[Chem. Pharm. Bull. 36(3)1086—1094(1988)]

Highly Sensitive Immunoliposome Assay of Theophylline

USA GLAGASIGIJ, YUKIO SATO,* and YASUO SUZUKI

Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980, Japan

(Received August 5, 1987)

A highly sensitive and reproducible immunoassay method for determination of theophylline was developed by using large unilamellar liposomes. Vesicles incorporating theophylline–phosphatidylethanolamine conjugate on the membrane surface as a sensitizer and including a fluorescent marker, calcein, were prepared by the reverse-phase evaporation method from mixtures of phosphatidylcholines containing various fatty acids and cholesterol. Competitive binding of specific antibody to the analyte drug and the sensitizer on the liposomal membrane resulted in lysis of liposomes, and consequently the amount of entrapped calcein that leaked out was inversely proportional to the concentration of the analyte. From studies of various parameters influencing liposome lysis, it was concluded that the chain length of fatty acid most strongly affected the calcein release. The proposed system is simple, rapid, precise, and sensitive to nanomolar concentrations of theophylline. Furthermore, the sensitized liposomes were stable and gave reproducible results. The principle of this assay should be applicable to routine analysis of a wide variety of drugs in biological samples for the purpose of clinical diagnosis or monitoring.

Keywords——immunoliposome; theophylline; large unilamellar vesicle; calcein; liposome lysis

Introduction

An important recent contribution of immunology to the biological sciences and clinical medicine has been the development of sensitive, specific, simple and rapid immunochemical assay methods for the determination of drugs in serum, urine, or tissues. Kinsky *et al.*[1] first showed that liposomes incorporating antigen or hapten on their membrane surface could bind with antibody, and the reacted vesicles were disrupted by the action of complement, resulting in the release of the marker trapped inside. Since then, liposomes have been extensively used as a detector of immune reactions. Many trapped markers such as enzyme,[2,3] dye,[4] electron-spin labels,[5,6] fluorogenic substrate,[7] radioactive materials[8] and fluorophore[9] have been utilized for immune reactions.

We present here a modified and improved method for routine clinical immunoassay of theophylline. Theophylline is widely used in asthma treatment but its therapeutic range is very narrow and control of its plasma level is necessary. Haga *et al.*[3] have reported the use of multilamellar liposomes for determining theophylline. Their systems, however, show poor reproducibility owing to the multiple layer structure in vesicles and also the use of a complex enzyme assay system. In this study, a very simple and reproducible method is introduced by using large unilamellar liposomes prepared by means of the reverse-phase evaporation method[10,11] and trapping of a highly fluorescent molecule, calcein. Calcein trapped at high concentration in the liposomes shows little fluorescence due to its self-quenching. Upon immunolysis, the released calcein is diluted in external medium, and shows markedly enhanced fluorescence, allowing convenient detection. A high encapsulated volume and the single-lamellar property of reverse-phase vesicles allow the trapping of a large amount of marker, leading to increased sensitivity. The proposed assay system requires no separation

procedure, and theophylline can be detected easily at nanomolar concentrations by this method.

Experimental

Materials——Dilauroylphosphatidylcholine (DLPC, C 12:0), dimyristoylphosphatidylcholine (DMPC, C 14:0), dipalmitoylphosphatidylcholine (DPPC, C 16:0), and distearoylphosphatidylcholine (DSPC, C 18:0) were purchased from Nippon Oil and Fats Co., Ltd. (Tokyo). They were used without further purification. Cholesterol was obtained from Koso Chemical Co., Ltd. (Tokyo), and was recrystallized three times from methanol. L-α-Phosphatidylethanolamine (PE) and calcein were from Sigma Chemical Co. (St. Louis). Calcein was purified by chromatography on Sephadex LH-20. *N*-Hydroxysuccinimide was obtained from Tokyo Kasei Kogyo Co., Ltd. Triethylamine and uric acid were purchased from Wako Pure Chemical Industries Ltd. (Tokyo). Caffeine was from Kanto Chemical Co. (Tokyo). *N,N'*-Dicyclohexylcarbodiimide, theophylline, xanthine, and theobromine were purchased from Nakarai Chemicals, Ltd. (Tokyo). All other reagents used were of analytical grade. Phospholipids were routinely checked for purity by chromatography on silica gel thin-layer plates with chloroform: methanol: water (65:30:5, v/v) as a solvent system and visualized by exposure of the plate to iodine vapor.

