Immunoassay Using Ion Selective Electrode and Protein Pendant Liposomes

Hirohisa ABE*, Masamitsu KATAOKA*, Tatsuji YASUDA and Yoshio UMEZAWA***

**Department of Chemistry, Faculty of Science, Hokkaido University, Sapporo 060*
***Laboratory of Biological Products, Institute of Medical Science, The University of Tokyo, Shiroganedai, Tokyo 108*

Immunoassay for an anti-human IgG antibody and a human IgG is presented. Thin-layer potentiometry, i.e., a combination of fluoride ion selective electrode and silver chloride coated silver-plate reference electrode was used to monitor fluoride release from the multilamellar liposome. Human IgG was attached to the surface of liposome membrane by the use of cross-linking reagent N-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate (SPDP). The antigen/antibody/complement reaction triggered the formation of "channel-like" holes across the liposome membrane which enabled entrapped fluoride anion flow through the hole. The fluoride ion release was specific for anti-human IgG and depended on the presence of complement. By the use of human IgG pendant liposomes, determination of about 10^{-14} mol of anti-human IgG (rabbit) and 10^{-14} mol human IgG was feasible.

Keywords Ion channel, immunosensor, biosensor, thin-layer potentiometry, human IgG

The ion channel is a very general system in biological activities, *i.e.*, hormones, nerve systems and others. Unique feature of the ion channel in biological cell membranes is a selective recognition of substrates and the following amplification of its information by channel switchings. Therefore, it seems natural to take advantage of this principle as a new philosophy of chemical sensors.

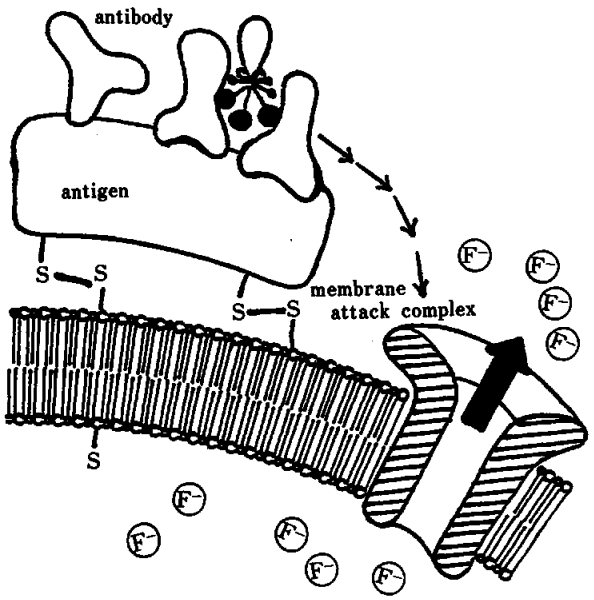

Fig. 1 Schematic diagram of the formation of "channel-like" holes across the liposome membrane. The antigen/antibody/complement reaction triggers the formation of "channel-like" holes which enable entrapped fluoride anions flow through the hole.

It has been known in biochemistry and well understood recently that the antigen/antibody/complement reaction triggers the formation of "channel-like" holes across the liposome membrane (Fig. 1). This phenomenon is not exactly the one what is called "ion channel" in the biological definition, but could be regarded as a model system for it. The combination of this channel-forming phenomenon with an ion selective electrode provides an unique electrochemical immunoassay. In the present study, we have attached with covalent bondings human IgG and other proteins on the surface of liposome membranes (Fig. 2). This is an extension of our previous work of "ion selective immuno-electrode", where only lipid haptens could be used for immunologically sensitizing the membrane surface.[1-3] The pendant protein on the membrane surface undergoes a specific antigen/antibody/complement reaction which causes the formation of "ion channels". As a result of this, trapped marker ions in the interior of liposome vesicles are released and are readily detected by a corresponding ion selective electrode.

