Vol. 95, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1980 Pages 187-192

LIPOSOME IMMUNOSENSOR FOR THEOPHYLLINE

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Received April 19,1980

SUMMARY

A new simple, sensitive liposome immunosensor (LIS) has been developed by combining the advantages of spin membrane immunoassay (SMIA) and enzyme immunosensor (EIS). The LIS system is composed of an oxygen electrode and sensitized liposomes. It records liposome lysis induced by specific anti-theophylline antibodies and complement which is monitored by the release of entrapped enzymes instead of spin labeles. A sensitive detection was performed because of the amplification of antigen-antibody reaction by liposome lysis and enzymatic reaction. The method offers a simple and sensitive quantitative detection of theophylline down to 4×10^{-9} M (0.7 ng/ml).

INTRODUCTION

Spin membrane immunoassay (SMIA)[2] has been devised by Humphries et al. (1), Wei et al.(2), Rosenqvist et al.(3), and recently by Hsia et al.(4,5). SMIA is sensitive, reproducible, and requires no separation procedures. On the other hand, it has several disadvantages including use of ESR apparatus and quenching of spin labels by biological fluids. Enzyme immunosensor (EIS), which has been developed by Suzuki et al.(6), offered an instantaneous assay method and a simple system easy to handle. However, the sensitivity of EIS method is not enough to measure a nanogram level of drugs.

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[2]Abbreviations used are: SMIA, spin membrane immunoassay; ESR, electron spin resonance; EIS, enzyme immunosensor; LIS, liposome immunosensor; RIA, radio immunosaaay; PC, phosphatidylcholine; Chol, cholesterol; DCP, dicetylphosphate; HPO, horseradish peroxidase.

0006-291X/80/130187-06$01.00/0

In the present report, a new simple and sensitive liposome immunosensor (LIS) is described which has been developed by combining the advantages of SMIA and EIS. The method is based on the liposome lysis induced by specific anti-theophylline antibodies and complement which is monitored by the release of entrapped enzymes. The rate of oxygen depletion, which is directly proportional to the amount of released enzymes in the presence of the substrate, is amperometrically monitored by a Clark oxygen electrode (7,8). In the system using theophylline as a model compound, it was demonstrated that nanogram/ml of theophylline could be detectable.

MATERIALS AND METHODS

5,6-diamino-1,3-dimethyluracil was purchased from Aldrich. Additional reagents were obtained from the following sources: bovine serum albumine (BSA), L-α-phosphatidylethanolamine (PE), L-α-phosphatidylcholine (PC), cholesterol (Chol), dicetylphosphate (DCP), β-nicotinamide adenine dinucleotide reduced (NADH) and horseradish peroxidase (HPO) from Sigma, guinea pig complement from Miles.

Preparation of antibody. Antisera to theophylline have been obtained by immunizing rabbits with a conjugate of 8-(3-carboxypropyl)-1,3-dimethylxanthine and BSA. 8-(3-carboxypropyl)-1,3-dimethylxanthine was synthesized according to Cook et al.(9). N-hydroxysuccinimide ester method (10) was used for the coupling reaction of hapten to BSA. Incorporation of hapten was determined by differential ultraviolet spectrophotometry (Erlanger et al. (11)) at 274 nm. Two male New Zealand White rabbits were immunized with the antigen. An initial intradermal administration of 2 mg of the antigen in 1 ml of 1 : 1 mixture of 0.9 % saline in Freund's complete adjuvant was followed by every two weeks injection. Antibodies were obtained in two of two rabits by 4 months after the initial immunization. The titer ranged 1 : 800 to 1 : 1300 at this time period.

Synthesis of sensitizer. Theophylline-PE composite was prepared by acylation of phosphatidylethanolamine with the acid chloride of theophylline hapten. Theophylline-PE was isolated by chromatography on preparative silica gel plates after initial extraction under conditions in which most of the excess acid chloride of theophylline hapten was converted to a compound soluble in methanol-water. Silicagel plates were developed in a system of chloroform-methanol-water (70 : 30 : 5). Only one of five bands (R_f of about 0.7) was trasferred to a Unisil chromatography column. The eluate was taken to dryness under reduced pressure.