Buffers——The following buffer systems were used throughout. Tris buffer: 20 mM Tris–HCl, 150 mM NaCl, pH 7.4; $Tris^{2+}$ buffer: same as Tris buffer but in addition it contained 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$, which was used in assay systems involving antigen–antibody reaction.

Antibody and Complement——Rabbit anti-theophylline antiserum was prepared by the method of Itagaki *et al.*[12] Protein (0.4 mg) was dissolved in 1 ml of $Tris^{2+}$ buffer and decomplemented by heating at 56 °C for 30 min. Guinea pig complement was obtained from the Research Laboratory of Microbiology, Osaka University. Dissolved complement was stored at 4 °C until use (not more than a few days). Inactivated complement was obtained by heating for 30 min at 56 °C to destroy the cytolytic activity of complement.

Synthesis of Theophylline–Phosphatidylethanolamine Conjugate (TP–PE)——Sensitizer [8-(3-phosphatidyl-ethanolamidopropyl)-1,3-dimethylxanthine] was synthesized by the method of Hashimoto *et al.*[13] with a minor modification. In brief, 444 mg of 8-(3-carboxypropyl)-1,3-dimethylxanthine (1.66 mmol), which had been synthesized by the method of Cook *et al.*,[14] was added in small portions while stirring to 11 ml of a mixture of chloroform: methanol (1:1, abbreviated as C/M) containing 0.74 mg of triethylamine (1.87 mmol), which had been freshly distilled over phenyl isocyanate. The following solutions were then added sequentially with stirring; 231 mg of PE (0.334 mmol) in 30 ml of C/M; 107 mg of *N*-hydroxysuccinimide (0.93 mmol) in 2 ml of C/M; 191.3 mg of *N,N'*-dicyclohexylcarbodiimide (0.93 mmol) in 2 ml of C/M. After a 10 h incubation at room temperature under nitrogen gas in the dark, the reaction mixture was taken to dryness under reduced pressure at 30 °C. The residue was purified by chromatography on a column of silica gel (100 g, *ca.* 2.7 × 38 cm) which had previously been washed with a large volume of C/M (5:1). The first fraction, eluted with 200 ml of C/M (5:1), was discarded. The second fraction, eluted with 200 ml of C/M (2:1), was evaporated under reduced pressure at 30 °C and the residue was redissolved in 2.6 ml of C/M (2:1). This sample was chromatographed on thin-layer preparative plates (Silica gel 60 F_{254}, 20 × 20 cm, 1 mm thickness, E. Merck, Darmstadt) by using C/M (5:1) as the developing solvent. After development, five distinct bands were visualized under ultraviolet (UV) light (254 nm) with approximate *Rf* values of 0.14, 0.23, 0.55, 0.64, and 0.80. Only one of these bands (*Rf* of 0.23) contained significant amounts of phosphate as determined by the method of Bartlett.[15] This band was scraped from the plates and extracted twice with 80 ml of C/M (2:1). After the removal of silica gel fines by centrifugation at 28000 × *g* for 20 min, the solvent was evaporated off and the powder obtained was stored at −20 °C under nitrogen.

From phosphate determination, the yield was 87.9% (based on the amount of PE added). Its UV spectrum showed a single peak at 274 nm due to the xanthine chromophore of the theophylline analog. No contaminants could be detected by thin-layer chromatography in several solvent systems and with either iodine vapor or ninhydrin spray for detection.

Preparation of Large Unilamellar Sensitized Liposomes——The reverse-phase evaporation method described by Szoka and Papahadjopoulos[10] and Düzgünes *et al.*[11] was applied with minor modifications to prepare large unilamellar liposomes (REVs; reverse-phase evaporated vesicles). Phospholipid mixtures composed of 20 μmol of phosphatidylcholine, cholesterol, and TP–PE (molar ratio 1:1:0.01) were dissolved in a mixture of 3 ml of isopropyl ether (washed three times with water just prior to use to remove any peroxides) and 1.8 ml of chloroform. To this solution, 1 ml of 20 mM Tris–HCl, pH 7.4, containing 58 mM calcein was added and the mixture was then vigorously vortexed under nitrogen for 10 min and followed by a 5 s sonication in a bath-type sonicator. This procedure was repeated twice to obtain a good emulsion. The organic solvent was evaporated off under slightly reduced pressure until a clear suspension was obtained. Tris buffer (1 ml) was added again and any residual organic solvent was eliminated by continuing evaporation under highly reduced pressure for another 1 h. All of the experiments were performed at temperatures above the transition temperature of each phospholipid *e.g.*, 0 °C for DLPC, 23 °C for DMPC, 41 °C for DPPC, and 58 °C for DSPC.[16] After removing untrapped calcein from the vesicles by repeated

