An Improved Chemiluminescence-Based Liposome Immunoassay Involving Apoenzyme

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An improved liposome immunoassay system (LIS) combining the chemiluminescence-based LIS with an apoenzyme reactivation immunoassay system (ARIS) was developed. A low-molecular-weight co-factor, FAD (flavin adenine dinucleotide), was incorporated into liposomes instead of the high-molecular-weight enzyme GOD (glucose oxidase). FAD released from liposomes by cytolysin–hapten conjugates bound to Apo-GOD and regenerated GOD. The system allowed detection of 10 pM digoxin, the model analyte and was linear over 10 pM to 13 nM digoxin. This sensitivity was about 300 times higher than that of the homogeneous system using GOD-containing liposomes and 30 times higher than that of the heterogeneous system which we reported previously. The time required for incubation during the detection of digoxin was reduced from 20 to 3 h.

Keywords liposome immunoassay; chemiluminescence; digoxin; apoenzyme reactivation; melittin; flavin adenine dinucleotide

In the previous paper we reported a new liposome immunoassay system (LIS) with a chemiluminescence reaction monitored *via* an optical fiber and photomultiplier tube.[1] Glucose oxidase (GOD)-containing liposomes were lysed by ouabain–melittin conjugates proportional to the amount of antigen, and the activity of GOD released was measured by chemiluminescence detection. To release entrapped GOD (molecular weight, about 180000) from liposomes, we required higher concentrations of ouabain–melittin conjugates than for the release of low-molecular-weight markers. The concentration of ouabain–melittin conjugates necessary for releasing entrapped GOD was considered to be the main factor determining the detection limit, since a higher concentration of the conjugates led to a higher concentration of the antigen to be measured.

In this report, we describe an improved liposome immunoassay, developed by combining the liposome immunoassay with a chemiluminescence detection and apoenzyme reactivation immunoassay system (ARIS). Flavin adenine dinucleotide (FAD) was incorporated into liposomes instead of GOD. FAD released by ouabain–melittin conjugates reactivated Apo-GOD. The GOD activity, which was proportional to the amount of antigen, was measured by chemiluminescence detection. The sensitivity was greatly improved by using FAD-containing liposomes instead of GOD-containing liposomes.

Materials and Methods

Materials Egg yolk L-α-phosphatidylcholine (PC), melittin (from bee venom), cholesterol (CH), GOD (from *Aspergillus niger*), FAD, microperoxidase (from equine heart cytochrome c, MP-11), albumin, bovine serum (BSA), horseradish peroxidase, ouabain, and *o*-dianisidine were obtaind from Sigma Chemical Co. (St. Louis, MO). Additional reagents and materials were obtained as follows: digoxin, sodium cyanoborohydride, sodium metaperiodate from Nacalai Tesque, Inc. (Kyoto); monoclonal anti-digoxin immunoglobulin G (IgG1-K) from Cambridge Medical Diagnostics (Billerica, MA); isoluminol from Tokyo Kasei Kogyo Co., Ltd. (Tokyo); PL HQ-Auto 15 from Nissui Seiyaku Co. (Tokyo); Markit digoxin from Dainippon Pharmaceuticals Co., Ltd. (Osaka); Sephadex G-25, G-50 and Sepharose 4B from Pharmacia Fine Chemicals (Uppsala, Sweden); Dowex 1-X8 from Munomachi Kagaku Kogyo Ltd. (Tokyo); Nuclepore from Nomura Micro Science Co., Ltd. (Tokyo). Human serum was obtained from a normal volunteer. Other chemicals used were commercial products of high purity and all operations utilized freshly redistilled water.

Preparation of Liposomes Multilamellar liposomes (MLV) containing FAD were prepared as previously described.[2] Untrapped FAD was removed by gel chromatography on a Sepharose 4B column (1.2 × 15 cm), equilibrated in buffer I (0.1 M phosphate buffer, pH 7.0). Liposomes eluting in the void volume of the column were pooled. 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 hepten in the conjugation because of its increased water solubility as described before.[1] Ouabain–melittin conjugates were prepared without [^{3}H]ouabain according to the method of Litchfield *et al.*[3] From the spectrophotometric measurement of melittin and determination of ouabain by the phenol–sulfuric acid reaction, we calculated that an average of 3.58 molecules of ouabain were conjugated per melittin molecule.

