A Liposome Immunoassay Based on a Chemiluminescence Reaction

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A new, sensitive immunoassay involving the combination of a liposome immunoassay with chemiluminescence detection is reported. Lysis of the liposomes with cytolysin–hapten conjugates released entrapped glucose oxidase. Chemiluminescence produced by reaction of the enzymatically formed hydrogen peroxide and isoluminol was monitored *via* an optical fiber and photomultiplier. Without a bound/free separation procedure, this chemical amplification of the immune reaction by liposome lysis and enzymatic reaction allowed detection of 1.7 ng/assay tube of digoxin, the model analyte. When liposome-coated microplates were used to separate intact liposomes from released enzyme, the sensitivity of detection was improved to 165 pg/assay tube.

Keywords liposome immunoassay; digoxin; optical fiber; melittin; chemiluminescence

Liposome immunoassays (LIAs) have been studied extensively, since their introduction by Kinsky *et al.*[1] The mode of assay, releasing entrapped signal-generating materials from liposomes, is based on complement-mediated lysis,[2-7] cytolysin-mediated lysis,[8] or destabilization of the liposomal membrane.[9,10] The quantity of released signal-generating materials is proportional to the concentration of analytes. Monitoring is generally by spectrophotometric or fluorimetric methods. Since the antigen–antibody complexing reaction is amplified by the liposome lysis or destabilization, the sensitivity of LIAs is generally higher than that of corresponding enzyme immunoassays. For some analytes in biological fluids, however, the detection limit of current systems is still inadequate.

In this paper we describe a sensitive, new liposome immunoassay with a chemiluminescence reaction monitored *via* an optical fiber and photomultiplier tube. Digoxin was chosen as the model analyte and the liposomes were lysed by melittin–ouabain conjugates. The principle of the assay was described by Litchfield *et al.*[8]

Materials and Methods

Materials Egg yolk L-α-phosphatidylcholine (PC), cholesterol (CH), melittin (from bee venom), glucose oxidase (GOD, from *Aspergillus niger*), microperoxidase (from equine heart cytochrome c), bovine serum (calf) and ouabain were obtained from Sigma Chemical Co. (St. Louis, MO). Additional reagents and materials were obtained as follows: monoclonal anti-digoxin immunoglobulin G (IgG) from Cambridge Medical Diagnostics (Billerica, MA); [^{3}H]digoxin (22 Ci/mmol) from NEN Research Products (Boston, MA); digoxin from Nacalai Tesque, Inc. (Kyoto); isoluminol from Tokyo Kasei Kogyo Co., Ltd. (Tokyo); Soluene-350 from Packard-Becker B. V. Chemical Operations (The Netherlands); Scintisol EX-H from Dojindo Laboratories (Kumamoto, Japan); PL HQ-Auto 15 from Nissui Seiyaku Co. (Tokyo); Sephadex G-25 and Sepharose 4B from Pharmacia Fine Chemicals (Uppsala, Sweden); Dowex 1-X8 from Muromachi Kagaku Kogyo Ltd. (Tokyo); Nuclepore from Nomura Micro Science Co., Ltd. (Tokyo); microplates from Terumo Co., Ltd. (Tokyo). Other chemicals used were commercial products of high purity and all operations utilized freshly redistilled water.

Preparation of Liposomes Multilamellar liposomes (MLV) were prepared as previously described.[11] Briefly, 20 μmol of PC and 10 μmol of CH were dissolved in 5 ml of $CHCl_3$. The mixture was dried to a thin lipid film by evaporation and residual traces of organic solvent were removed in a vacuum. The dry lipid film was dispersed in 2 ml of buffer I (0.05 M Tris, pH 7.0) containing 20 mg of GOD, with vortexing at room temperature under N_2. The mixture was sonicated in a N_2 stream for 5 min at 0 °C in a probe-type sonicator (Tomy Seiko, UR-200R). The suspension was then placed in a 25 mm diameter Millipore ultrafiltration cell (Bedford, MA) fitted with a 0.4 μm pore-size Nuclepore membrane (Pleasanton, CA) and extruded twice. Untrapped GOD was removed by gel chromatography on a Sepharose 4B column (1.5 × 20 cm), equilibrated in buffer I. Liposomes eluting in the void volume of the column were pooled. To this solution (2.5 ml), 0.6 U of chymotrypsin (0.5 ml) was added and the mixture was incubated at pH 8.0 and 25 °C for 30 min. The mixture was then re-filtered by gel chromatography on a Sepharose 4B column. Phospholipid concentration in the vesicle preparations was measured by enzymatic choline determination using a PL HQ-Auto 15 Kit.