centrifugation at 28000×*g* for 30 min in Tris buffer, the resulting pellet was suspended in 2 ml of Tris buffer and stored under nitrogen in the dark at room temperature until use. The phospholipid content of the liposomes was determined by digestion into inorganic phosphate.[15] An aliquot was then taken and diluted to 0.46 mM just prior to use in all experiments.

Standard Assay System——Immune damage to liposomes was determined by measuring the amount of calcein released. Briefly, 20 μl of 0.46 mM liposomes was incubated with appropriate quantities of antibody and 80 μl of complement in a final volume of 1 ml of $Tris^{2+}$ buffer unless otherwise indicated. After further incubation with shaking at 25 °C for 30 min, the reaction mixtures were diluted with 1.5 ml of $Tris^{2+}$ buffer and the fluorescence intensity of the calcein released was determined by excitation at 494 nm, with emission at 512 nm. The total calcein fluorescence in liposomes was measured after the addition of sodium deoxycholate to a final concentration of 0.19%. The percentage of dye released was defined as:

$$\% \text{ trapped calcein released} = [(F - F_0)/(F_t - F_0)] \times 100$$

where F_0 and F are the calcein fluorescence of the sample before and after the interaction with the antibody, respectively, and F_t is the total calcein fluorescence after lysis of liposomes with sodium deoxycholate. Untrapped calcein was less than 5% of the total calcein fluorescence. The controls were composed of liposomes without sensitizer (TP–PE) or with sensitized liposomes but incubated with normal rabbit serum instead of antibody.

Results

Effects of Acyl Chain Length of Phospholipid on Immune Damage to Liposomes

The effects of different acyl chain lengths of phospholipid in liposomes on immune damage were examined by measuring complement-mediated immune lysis. Liposomes composed of DLPC, DMPC, DPPC, and DSPC with an equimolar quantity of cholesterol and 1 mol% sensitizer (related to phospholipid), and trapped calcein inside were incubated with various quantities of antibody against theophylline and complement. As shown in Fig. 1, a proportional rise in the amount of calcein released was observed when the concentration of antibody was increased. Decreasing the fatty acyl chain length resulted in an increase of calcein release, and saturation of the release was dependent on acyl chain length. Liposomes prepared from the shortest acyl chain length phospholipid (DLPC) showed maximum lysis of about 36% while the long ones (DPPC and DSPC) gave about 22.5%. Less than 1.5% of calcein was released from all kinds of liposomes when normal rabbit serum was used as the control (data shown only for DLPC and DSPC). To obtain the highest sensitivity in assaying theophylline, liposomes prepared from DLPC were incubated with a saturating concentration of antibody (125 μl) for the subsequent experiments.

Effects of Epitope Density of Sensitizer on Immunogenicity

The amount of sensitizer on liposome membrane is an important factor in the reactivity of liposomes with antibody. In order to determine the optimum incorporation of sensitizer,

