corresponding to antigens were used. (The rabbit (anti-DNP) Fab'-enzyme complex was used for DNP-human IgG.) With the silicone pieces corresponding to antigens, 0.3 and 0.03 fmol of human IgG and ornithine $\delta$-aminotransferase from rat liver, respectively, could be determined. With the rabbit (anti-DNP) IgG- or with the rabbit (anti-human IgG) IgG-loaded silicone pieces, 0.2 or 0.07 fmol of DNP-human IgG could be determined. The preparation of DNP-human IgG assayed contained 8.2 DNP residues per molecule, and the sensitivity was less for that containing 3.9 or 13 DNP residues per molecule. The (anti-DNP) serum used might have contained the (anti-bovine serum albumin) IgG, since the anti-serum was produced using DNP-bovine serum albumin. However, a possible interference would have been prevented by the presence of excess bovine serum albumin in the assay mixture. This is supported by the finding that the enzyme activities bound in the absence of antigens were similar in the assays performed for DNP-human IgG and ornithine $\delta$-aminotransferase from rat liver (fig.1). The enzyme activity bound to the non-specific rabbit IgG-loaded silicone pieces did not increase with the increase of antigens added, indicating that the present assays are specific for each antigen.

Silicone pieces can be readily prepared in the laboratory and are much cheaper than polystylene tubes which have been used for the sandwich immunoassay [1,2]. The assays with the silicone pieces are less tedious than those with Sepharose 4B, which require washing by centrifugation [6,7].

When silicone pieces were loaded with rabbit antibody IgG by treating with the concentration of

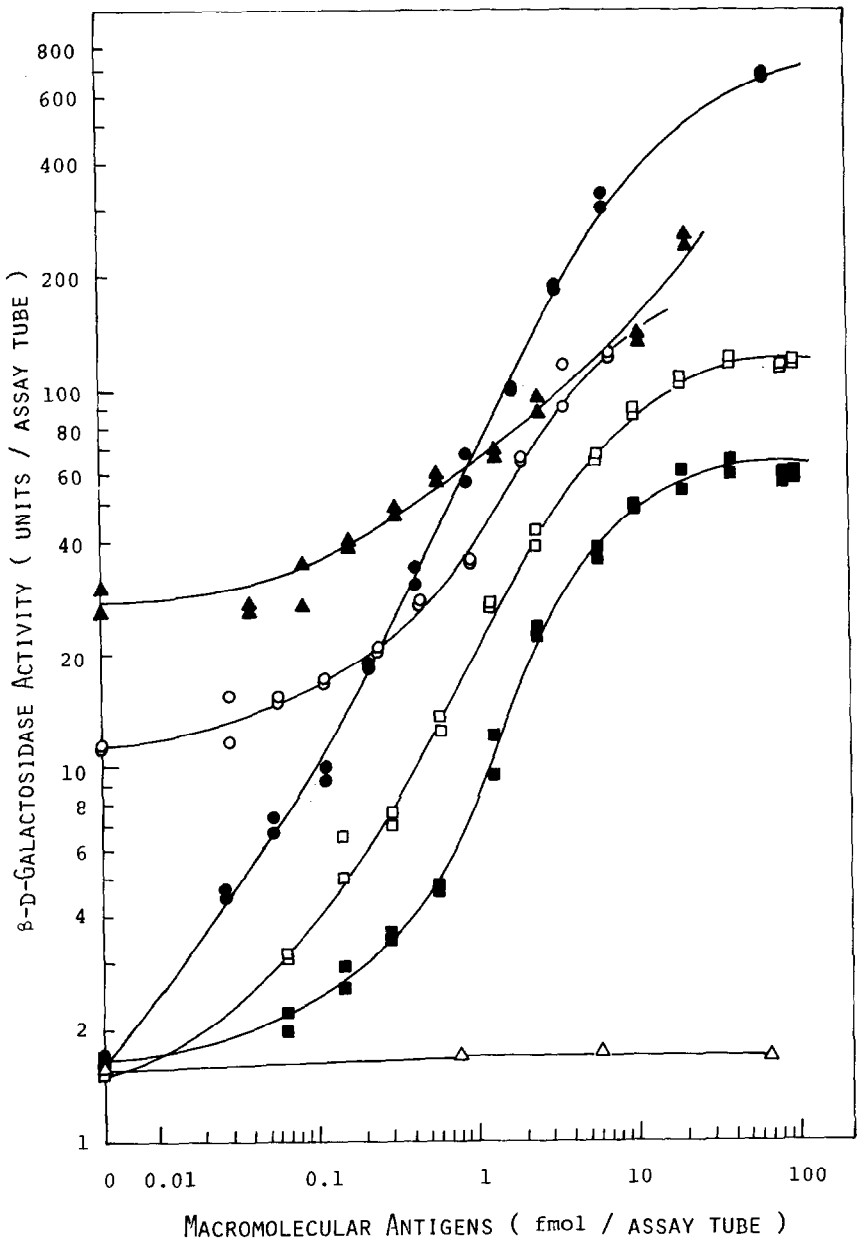


Fig.1. Solid phase sandwich immunoassays of macromolecular antigens. The solid phases used are: the rabbit (anti-human IgG) IgG-loaded silicone pieces for human IgG (closed triangles); the rabbit (anti-ornithine δ-aminotransferase from rat liver) IgG-loaded silicone pieces (closed circles), the rabbit (anti-ornithine δ-aminotransferase from rat liver) IgG-coupled Sepharose 4B (open circles) or the non-specific rabbit IgG-loaded silicone pieces (open triangles) for ornithine δ-aminotransferase from rat liver; the rabbit (anti-DNP) (closed squares) or the rabbit (anti-human IgG) (open squares) IgG-loaded silicone pieces for DNP-human IgG. All the silicone pieces used were prepared by treating with the concentrations of 1.0 mg/ml of the corresponding IgG fractions. In all the assays performed, the rabbit antibody (Fab')-β-D-galactosidase complexes corresponding to antigens were used. (The rabbit (anti-DNP) Fab'-enzyme complex was used for DNP-human IgG.)