Preparation of Apo-GOD Apo-GOD was prepared according to the method of Morris *et al.*[4] Briefly, 80 mg of GOD was dissolved in 14 ml of 0.1 M acetate buffer (pH 4.0). Then, 6 ml of glycerol was added and the pH of the solution was adjusted to 1.4 by addition of 2.5% H_2SO_4 on an ice bath with vigorous stirring. After incubation for 2 h at 0 °C, the solution was gel-filtered at 4 °C on a Sephadex G-50 column equilibrated with 30% (v/v) glycerol solution (pH 1.4). The GOD-fraction was collected and added to 4 ml of 0.4 M phosphate buffer (pH 8.0) containing 200 mg of BSA and 400 mg of activated charcoal, followed by adjustment of the pH to 7.0 with 2 M NaOH. The suspension was stirred for 60 min at 0 °C, then ultrafiltered twice with 0.2 μm pore-size polycarbonate membrane to remove charcoal. Finally, 10% (w/v) sodium azide in water was added to the solution to give a final concentration of 0.1% (w/v). The Apo-GOD solution was stored at 4 °C. The concentration of Apo-GOD was assessed in terms of the concentration of FAD-binding site, assuming equimolecular binding of FAD and Apo-GOD. Equal quantities of 10-fold diluted Apo-GOD solution and $1.28 \times 10^{-9} - 1.28 \times 10^{-5}$ M FAD solution were mixed and the resulting solution was incubated at room temperature for 1 h. Activity of the regenerated GOD was measured as follows: 6.6 mg of *o*-dianisidine was dissolved in 1 ml of water. Then 500 μl of this solution was diluted to 50 ml with 0.05 M sodium acetate buffer (pH 5.1). Immediately before use, pure oxygen gas was passed through the *o*-dianisidine solution for 5 min for O_2 saturation. Sample solution (100 μl) was added to the mixture of 2.4 ml of *o*-dianisidine solution, 500 μl of 10% (w/v) of glucose and 100 μl of peroxidase solution (60 purpurogallin units/ml). The resulting solution was mixed quickly and the increase of absorbance at 500 nm was recorded for 5 min. The ΔA_{500}/min was plotted *vs.* FAD concentration using the maximum linear rate. The concentration of FAD-binding site was determined from the FAD concentration at which ΔA_{500}/min was saturated.

Determination of FAD Concentration A solution of FAD at various concentration (200 μl) and 600 μl of 0.2 M glucose in buffer I were added to 150 μl of buffer I and mixed. Then 50 μl of 50 nM Apo-GOD (final concentration, 2.5 nM) was added and the mixture was incubated at 15 °C for 3 h. 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 chemiluminescent reagent (CL-reagent), and the intensity of chemiluminescence was measured. The CL-reagent contained 100 μl of 100 μM isoluminol, 100 μl of 10 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) BSA and 0.1% (w/v) NaN_3.

Chemiluminescence Analysis Light emission was measured, and luminescence counts were memorized and analyzed as previously described.[1]

Lysis of Liposomes by Ouabain–Melittin Conjugates A solution of ouabain–melittin conjugates (50 μl; 4.3×10^{-8}—2.15×10^{-5} M) was added to 100 μl of buffer I and mixed. To this mixture, 50 μl of liposome solution (concentration of PC, 1 nmol/ml) was added and the resulting solution allowed to stand at room temperature for 5 min. The concentration of FAD released from liposomes was determined by the chemiluminescence measurement as described above. The percentage of lysis was calculated from Eq. 1.

$$\% \text{ of lysis} = (L_S - L_B)/(L_T - L_B) \times 100 \quad (1)$$

L_S is the chemiluminescence intensity of sample solution and L_B is the chemiluminescence intensity of reference solution which contains 50 μl of liposome solution and 150 μl of buffer I. L_T is the total chemiluminescence intensity when liposomes are lysed completely by excess melittin (50 μl of 3.6 μM melittin, final concentration, 0.9 μM).