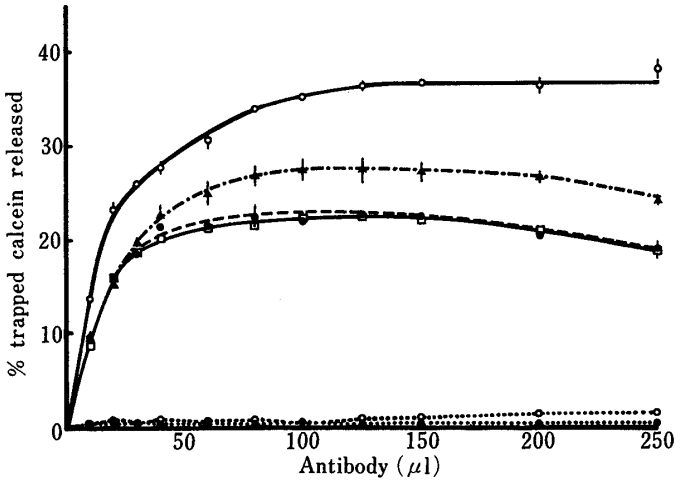

Fig. 1. Effect of Phospholipid Side-Chain Length on Immunolysis of Liposomes

Calcein release from REVs was measured at various concentrations of antibody. Normal rabbit serum was used as the control (indicated by dotted lines).
○—○, DLPC; ▲---▲, DMPC; □—□, DPPC; ●---●, DSPC.
Each point represents the mean of three samples ±S.D.

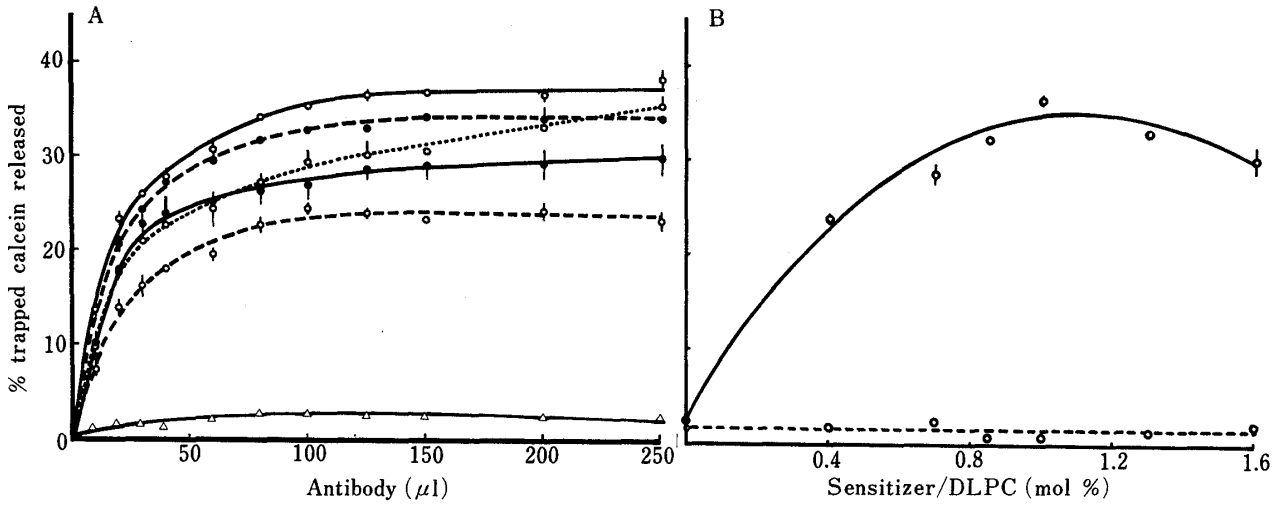

Fig. 2. Effect of Sensitizer Concentrations on the Immunolysis of DLPC-REVs

(A) Antibody dependence of calcein release: △—△, $s=0$; ○---○, $s=0.4$; ●—●, $s=0.7$; ○—○, $s=1.0$; ●---●, $s=1.3$; ○···○, $s=1.6$. The s value indicates the mole percent of TP-PE with respect to DLPC.

(B) Sensitizer dependence of calcein release: the broken line indicates the control (normal rabbit serum).

Each point represents the mean of two samples ± S.D.

liposomes containing various amounts of TP-PE relative to DLPC ($s=0.4$—1.6; s, mol percent sensitizer to DLPC) were prepared, and calcein released was determined in the presence of various amounts of antibody. As shown in Figs. 2A and 2B, control liposomes lacking sensitizer released less than 3% of trapped calcein when complement was added. The marker release increased in parallel with sensitizer concentration. The maximum calcein release (36%) was obtained at 1.0 mol% concentration of sensitizer. A further increase of sensitizer resulted in a decrease of immunolysis (Fig. 2B). Substitution of normal rabbit serum for antibody gave no detectable release of calcein.