20, 200 or 2000 μg/ml of rabbit antibody IgG fractions, the enzyme activities bound in the presence of antigens were not affected by the concentrations used. After the rabbit antibody (IgG)-loaded silicone pieces were washed up to 5 times by shaking in Buffer A or in 0.25 M sodium phosphate buffer, pH 7.5 containing non-specific rabbit IgG fraction (2 mg/ml) or bovine serum albumin (10 mg/ml) at 37°C for 4 h, they showed the same ability to bind the rabbit antibody (Fab')-β-D-galactosidase complexes in the presence of antigens as before washing. These findings indicate that a wide range of the concentrations of rabbit IgG is effective for its stable loading, although its amount loaded remains to be determined.

The non-specific binding in the assay with silicone pieces was about one seventh of that with Sepharose 4B, even if the amount of Sepharose 4B used was reduced to the minimum that gave reproducible results. This may be due in part to the fact that small quantities of Sepharose 4B can not be transferred from one test tube to another with reproducible results, while the silicone pieces can be easily transferred. With Sepharose 4B, 0.2 fmol of ornithine δ-aminotransferase from rat liver could be determined, while 0.03 fmol could be determined using the silicone pieces (fig.1). The non-specific binding in the assay of human IgG was much higher than those in the assay of other antigens and tended to increase with time of incubation. This may be due to the cross reaction between rabbit IgG on the solid phase and rabbit (anti-human IgG) Fab' of the complex. DNP-human IgG, not human IgG, may be an adequate model antigen for further improvements of the assay, since DNP-human IgG and antibody against it are readily prepared and commercially available, respectively.

The assay of DNP-human IgG with the rabbit (anti-DNP) IgG-loaded silicone pieces was less sensitive than that with the rabbit (anti-human IgG) IgG-loaded silicone pieces (fig.1.), although the same amount of the complex was used for both assays. To understand the reason for this, various amounts of the antigen (less than 20 fmol) were subjected to the sandwich immunoassay using the (anti-DNP) or the (anti-human IgG) IgG-loaded silicone pieces (first assay) and, after removing those pieces, the incubation media were again subjected to the sandwich assay (second assay). The enzyme activities bound in the second assay for both assays were similar to those in the absence of the antigen, indicating that the antigen to be assayed was completely adsorbed in the first assay for both assays. (When the non-specific rabbit IgG-loaded silicone pieces were used in the first assay, the enzyme activities bound in the second assay were equal to those in the first assay with the rabbit antibody (IgG)-loaded silicone pieces.) This indicates that the sensitivities in the present assay of DNP-human IgG depend upon the ability of the antigen, adsorbed on the solid phase, to bind the complex but not upon the efficiency of adsorption.

Our preliminary experiments suggested that the antibody (IgG)-loaded silicone pieces are applicable not only to the sandwich immunoassay but also to the competitive immunoassay of antigens and haptens and the assay of antibodies.

## References

[1] Belanger, L., Sylvestre, C. and Dufour, D. (1973) Clin. Chim. Acta 48, 15–18.
[2] Stimson, W. H. and Sinclair, J. M. (1974) FEBS Lett. 47, 190–192.
[3] Maiolini, R. and Masseyeff, R. (1975) J. Immunol. Method. 8, 223–234.
[4] Kato, K., Hamaguchi, Y., Fukui, H. and Ishikawa, E. (1975) J. Biochem. 78, 423–425.
[5] Kato, K., Hamaguchi, Y., Fukui, H. and Ishikawa, E. (1976) Eur. J. Biochem. 62, 285–292.
[6] Kato, K., Hamaguchi, Y., Fukui, H. and Ishikawa, E. (1975) FEBS Lett. 56, 370–372.
[7] Kato, K., Fukui, H., Hamaguchi, Y. and Ishikawa, E. (1976) J. Immunol. 116, 1554–1560.
[8] Palmer, J. L. and Nisonoff, A. (1963) J. Biol. Chem. 238, 2393–2398.
[9] Eisen, H. N., Carsten, M. E. and Belman, S. (1954) J. Immunol. 73, 296–308.
[10] Kekwick, R. A. (1940) Biochem. J. 34, 1248–1257.
[11] Levy, H. B. and Sober, H. A. (1960) Proc. Soc. Exp. Biol. Med. 103, 250–252.
[12] Matsuzawa, T., Katsunuma, T. and Katunuma, N. (1968) Biochem. Biophys. Res. Commun. 32, 161–166.
[13] Lowry, O. H., Rosebrough, N. J., Farr, A. A. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[14] Arnon, T. and Shapira, E. (1967) Biochemistry 6, 3942–3950.