Preparation of Ouabain–Melittin Conjugates Ouabain was used instead of digoxin hapten in the conjugation because of its increased water solubility. Ouabain–melittin conjugates were prepared without [^{3}H]ouabain according to the method of Litchfield *et al.*[8] From the spectrophotometric measurement of melittin and the determination of ouabain by means of the phenol–sulfuric acid reaction, we calculated that an average of 4.25 molecules of ouabain were conjugated per melittin molecule.

Measurement of GOD Activity A solution of various concentrations of GOD (50 μl) was added to 450 μl of buffer I and mixed. Then 500 μl of 0.5 M glucose in 0.05 M acetate buffer (pH 5.0) was added and the mixture was incubated for 20 h at 4 °C. The enzymatic reaction was stopped by putting the test tube in ice. Aliquots of the reaction mixture (50 μl) were injected into a chemiluminescence-measuring cell containing 350 μl of chemiluminescence reagent (CL-reagent), and the intensity of chemiluminescence was measured. The CL-reagent contained 100 μl of 100 μM isoluminol, 100 μl of 10.6 μM microperoxidase and 150 μl of assay buffer. The assay buffer was 0.05 M phosphate-buffered saline (pH 8.0) containing 0.9% (w/v) NaCl, 0.01% (w/v) bovine serum albumin (BSA) and 0.1% (w/v) NaN_3.

Lysis of Liposomes by Melittin or Ouabain–Melittin Conjugates A solution of melittin or ouabain–melittin conjugates (50 μl; 6.7×10^{-9} – 1×10^{-6} M) was added to 400 μl of buffer I and mixed. To this mixture, 50 μl of liposome solution (concentration of PC, 10 nmol/ml) was added. Further procedures were as described above.

Chemiluminescence Analysis Light emission was measured *via* an optical fiber (ESKA Lightguide, B10CC-2, Mitsubishi Rayon Co., Ltd., Tokyo) using a highly sensitive photomultiplier tube with a preamplifier and a photon counter (R1635-02, C716-07, C1230, Hamamatsu Photonics K. K.). Luminescence counts were memorized on a PC-8801 microcomputer (NEC Co.) through an A/D converter (DAS-1280 BPC, Microscience Co. Ltd. Tokyo) and integrated over 8 s (from 2 to 10 s after the injection of enzymatic reaction product).

Assay Procedure A liposome-coated and a BSA-coated microplate were prepared as follows: a 300 μl aliquot of liposome solution (concentration of PC, 1 μmol/ml) or BSA in buffer I (5 mg/ml) was added to each microplate well (Petray 96U, Terumo Co., Ltd., Tokyo). The plate was kept overnight at 4 °C. The liposome solution was removed and the plate was washed 3 times with buffer I. The BSA-coated plate was washed 3 times with NaCl solution (0.9% (w/v)) containing 0.025% (v/v) Tween 20, then 3 times with buffer I.

Homogeneous Assay Procedure: Standard digoxin solution (50 μl) was mixed with 50 μl of 100-fold-diluted monoclonal anti-digoxin IgG (affinity constant 5.4×10^9 l/mol) and the mixture was incubated at room temperature for 5 min. Then, 50 μl of ouabain–melittin conjugate (200 nM) was added and the mixture incubated for another 5 min. Three hundred microliters of buffer I and 50 μl of liposomes (0.5 nmol of PC) were added.

Then, 500 μl of 0.5 M glucose was added and the mixture incubated at 4 °C overnight. A 50 μl aliquot of the reaction mixture was then analyzed by chemiluminescence.