Inhibition of Liposome Lysis by Antibody A solution of monoclonal anti-digoxin antibody diluted from 300 times to 30000 times (50 μl) and 50 μl of buffer I were mixed. Then, 50 μl of 0.86 μM ouabain–melittin conjugate (concentration at lysis, 0.215 μM) was added. To this mixture, 50 μl of liposome solution (concentration of PC, 1 nmol/ml) was added and the resulting solution was allowed to stand at room temperature for 5 min. The concentration of FAD released from liposomes was determined by chemiluminescence measurement as described above. The percentage inhibition was calculated from Eq. 2.

$$\% \text{ inhibition} = (L_{OM} - L_S)/(L_{OM} - L_B) \times 100 \quad (2)$$

where L_{OM} is the chemiluminescence intensity of the solution which contains 50 μl of buffer I instead of antibody.

Assay Procedure Standard digoxin solution was prepared by dissolving 1 mg of digoxin in 1 ml of dimethylformamide (DMF) and diluting the solution with buffer I. A 50 μl aliquot of standard solution or sample solution was mixed with 50 μl of 1000-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 (concentration at lysis, 0.215 μM) was added and the resulting solution was incubated for another 5 min. Next, 50 μl of liposome solution (concentration of PC, 1 nmol/ml) was added and the mixture allowed to stand at room temperature for 5 min. The concentration of FAD released was determined by chemiluminescence intensity (L_S) as described above. The percentage of lysis at various concentrations of digoxin was calculated from Eq. 1.

Results and Discussion

CL-Reagent The optimal concentrations of isoluminol and microperoxidase to obtain a maximum S/N ratio of chemiluminescence were 100 and 10 μM, respectively, as previously[1] described.

Effect of Apo-GOD and Glucose Concentrations on the Chemiluminescence Intensity From the time course of the reactivation of Apo-GOD with FAD, Morris *et al.*[4] found that after 1 h, 16 h and 5 d, the recoverable activity was 50%, 70% and almost 100%, respectively. They established that the concentration of FAD-binding site could be detemined after an incubation time of at least 60 min, despite the fact that up to 5 days' total incubation was needed to fully recover glucose oxidase activity. In this study we incubated Apo-GOD with FAD for 60 min to estimate the concentration of FAD-binding site of Apo-GOD. The concentration of Apo-GOD prepared was determined to be 10 μM from the FAD concentration at which ΔA_{500}/min was saturated. The Apo-GOD prepared was diluted with 30% (v/v) glycerol in buffer I, since it was reported[4] that the presence of glycerol in an acid dissociation medium has a marked stabilizing effect on the Apo-GOD. The effect of Apo-GOD concentration on chemiluminescence intensity was studied in the presence of 1.2×10^{-8} M FAD (final concentration, 2.4×10^{-9} M). This concentration was estimated from the entrapment efficiency and amount of the liposomes assuming their complete lysis. The chemiluminescence intensity (L_S) increased linearly with increasing concentration of Apo-GOD. On the other hand, the background (L_B) also increased with increasing concentration of Apo-GOD. However, the ratio of L_S/L_B had a minimum at 2.5×10^{-9} M (data not shown). Thus, we used 2.5×10^{-9} M Apo-GOD in subsequent experiments. The effect of glucose concentration of L_S was then investigated. L_S increased with increasing concentration of glucose and leveled off at 0.2 M. Therefore, we used 0.2 M glucose in subsequent experiments.

Effect of pH, Temperature and Incubation Time The effect of pH on L_S was studied in 0.1 M phosphate buffer (1 ml) of various pH values containing 2.4 nM FAD (final concentration), 2.5 nM Apo-GOD (final concentration) and 600 μl of 0.2 M glucose. The chemiluminescence intensity had a maximum between pH 7.0 and 7.5 (data not shown). Considering the stability of FAD and Apo-GOD, we set the pH of the incubating solution at 7.0. The effect of incubation time and incubation temperature on L_S was investigated in buffer I (1 ml, pH 7.0) at 4, 15 and 25 °C. Apo-GOD was very unstable at 37 °C and inactivated rapidly (data not shown). At each temperature L_S increased with time. Maximum chemiluminescence intensity was obtained over 3 h at 15 and 25 °C, and over 4 h at 4 °C. Lower temperature and shorter incubation time would be advan-