Experimental

Buffers

Hepes-buffered salt solution was prepared by

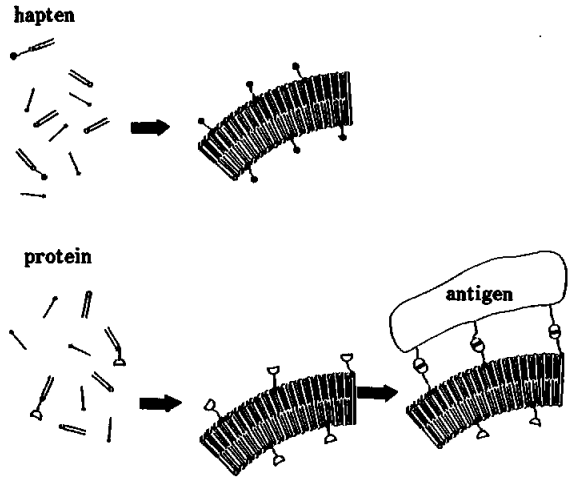

Fig. 2 Immunological sensitization of the liposome membrane surface by different two methods. (1) Lipid haptens can be liposome constituents by themselves, and (2) protein antigens, which is the general case, are chemically bonded on the membrane surface.

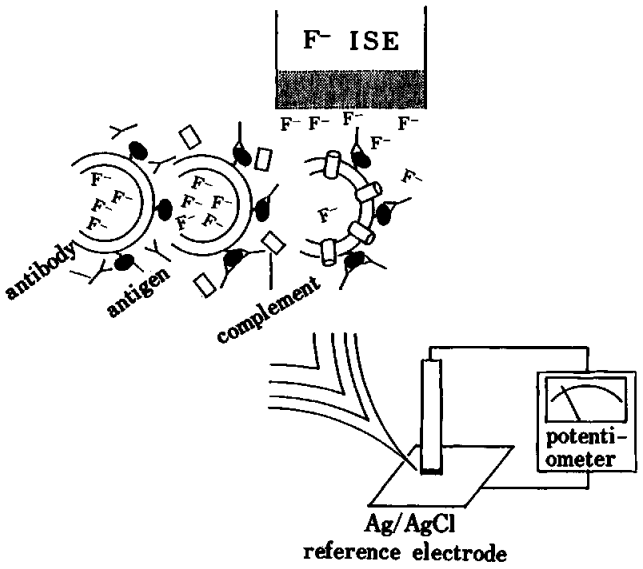

Fig. 3 Release of marker ions (F^- ions) through membrane channels upon antigen/antibody/complement reaction and following detection of F^- ions in micro volume sample solutions by thin-layer potentiometry.

dissolving 8.0 g of NaCl, 0.4 g of KCl, 0.25 g of Na_2HPO_4 and 2.38 g of 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) to 1000 ml of water. The pH was adjusted to 7.45 with 0.1 M NaOH.

The potassium fluoride-Hepes-buffered solution was prepared by dissolving 14.1 g of $KF{\cdot}2H_2O$, 2.38 g of Hepes to 1000 ml of water. The pH was adjusted to 7.45 with 0.1 M NaOH.

Acetate buffered saline (pH 4.50) and gelatin veronal-buffered saline (GVB^-) was prepared as mentioned before.[4] GVB^- was supplemented with 35 mM $MgCl_2$ and 0.15 mM $CaCl_2$ for assay procedures (GVB^{++}).

Lipid

Cholesterol and dipalmitoylphosphatidylethanolamine (DPPE) were obtained from Sigma (St. Louis, MO). Dimyristoylphosphatidylcholine (DMPC) was purchased from Nippon Yushi Co. (Tokyo). DTP-DPPE was prepared by the method described previously.[5] Each lipid solution in chloroform was stored in a refrigerator at −20°C under nitrogen atmosphere.

Immunological reagents

A rabbit anti-human IgG antibody (IgG fraction) was purchased from Miles-Yeda (Elkhart, IN). Monoclonal anti-human IgA, IgG and IgM antibodies were purchased from Diagnostic Technology(Hauppauge, NY). The complement used in this study was guinea pig serum, which was stored at −80°C. Specific pathogen free (SPF) complement was obtained from SPF guinea pig bought from Shizuoka Laboratory Animal Center (Hamamatsu).