Assay Procedure Using Liposome-Coated Microplate: Standard digoxin solution (50 μl) and 50 μl of 100-fold-diluted anti-digoxin IgG were mixed in the BSA-coated microplate well, and incubated at room temperature for 5 min. To this mixture, 50 μl of ouabain–melittin conjugate (200 nM) was added. After a second 5 min incubation at room temperature, 150 μl of buffer I was added. Aliquots of the final mixture (250 μl) were transferred to the liposome-coated microplate wells and diluted with 50 μl of buffer I. Supernatant containing the released GOD (200 μl) was added to 500 μl of 0.5 M glucose solution in a test tube and the mixture was incubated at 4 °C overnight. The percentage of lysis was calculated from Eq. 1.

$$\% \text{ of lysis} = (L_s - L_f)/(L_t - L_f) \times 100 \quad (1)$$

L_s is the chemiluminescence intensity of the standard or sample solution and L_f is the chemiluminescence intensity of control solution which contains 50 μl of buffer I instead of the ouabain–melittin conjugate. L_t is the total chemiluminescence intensity when liposomes are lysed completely by sodium deoxycholate (0.4% (w/v)).

Results and Discussion

CL Solution and Enzymatic Reaction We investigated the optimal concentration of CL solution to obtain a maximum S/N ratio of chemiluminescence and the optimal concentration of glucose in the presence of 5×10^{-5} U of GOD. The optimal concentrations of isoluminol and microperoxidase were 100 and 10.6 μM, respectively. The unit of GOD used was estimated from the entrapment efficiency and amount of the liposomes. The chemiluminescence intensity increased with increasing concentration of glucose and leveled off at 0.5 M. Thus, we used 0.5 M glucose in 0.05 M acetate buffer (pH 5.0), which was optimal for the enzymatic reaction. Then, the effect of incubation time and incubation temperature on the chemiluminescence intensity was examined. At 25 and 35 °C, the chemiluminescence intensity decreased rapidly. At 15 °C, the chemiluminescence reached a maximum at about 2 h, then gradually decreased. At 4 °C, the chemiluminescence intensity continuously increased with time up to 20 h (the maximum in this study). Consequently, in subsequent investigations, we incubated GOD with glucose at 4 °C for 20 h.

Determination of GOD by CL Assay The dose–response curve for GOD was linear over the range from 30 to 500 μU/ml. The CL assay could detect concentrations as low as 1 fM GOD.

Lytic Activity of Ouabain–Melittin Conjugate Lysis of the GOD-entrapped liposomes by native melittin and the ouabain–melittin conjugate is shown in Fig. 1. Melittin alone induced partial lysis at 5×10^{-8} M and total lysis at 2×10^{-7} M, while ouabain–melittin conjugate induced partial lysis at about 1×10^{-8} M and total lysis at 2×10^{-7} M. This enhanced lytic action may be attributed to the increased hydrophobicity of melittin conjugate, as pointed out by Litchfield *et al.*[8] Inhibition of the conjugate-induced lysis of liposomes by monoclonal anti-digoxin IgG is shown in Fig. 2 (curve a). Lysis of the liposomes was inhibited by 5% at 1000-fold dilution, and by 82% at 100-fold dilution. At the highest IgG concentration, the lysis was bout 4% . This may be attributed to the instability of the liposomes. Thus, we used 100-fold-diluted anti-digoxin IgG in the subsequent assay. The conjugate-induced lysis of liposomes was also inhibited nonspecifically by serum. Figure 2 (curve B) shows the inhibition by bovine serum. The lysis was about 85% at 100-fold dilution.

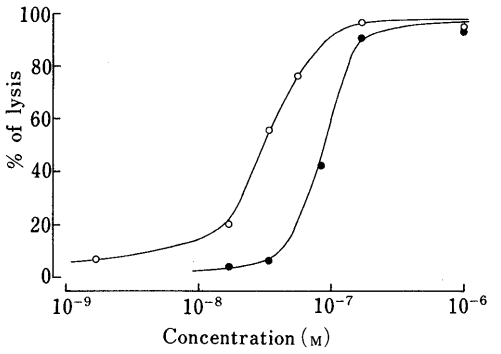

Fig. 1. Dose-Dependent Lysis of GOD-Entrapped Liposomes by Native Melittin and Ouabain–Melittin Conjugate

Fifty microliters of GOD-entrapped liposomes (0.5 nmol of PC) was added to 0.05 M Tris–HCl buffer solution (pH 7.0, 450 μl) containing various amounts of native melittin (●) or ouabain–melittin conjugate (○). Then, 500 μl of 0.5 M glucose was added to the mixture and the whole was incubated at 4 °C for 20 h. The % of lysis was calculated according to Eq. 1.