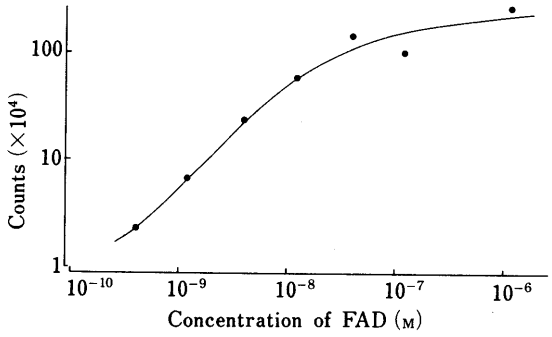

Fig. 1. Dose–Response Curve for FAD

The indicated concentration of FAD (200 μl) and 600 μl of 0.2 M glucose were mixed with 150 μl of buffer I. Then 50 μl of Apo-GOD (final concentration: 2.5 nM) was added and the mixture was incubated at 15°C for 3 h.

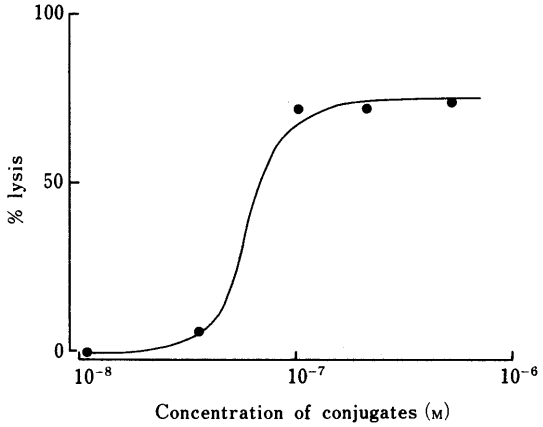

Fig. 2. Dose-Dependent Lysis of FAD-Entrapped Liposomes by Ouabain–Melittin Conjugate

The indicated concentration of ouabain–melittin conjugate (50 μl) and 100 μl of buffer were mixed with 50 μl of liposome suspension (concentration of PC: 1 nmol/ml) and the mixture was incubated at room temperature for 5 min. Then 600 μl of 0.2 M glucose, 150 μl of buffer I and 50 μl of Apo-GOD (final concentration: 2.5 nM) were added and the mixture was incubated at 15°C for 3 h.

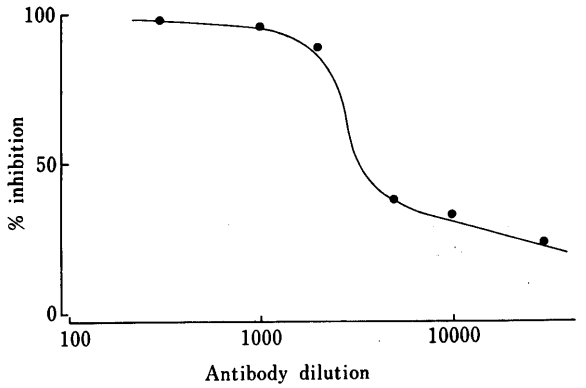

Fig. 3. Inhibition of Ouabain–Melittin Conjugate-Induced Liposome Lysis by Monoclonal Anti-digoxin IgG

Monoclonal anti-digoxin IgG (50 μl) diluted as indicated and 50 μl of buffer I were incubated with 50 μl of ouabain–melittin conjugate (concentration at lysis: 0.215 μM) for 5 min prior to the addition of FAD-entrapped liposomes (concentration of PC: 1 nmol/ml). The % inhibition was calculated according to Eq. 2.