Preparation of liposomes

The multilamellar liposomes were prepared from chloroform solutions of DMPC, cholesterol and DTP-DPPE in molar ratio of 1:1:0.06. After evaporation of the chloroform, 0.2 M KF solution was added to the dried lipid film. After incubated at 50°C for 1 min, the lipid film was dispersed by vigorous vortexing. The liposomes were collected by centrifugation at 15000 r.p.m. for 20 min. The pellet of liposomes was suspended in potassium fluoride-Hepes buffered solution and stored at 4°C until the coupling reaction described below. Human IgG was coupled to liposomes with a crosslinking reagent, *N*-hydroxysuccinimidyl 3-(2-pyridylthio)propionate (SPDP) and a reducing agent, dithiothreitol (DTT). In this coupling reaction, normal S-S bonds in protein molecules were not reduced.[5,6] Therefore, the affinity of modified human IgG against antibody was not changed. Modified human IgG with SPDP and DTT was prepared by the method proposed by Ishimori *et al.*[5] Two ml portion of the liposomes suspension was added to the same volume of modified human IgG and reacted overnight at room temperature with slow shaking. Untrapped marker ions, fluoride ions, were removed by repeated centrifugation at 15000 rpm for 20 min in GVB^{++}. The final pellet of liposomes was suspended in 1.2 ml of GVB^{++} and stored at 4°C. Estimation of the amount of pendant IgG was carried out by measuring proteins[7] in the supernatant before and after the coupling reaction. About five hundred microgram of human IgG was coupled to liposomes from 5 μmols of lipids.

Standard assay procedure

Twenty-five microliters of IgG fraction of rabbit anti-human IgG antibody and 25 μl of complement diluted 50 times with GVB^{++} was added to a 25-μl liposome aliquot. The mixture was incubated for 60 min at 37°C

in a moist chamber and then the reaction was stopped by cooling at 4° C. A resulting solution (25 μl) was placed on an Ag/AgCl plate reference electrode and a fluoride ion selective electrode (ISE) was lowered onto the sample droplet (Fig. 3). With this arrangement, thin-layer potentiometry, the necessary sample volume can be reduced to a greater extent without miniaturizing the ISE itself. The e.m.f. was measured by a milivolt meter (Model HM-18E, TOA Electoric Co., Tokyo) after 1 min when an equilibrium potential be attained. The total releasable F^- in the liposomes was determined by lysing with 25 μl of a 9% Triton X-100 solution. Marker release % was estimated as follows:

$$\text{Marker release}(\%) = \frac{\text{experimental release} - \text{blank}}{\text{total release} - \text{blank}} \times 100$$

where "blank" was obtained by simply replacing the antibody solution with the same volume of GVB^{++} solution in the above standard assay procedure.

Results and Discussion

Permeability of antigen-sensitized liposomes was increased by complement mediated antigen antibody reaction. The marker ions, fluoride ions, thus released to a dilute solution were measured by a thin-layer potentiometry with a fluoride ion selective electrode, which is a direct but much amplified measure of the antigen and/or antibody to be assayed.

Effect of complement concentration

Effect of complement concentration on release of trapped marker ions was examined by changing its concentration with fixed concentrations of antibody and liposome suspensions. The results are shown in Fig. 4. The marker release increased with increasing the complement concentration. However, too high complement concentration seemed to cause non-specific change in permeation rate of marker ions through the liposome membrane. Thus, the 100-times dilution of complement was employed as a complement source.

Dependence of antibody concentration

The dependence of the extent of the marker ion release on the concentration of IgG antibody was examined at given amounts of complement and IgG antigen. As shown in Fig. 5, the marker release starts to occur when the antibody concentration becomes greater than 2×10^{-3} mg/ml and gradually levels off at about 30% marker release above 2×10^{-1} mg/ml of antibody. Using Fig. 5 as a calibration curve, one can determine the anti-human IgG antibody level of 2×10^{-3} through 2×10^{-1} mg/ml.