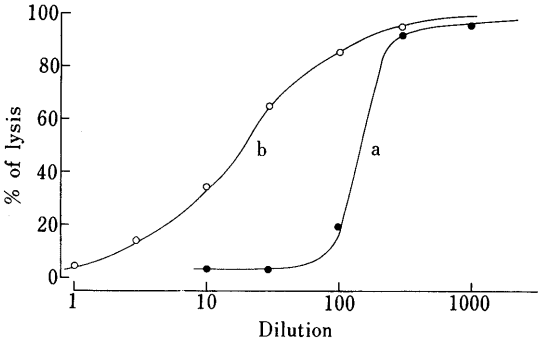

Fig. 2. Inhibition of Ouabain–Melittin Conjugate-Induced Liposome Lysis by Monoclonal Anti-digoxin IgG (a) and Bovine Serum (b)

Monoclonal anti-digoxin IgG (50 μl, curve a) or bovine serum (50 μl, curve b) was diluted as indicated, and incubated with 50 μl of ouabain–melittin conjugate (200 nM) for 5 min at room temperature prior to the addition of GOD-entrapped liposomes (0.5 nmol of PC in 350 μl of buffer solution).

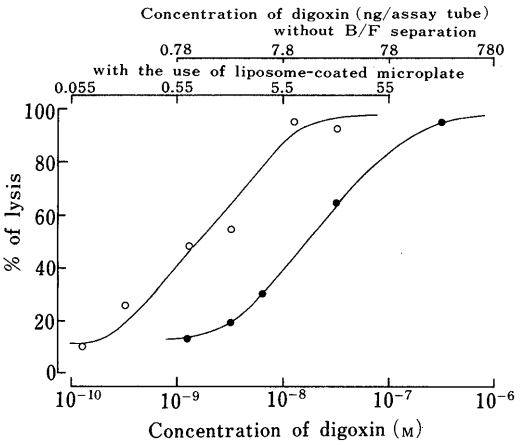

Fig. 3. Dose–Response Curve for Digoxin without a Bound/Free Separation Procedure and with the Use of a Liposome-Coated Microplate

Assay conditions were as described in Materials and Methods. Concentrations shown are those in the final assay volume. ○, with the use of a liposome-coated microplate; ●, without B/F separation procedure.

Assay for Digoxin The dose–response curve for digoxin is shown in Fig. 3. A linear relationship was obtained over the range from 3 to 100 nM digoxin. The detection limit was 3 nM, which is 3 times more sensitive than that previously

reported.[8]

In order to improve the background, we tried to separate the released GOD from that entrapped in intact liposomes by the use of a liposome-coated microplate. The dose–response curve for digoxin with the use of a liposome-coated microplate is also shown in Fig. 3. In this assay, 0.3 nM (165 pg/assay tube) digoxin was readily detectable.

In the present study we used commercial melittin, which contained phospholipase A_2 as an impurity (less than 20 units per mg of protein), without further purification. If free phospholipase A_2 exists in the conjugate, the lytic activity of the melittin–ouabain conjugate is not properly inhibited by anti-digoxin IgG. Moreover, it has been reported that melittin enhanced the lytic activity of phospholipase A_2.[12] However, in our study, the lytic activity of the conjugate was inhibited by anti-digoxin IgG (lysis was about 4% at the maximum concentration of IgG, Fig. 2). It is considered that free phospholipase A_2 was coupled with ouabain and the lytic activity of the phospholipase A_2–ouabain conjugate produced was also inhibited by anti-digoxin IgG. Hence, the effect of contaminating phospholipase A_2 was considered negligible.

Lysis of the liposomes by melittin–ouabain conjugate was sensitive to the surface condition, namely the adsorption of protein. In the preparation of GOD-entrapped MLV by Bangham's method, GOD was also incorporated in the lipid membrane or adsorbed on the liposomal surface. The adsorbed GOD inhibited the lytic activity. In order to digest adsorbed GOD, the MLV were treated with chymotrypsin. Lysis of the MLV by melittin–ouabain conjugate (1.1×10^{-6} M) was 57% before and 100% after the digestion by chymotrypsin, respectively. In contrast, the digestion of GOD-entrapped small unilamellar vesicles (SUV) by chymotrypsin affected the % of lysis very little (data not shown). The radius of curvature of SUV is smaller than that of MLV, which may lead to smaller amount of adsorption of GOD on the liposomal surface and hence to a reduced effect of chymotrypsin treatment.