Dependence of Liposome Lysis on Complement Concentration

Guinea pig serum is suspected to contain various kinds of nonspecific factors that lyse liposome. Figure 3 demonstrates that in the presence of a saturating amount of antibody (125 μl), the immunolysis of DLPC-liposomes sensitized with TP-PE ($s=1.0$) was dependent on the amount of complement serum. At the concentration range of 0—80 μl, a proportional rise in calcein released was observed. Above 80 μl, a gradual but erratic increase occurred. Hence, 80 μl of complement serum was used in the following experiment. On the other hand, less than 3% of calcein released was observed when 170 μl of heat-inactivated serum was employed. It should be noted that under the conditions of this assay, antiserum per se did not cause a release of marker without complement serum.

Effect of Incubation Time on Immunolysis

The calcein release caused by immunolysis for various periods is illustrated in Fig. 4. The lysis occurred suddenly and reached a plateau after incubation of 10 min. No increase in the lysis was observed on longer incubation time. Consequently, a 30 min incubation period was selected for subsequent experiments.

Effect of Temperature on Immunolysis

Incubation temperature sometimes has a marked influence on immune lysis of liposomes.[17] As shown in Fig. 5, calcein release was also temperature-dependent. When the reaction mixture was incubated at various temperatures, the calcein released was proportional to the temperature in the range of 15 to 25 °C and gradually decreased at higher temperatures.

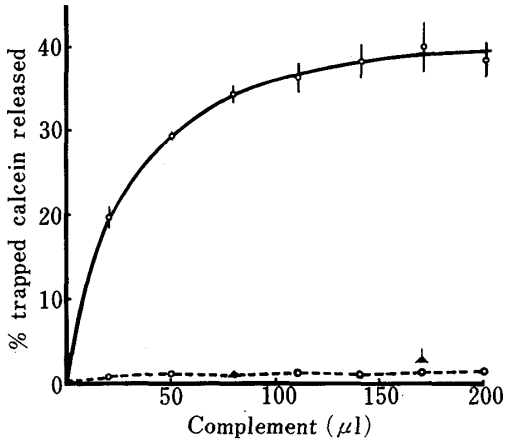

Fig. 3. Effect of Complement Concentration on Calcein Release from DLPC-REVs Containing 1 mol% TP–PE

The broken line indicates the control (normal rabbit serum) and the symbol ▲ indicates heat-inactivated complement. Each point represents the mean of four samples ± S.D.

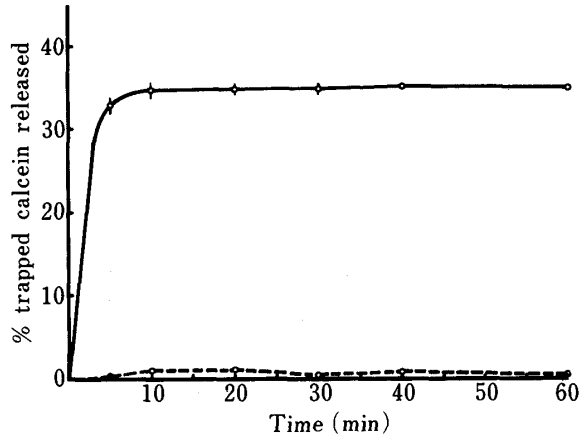

Fig. 4. Effect of Incubation Time on the Immunolysis of DLPC-REVs Containing 1 mol% TP–PE

The reaction mixtures containing liposomes, antibody and complement were incubated at 25 °C. The broken line indicates the control (normal rabbit serum). Each point represents the mean of four samples ± S.D.

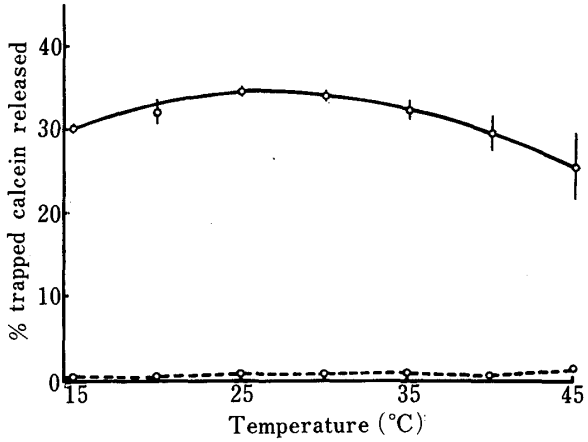

Fig. 5. Effect of Incubation Temperature on the Immunolysis of DLPC-REVs Containing 1 mol% TP–PE

The reaction mixtures containing liposomes, antibody and complement were incubated for 30 min. The broken line indicates the control (normal rabbit serum). Each point represents the mean of four samples ± S.D.