Antibody specificity for "channel" formation

The cross-reactivity in immuno-reactions often causes some non-specificity among analogous antigen and antibodies. Antibody specificity, that is the so-called interference in analytical sense, was examined in different two ways: Each anti-human IgA or IgM antibody was reacted together with complement with IgG pendant liposomes in the 1) absence and 2) presence of anti-human IgG antibody, respectively. 1) Twenty-five microliters of liposome suspention and 25 μl of complement diluted 50 times with GVB^{++} were added to each antibody solution. The mixture was incubated for 60 min at 37° C. The results with a 100-times dilution of complement are shown in Fig. 6. It was found that the human IgG (as protein antigen) pendant on the liposome was virtually specific to anti-human IgG

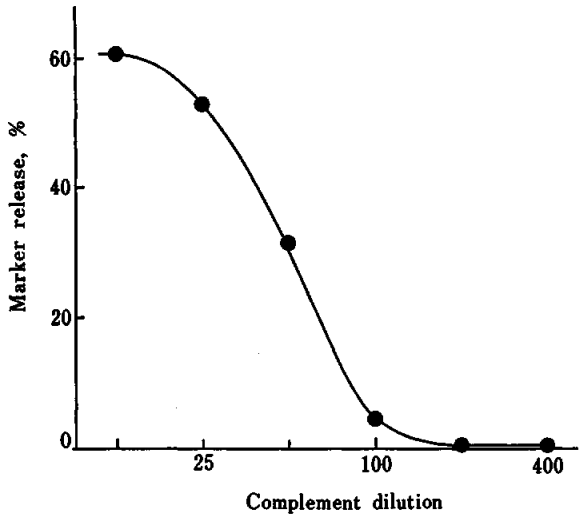

Fig. 4 Effect of complement concentration on the marker release through IgG pendant liposome membranes in the absense of antibody.

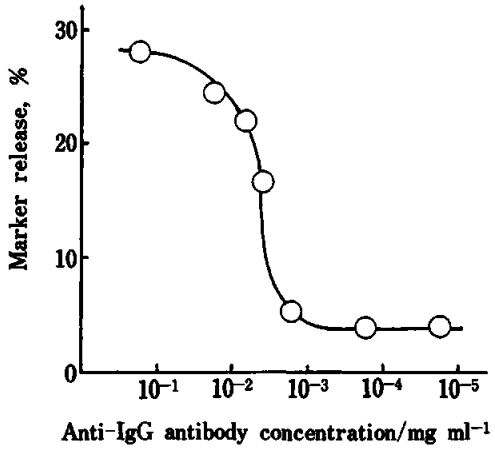

Fig. 5 Effect of anti-human IgG antibody concentration on the release of marker ions through the IgG pendant liposome membranes upon complement mediated immunoreaction. Complement, 100 times dilution with GVB^{++}; incubation, 37° C for 1 h.

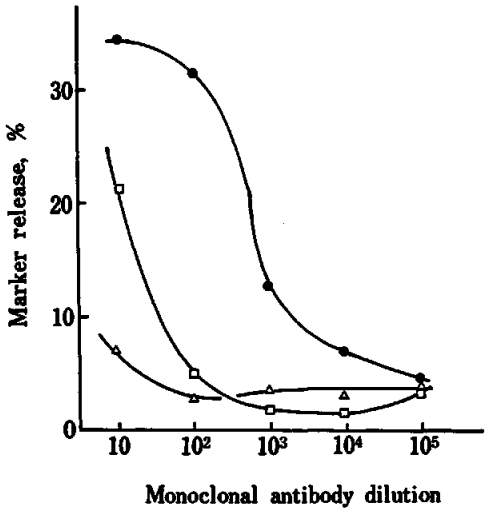

Fig. 6 Antibody specificity of the release of marker ions through the IgG pendant liposome membranes upon complement mediated immunoreaction. (●) monoclonal anti-human IgG antibody, (□) monoclonal anti-human IgM antibody, (△) monoclonal anti-human IgA antibody. Complement dilution: 100 times with GVB^{++}.

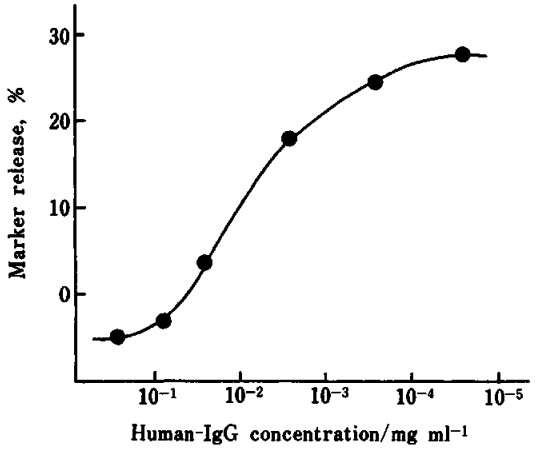

Fig. 7 Inhibition by aqueous free humn IgG of marker release. A known amount of anti-human IgG antibody was first "titrated" with a varying concentration of human IgG (incubation; room temperature, 90 min). Then, each reaction mixture was "back-titrated" with a known amount of IgG pendant liposomes in the presence of complement (incubation; 37°C, 60 min).