It was found in our study that the lytic activity of the melittin–ouabain conjugate was also significantly affected by serum. It was greatly decreased when anti-digoxin antiserum was used as a source of antibody or when bovine serum was added to the conjugate solution (Fig. 2, curve b). Conversely, the lytic activity was not affected when the monoclonal anti-digoxin IgG was used as a source of antibody. Serum protein may cause some inhibitory effects on the lytic action of melittin–ouabain conjugate. For a practical determination of digoxin in a serum sample, therefore, it is necessary to dilute the sample. We observed very little influence of serum on the lytic activity of our conjugate when the serum samples were diluted 100 times.

An important feature of the liposome immunoassay using cytolysin–antigen conjugate is that stable liposomes can be used to measure several different analytes instead of using sensitized liposomes. In this type of assay, however, the molecular weight of the antigen linked to the cytolysin must be low, otherwise the lytic function of the conjugate may be prevented by steric hindrance. The molecular size of the marker entrapped in the liposomes is also important. Katsu *et al.*[13] have reported that the size of membrane lesion increased with increase in the concentration of melittin. We needed a higher concentration of melittin for releasing entrapped GOD than was necessary for releasing low molecular weight marker (6-carboxyfluorescein, data not shown). The higher concentration of melittin, leading to a higher concentration of hapten, results in lower sensitivity. The concentration of melittin–ouabain conjugate necessary for releasing entrapped GOD is considered to be the main factor determining the detection limit. Further studies are under way to incorporate a low-molecular-weight co-factor substance (*e.g.* flavine adenine dinucleotide, FAD) into liposomes, and regulate GOD allosterically, instead of entrapping the enzyme itself.

In summary, we have developed a liposome immunoassay with a chemiluminescence detection. With appropriate modifications, the assay may be used to determine a variety of haptens with high sensitivity and specificity.

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References

1) S. C. Kinsky, J. A. Haxby, D. A. Zopf, C. R. Alving and C. B. Kinsky, *Biochemistry*, **8**, 4149 (1969).
2) F. S. Ligler, R. Bredehorst, A. Talebian, L. C. Shriver, C. F. Hammer, J. P. Sheridan, C. Vogel and B. P. Gaber, *Anal. Biochem.*, **163**, 369 (1987).
3) M. Umeda, Y. Ishimori, K. Yoshikawa, M. Takada and T. Yasuda, *J. Immunol. Methods*, **95**, 15 (1986).
4) D. W. Bowden, M. Rising, G. Akots, A. Mules and R. J. Broeze, *Clin. Chem.*, **32**, 275 (1986).
5) U. Glagasigij, Y. Sato and Y. Suzuki, *Chem. Pharm. Bull.*, **36**, 1086 (1988).
6) Y. Ishimori, T. Yasuda, T. Tsumita, M. Notsuki, M. Koyama and T. Tadakuma, *J. Immunol. Methods*, **75**, 351 (1984).
7) R. L. Richards, R. C. Habbersett, I. Scher, A. S. Janoff, H. P. Schieren, L. D. Mayer, P. R. Cullis and C. R. Alving, *Biochim. Biophys. Acta*, **855**, 223 (1986).
8) W. J. Litchfield, J. W. Freytag and M. Adamich, *Clin. Chem.*, **30**, 1441 (1984).
9) R. J. Y. Ho and L. Huang, *J. Immunol.*, **134**, 4035 (1985).
10) P. Pinnaduwage and L. Huang, *Clin. Chem.*, **34**, 268 (1988).
11) M. Haga, F. Kato, M. Yoshida, F. Kohara and Y. Kato, *Chem. Pharm. Bull.*, **34**, 2979 (1986).
12) C. Mollay, G. Kreil and H. Berger, *Biochim. Biochem. Acta*, **426**, 317 (1976).
13) T. Katsu, C. Ninomiya, M. Kuroko, H. Kobayashi, T. Hirota and Y. Fujita, *Biochim. Biophys. Acta*, **939**, 57 (1988).