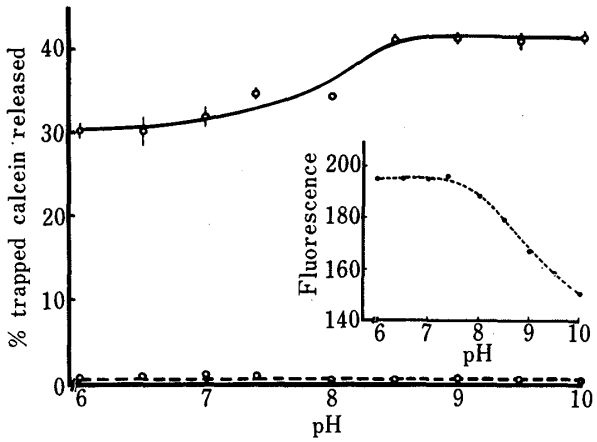

Fig. 6. The pH Profile for the Immunolysis of DLPC-REVs Containing 1 mol% TP–PE

The reaction mixtures containing liposomes, antibody and complement were incubated at 25 °C for 30 min. The broken line indicates the control (normal rabbit serum). Each point represents the mean of four samples ± S.D. The inset shows the effect of pH on fluorescence intensity of standard calcein (2×10^{-6} M) in $Tris^{2+}$ buffer.

No significant leakage of calcein was observed throughout this temperature range when normal rabbit serum was used instead of antibody. Because optimal lysis was obtained at 25 °C, this temperature was chosen as a standard assay condition.

pH Dependence of Immunogenicity

The influence of pH on immunolysis of liposomes was also studied. As illustrated in Fig. 6, the lysis increased until the pH reached 8.5. The fact that the fluorescence intensity of calcein was relatively constant in phosphate buffer between pH 6.0 and 8.5 makes it a useful fluorescent reagent in the physiological pH range.[18,19] However, under our experimental conditions, using $Tris^{2+}$ buffer, calcein showed a pH-dependent quenching as demonstrated in

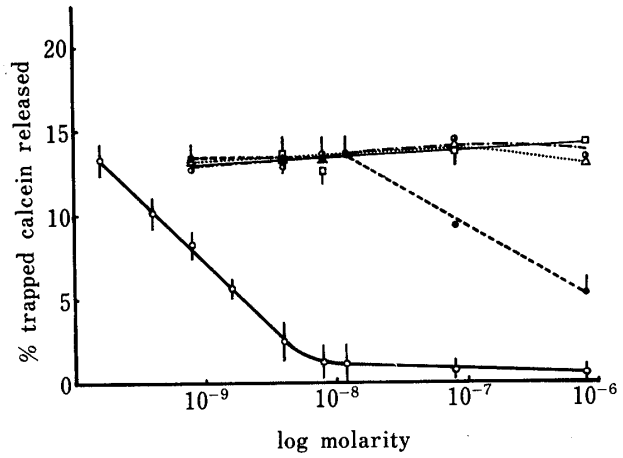

Fig. 7. Calibration Curve for Theophylline and Cross-Reactivity of Xanthine Derivatives in the Immunoliposome Assay

Various amounts of free theophylline were pre-incubated with 10 μl of antibody at 25 °C for 5 min, followed by further incubation for 30 min after addition of 20 μl of liposomes and 80 μl of complement. Each point represents the mean of three samples ± S.D. for theophylline and of two samples ± S.D. for the others.

○—○, theophylline; ●---●, caffeine; □—□, theobromine; △···△, uric acid; ◑---◑, xanthine.

Fig. 6 (inset). The fluorescence intensity of calcein remained constant from pH 6.0 to 7.5, and a further increase of pH resulted in decreasing fluorescence. Decreased fluorescence of calcein at pH above pH 8 results in a decrease of sensitivity even if immunolysis occurs effectively at these pHs. Thus, pH 7.4 was selected as the pH for the assay, since it is close to the physiological pH.