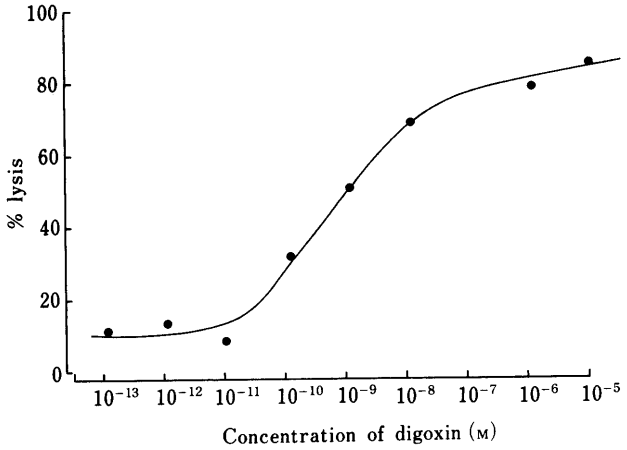

Fig. 4. Standard Curve for Digoxin

Assay procedures were as described in Materials and Methods. Concentration shown is that in the sample.

tageous to the stability of liposomes and rapid assay, respectively. We therefore chose 15 °C for 3 h.

Determination of FAD by CL Assay The dose–response curve for FAD was linear over the range from 0.4 to 12 μM (final concentration, 0.08—2.4 nM, Fig. 1).

Lytic Activity of Ouabain–Melittin Conjugates and Inhibition by Antibody Lysis of the FAD-containing liposomes by ouabain–melittin conjugates is shown in Fig. 2. The ouabain–melittin conjugates induced partial lysis at about 3.6×10^{-8} M and almost total lysis at 2.15×10^{-7} M. Thus, we identified the concentration of ouabain-melittin conjugate for total liposome lysis as 2.15×10^{-7} M. Katsu *et al.*[5] studied the mechanism of membrane lesion of human erythrocytes produced by melittin and reported that the size of membrane lesion increased with increasing concentration of melittin. From their results, the concentration of melittin corresponding to the size of the membrane lesion releasing an FAD molecule (18 Å; molecular weight, about 800) was estimated to be 0.25 μM. This concentration was in good agreement with our data. Time required for liposome lysis by melittin was 5 min in our experiment, which is consistent with the data reported by Dawson *et al.*[6] The results of inhibiting the conjugate-induced lysis of liposomes with monoclonal anti-digoxin IgG are shown in Fig. 3. Lysis of liposomes was inhibited by 25% at 30000-fold dilution and by 96% at 1000-fold dilution. Thus, we used 1000-fold-diluted anti-digoxin IgG in the following assay.

Assay for Digoxin The calibration curve for digoxin was calculated by the nonlinear least-squares method for logistic curve fitting with the use of MULTI[7] and is shown in Fig. 4. A linear relationship was obtained over the range from 10 pM to 13 nM digoxin. The detection limit (10 pM, 0.39 pg/assay tube) was 300 times or 30 times lower than that of the homogeneous system or the heterogeneous system using GOD-containing liposomes, respectively, which we reported previously.[1] The coefficients of variation values at 1.3×10^{-8} and 1.3×10^{-9} M were less than 2%.

In our previous assay system, relatively high concentrations were required to release high-molecular-weight GOD. The high concentration of melittin, leading to a high concentration of hapten, resulted in low sensitivity. In the present study, we incorporated a low-molecular-weight co-factor, FAD, into liposomes instead of GOD to improve the sensitivity. FAD released from liposomes bound to Apo-GOD and reactivated GOD, and greatly improved sensitivity was obtained by combining the liposome immunoassay system with an ARIS. Since the effect of intact liposomes which were not lysed by ouabain–melittin conjugate on the L_S was less than 6%, no steps were required to separate intact liposomes from free FAD solution. Therefore, a homogeneous assay was possible. Furthermore, the time required for incubation was reduced from 20 to 3 h. This provides a rapid method for the measurement of digoxin.

It was found in our previous study that serum protein could inhibit the lytic action of ouabain–melittin conjugate.[1] However, when the serum samples were diluted 100 times, very little influence of serum on the lytic activity was observed. In this assay system, we diluted the sample 100 times for the determination of digoxin in a serum sample, in spite of the loss of sensitivity (data not shown). It remained unclear whether the lytic activity of melittin itself was affected by serum protein or whether it became more difficult to lyse liposomes as a result of adsorption of serum protein. Further studies are under way to investigate the effect of serum protein.

This study introduces a new liposome immunoassay concept with potential practical application for the measurement of drugs in biological fluid.

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