antibody when the concentration of anti-human IgA and IgM antibodies are both of 10^2 dilution or less, so that monoclonal anti-human IgA and IgM antibodies did not interfere with the determination of anti-human IgG antibody in this concentration range. More than 20% of the marker release was observed at the anti-human IgM antibody concentration of 10-times dilution. This may be attributed to non-specific release of marker ions in the presence of large amount of anti-human IgM antibody. 2) There are some possibilities that anti-human IgA and IgM antibodies interfere with the formation of IgG/anti-human IgG antibody complex. The following experiment was also carried out to evaluate an extent of interference to determine anti-human IgG concentration. Marker release was observed with the mixture of IgG pendant liposome, and anti-human IgG antibody in the presence of an equal amount of anti-human IgA or IgM antibody. It was found that the marker release is not affected by the addition of anti-human IgA or IgM antibody.

Assay of human IgG

Inhibition of the immune reaction also occurs when a free antigen which has cross reactivity with the corresponding antibody coexists in solution. In the present study, the minimum amount of anti-human IgG antibody which is necessary for the maximum channel formation of liposomes was first reacted with known amount of a free human IgG in aqueous solution. The degree of inhibition of antibody activity caused by this reaction was then measured by adding the liposome aliquot. The antibody activity which remained after reaction with the free human IgG was measured by the degree of F^- release from the added liposomes in the presence of complement. The degree of inhibition thus measured is dependent on the free (solution) antigen concentration as shown in Fig. 7 and this fact can be used for the assay for IgG antigen.

Procedure for the determination of human IgG by the present assay system was as follows. A mixture of twenty-five microliters of anti-human IgG antibody (22 μg/ml) and 5 μl of adequately diluted human IgG ($0.8-2.5\times10^{-4}$ mg/ml) was incubated for 1.5 h at room temperature. Further incubation for 60 min at 37°C was carried out after addition of 25 μl of the liposome suspension and 25 μl of the 100 times diluted complement. The calibration graph for human IgG was shown in Fig. 7. The IgG concentration from 0.8 to 2.5×10^{-4} mg/ml was available for the determination in this assay system.

In conclusion, by the use of human IgG pendant liposomes, determinations of about 10^{-14} mol of anti-human IgG (rabbit) and 10^{-14} mol of human IgG were feasible. The present analytical system is a simple homogeneous system and the reaction time is much shorter than enzyme-linked immunosorbent assay.

As was described in this paper, this approach is still a "batch" system, in which the membrane reaction and its signal transduction by an ion selective electrode is not synchronized. Immobilized liposome and lipid bilayer membrane sensors are desired for an ideal "ion channel sensor".

The authors thank Dr. Y. Ishimori for experimental help. Y.U. also acknowledges The Ministry of Education, Science and Culture, and Shimadzu Science Foundation for the financial support.

References

1. Y. Umezawa, Proceedings of the International Meeting on Chemical Sensors, p. 705 – 710, Kodansha and Elsevier (1983).
2. K. Shiba, Y. Umezawa, T. Watanabe, S. Ogawa and S. Fujiwara, *Anal. Chem.*, **52**, 1610 (1980).
3. Y. Umezawa, S. Sofue and Y. Takamoto, *Talanta*, **31**, 375 (1984).
4. T. Yasuda, Y. Naito, T. Tsumita and T. Tadakuma, *J. Immun. Methods*, **44**, 153 (1981).
5. Y. Ishimori, T. Yasuda, T. Tsumita, M. Notsuki, M. Koyama and T. Tadakuma, *J. Immun. Methods*, **75**, 351 (1984).
6. J. Carlsson, H. Drevin and R. Axen, *Biochem. J.*, **173**, 723 (1978).
7. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).

(Received August 8, 1986)
(Accepted August 30, 1986)