Determination of Theophylline

From the various parameters studied, the most convenient standard conditions for assay of theophylline were decided to be as follows. The reaction mixture containing 20 μl of theophylline solution (10^{-6}—10^{-10} M) and 10 μl of antibody solution in a total volume of 900 μl in $Tris^{2+}$ buffer was preincubated for 5 min at 25 °C, followed by further incubation for 30 min after adding 20 μl of liposome suspension and 80 μl of complement serum. Repression of calcein release by competitive immune reaction between free theophylline and sensitizer on the vesicle membrane is shown in Fig. 7. The percent of marker released is inversely proportional to the amount of theophylline added. In this assay, 0.29 ng/ml of theophylline (1.6 nmol/l) was readily detectable. Theophylline exceeding 14.5 ng/ml (80 nmol/l) almost completely inhibited the lysis of vesicles. Under the same experimental conditions, theophylline in plasma could also be determined. Since plasma also had lytic activity against liposomes,[18,19] it was necessary to dilute the plasma sample about 20-fold before assay. The estimated values of theophylline in plasma agreed closely with the calibration data in buffers with coefficients of variation of 3.19, 9.93, 1.73, and 8.77% for theophylline concentrations of 0.72, 1.44, 2.9, and 7.2 ng/ml, respectively. The specificity of immunolysis was further investigated by inhibition studies with other xanthine derivatives. Theobromine, uric acid and xanthine produced no inhibition of lysis even at a high concentration of 8×10^{-6} M. However, a slight cross-reaction was observed in the case of caffeine which is structurally very similar to theophylline, but at a high concentration (> 120 nmol/l).

Stability of Sensitized Liposomes

The stability of sensitized liposomes containing trapped calcein was examined by measuring the amount of trapped marker and degree of immunolysis as a function of time. In the dark under an atmosphere of nitrogen, liposomes could be stored at room temperature for at least two months without any detectable release of fluorescent marker, and no decrease in the sensitivity to antibody. The result of a study on the stability will be reported elsewhere.

Discussion

Liposomes or lipid vesicles are being widely utilized today in biology and medicine as a

model for studying biological membranes,[20,21] microcapsules to deliver therapeutic agents selectively to cells and tissues,[22,23] and as a potential clinical tool in immunoassay.[4,24,25] Although the last application was reported early in 1969,[1] it has not been developed for routine analysis. Requirements for special instrumentation,[5,6] hazardous reagents,[8] or time-consuming procedures[26] make these assay methods impractical for normal laboratory situations.

The liposome immunoassay described herein makes use of the technique of fluorescence quenching which is highly sensitive and simple in principle and in the instrumentation required. Upon lysis of liposomes by immunological reaction, the entrapped dyes leak out from the liposomal compartment, and are diluted into the surrounding media, where upon fluorescence appears. Because of this, the liposome–antibody complexes, which still contain some trapped calcein, need not be removed from the solution containing released marker, and the entire series of reactions and measurements can be conducted conveniently in a single reaction vessel.

Calcein was selected as a trapped marker because it is stable, commercially available and well documented as to its high fluorescence. At concentrations greater than 4×10^{-4} M, a progressive fluorescence quenching takes place due to excimer formation.[27,28] Its fluorescence emission is free from interference by components present in the immune sera and complement.[19] The degree of release of trapped markers is dependent on the number of antigen–antibody complexes which are formed on the membrane and which lead to complement activation.[1] In this study, several factors that influence the degree of immunological reaction were investigated. These included fatty acyl chain length of liposomal phospholipids[29] and concentration of the hapten on the liposomal membrane.[1] The size of the liposomes affects the degree of signal amplification and the amount of trapped marker inside. It is, therefore, important to use a vesicle-forming procedure which produces large single-walled lipid vesicles for maximum amplification. The reverse-phase evaporation procedure used here clearly meets these requirements. Vesicles of fairly homogeneous size could be obtained with high entrapment efficiency (20%) and large internal aqueous volume (12 μl/μmol lipid), which are close to theoretical values for single-layered vesicles.[10,30] Furthermore, this procedure produces vesicles that are extremely stable to storage. Sonicated liposomes were also studied in the immunological assay system but no lysis was seen at any concentration of sensitizer used. This might be due to destruction of the sensitizer by ultrasonication when prolonged sonication was used,[31] because sensitized MLVs (multilamellar vesicles) which required no sonication showed lysis (data not shown). Nevertheless, the large number of stacked bilayers in multilamellar liposomes leading to low encapsulation efficiency (between 1 and 4 μl/μmol lipid) limited the utility of this system in theophylline immunoliposome assay.[32] The reverse-phase evaporation method also required sonication to produce a uniform and stable emulsion before evaporations, but in our experiments, the sonication time could be extended to 25 s without causing any decline in liposome lysis.

Phospholipid composition also influenced liposomal immunogenicity. Liposomes prepared with DLPC or DMPC were more immunogenic than those prepared with DPPC or DSPC, as can be seen from Fig. 1. In regard to the length of acyl chain of phospholipid, numerous studies have suggested that liposomes composed of phospholipids that are 'fluid' (low transition temperature) show better fixation of antibody and complement due to greater lateral hapten mobility.[33–35] At temperatures below the optimum, 25 °C, DLPC and DMPC exist in the fluid state. Therefore, lateral mobility of the hapten in DLPC- and DMPC-liposomes would contribute to the attachment of antibody because of the greater ability of hapten molecules to form complexes. Another effect is enhancement of hapten exposure to antibody binding sites.[17,36] Shin *et al.*[36] found that the amount of marker released from liposomes containing Forssmann antigen on treatment with antibody and complement was

inversely related to the fatty acyl chain length of phospholipids. This is attributable to an increase of membrane thickness in the case of the longer acyl chains. In the present investigation, the haptenic portion of the sensitizer should project much more clearly above the plane of the bilayer. Thus, the extended spacer arm of theophylline in PE over the phospholipid head group of DLPC minimizes the steric hindrance at the bilayer interface and thereby ensured favorable antibody binding activity.[37] At saturating levels of antibody and complement, the best lysis was obtained with DLPC-liposomes.

Epitope density of hapten molecules on liposome membrane is one of the important factors that governs the degree of immunolysis of liposomes.[1,24,34] As indicated in Figs. 2A and 2B, the immunolysis increased with the increment of hapten density in the presence of excess antibody and complement. Maximum lysis was obtained when the epitope density reached 0.01 (molar ratio to DLPC) followed by a decrease at higher sensitizer concentrations. The phenomena observed were similar to those described by Smolarsky *et al.*[24] and Okada *et al.*[38] The decrease of liposome lysis at high concentrations of hapten might be explained in terms of steric hindrance by the excess amount of hapten, interrupting the deposition of complement components into the liposomal membrane after the antigen–antibody reaction.[38]

The amount of marker released seems to be proportional to the amount of lesion sites formed by activated complement in the liposomal membrane owing to antigen–antibody binding.[39] Under the optimum conditions in this experiment, maximum lysis was only 36% though 100% lysis was expected for single unilamellar vesicles. This result, however, is consistent with data reported in several papers when large unilamellar vesicles (LUVs) were used as liposomes for studying immune lysis.[40–42] The mechanism might involve loss of complement channels from the liposomal membrane, as suggested by Richards *et al.*[41] Low surface curvature found in LUVs reduces the exposure of the hydrocarbon region of the membrane that is responsible for hydrophobic interaction with the protein segment of complement C_{5b-7},[42] leading to reduced or incomplete insertion of C_{5b-7} into the lipid membrane. As a result, loss of complement channels may occur easily in for LUVs. On the other hand, the high surface curvature of SUV (small unilamellar vesicle) is effective in binding and resulted in essentially 100% marker release.[7] Although marker release was only partial in REVs, this drawback could be eliminated by the high sensitivity arising from the high trapped volume of marker and the reproducibility of lysis.

Very recently, Canova-Davis *et al.*[43] have reported a complement-mediated release of enzyme molecules from REVs in the immunoassay system of theophylline. The proposed system in this study is more sensitive than theirs (Fig. 7). This assay format can in principle be extended for analyzing a wide variety of drugs by incorporating the hapten of interest into a liposome bilayer after conjugation with a suitable lipid, and thus the immunoliposome assay is a potentially useful alternative to other methods.

Acknowledgment This work was supported in part by a Grant-in-Aid for Scientific Research (No. 61127002) from the Ministry of Education, Science and Culture of Japan.

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