Preparation of sensitized liposomes. Liposomes were actively sensitized by incorporating the appropriate amount of sensitizer

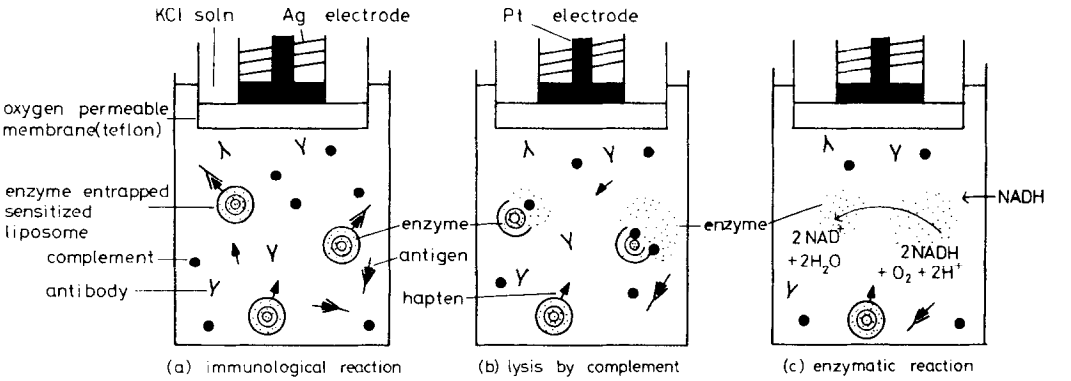

Fig. 1 Principle of Liposome Immunosensor (LIS)

into a basic lipid mixture containing PC, Chol, and DCP; molar ratio, 1 : 0.75 : 0.1. An aqueous solution (pH 8.0) of HPO containing 0.05 M NaCl, 1 mM $MgCl_2$, 0.15 mM $CaCl_2$ and 0.13 mM $MnSO_4$ was used as medium to swell liposomes. Untrapped enzymes were removed by two times of ultracentrifugation (78,000, 60 min).

Assay procedure. A homebrew Clark type oxygen electrode with the Beckman oxygen permeable teflon membrane was used. The sample solution (400 µl) containing theophylline antiserum (40 µl), guinea pig serum (as a source of complement) (40 µl) and co-factor was incubated at 30°C for 30 min. Then the electrode was immersed in the sample solution. After the electrode was equilibrated against the sample solution in equilibrium with atmospheric oxygen, a pulse of substrate (10 µl of 200 mM NADH) was introduced. The output current, reflecting the changed concentration of oxygen, was registered on a recorder. From the initial rate of current decrease, the concentration of theophylline was determined.

RESULTS

The principle of LIS is shown schematicaly in Fig.1. Unknown concentration of antigen and known quantity of antibody, complement and sensitized liposome were incubated. The competition between free antigen and hapten which was bound on the liposome, will take place for binding to the antibodies (Fig.1 a). The complement activated by antigen-antibody reaction lyses liposome and entrapped enzyme (HPO) is released (Fig.1 b). The enzymatic activity is directly proportional to the immune lysis of liposome, which relates inversely to the concentration of free antigen. When a pulse of substrate (10 µl of 200 mM NADH) is introduced, the enzyme catalyzes

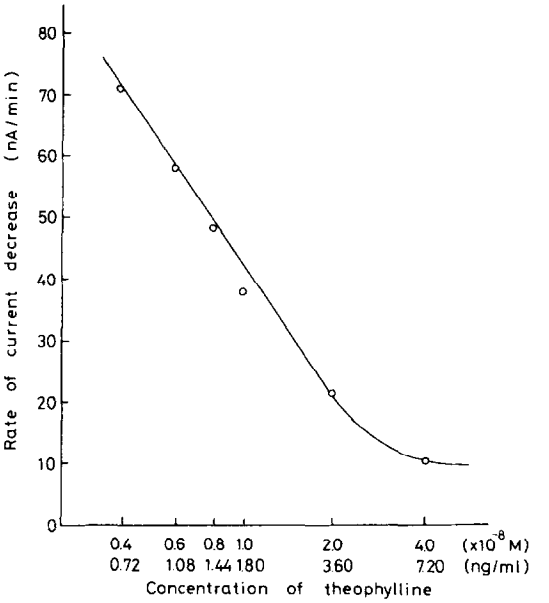

Fig.2. Standard Curve for the Assay of Theophylline. 40 μl of theophylline calibrators were mixed with 100 μl of liposome suspension, 60 μl of guinea pig complement, 60 μl of anti-theophylline antiserum and co-factors. The mixture was incubated at 30°C for 30 min and then 10 μl of NADH (200 mM) was added.

the following reaction (1)

$$2\ NADH + O_2 + 2\ H^+ \longrightarrow 2\ NAD^+ + 2\ H_2O \qquad (1)$$

and oxygen in the solution is consumed. The depletion of oxygen is detected by a Clark type oxygen electrode (Fig.1 c) and amperometric current is registered on a chart recorder. Because the reaction is very rapid, the effect of oxygen back-diffusion from the atmosphere on the initial rate is small. In order to perform high sensitive and reproducible assay, the optimal amount of antibody, guinea pig serum, was investigated. For liposomes sensitized with theophylline-PE, approximately 40 μl of anti-theophylline antiserum and 40 μl of guinea pig serum were required to produce complete lysis. In Fig.2 the initial rate of current decrease was plotted for respective concentration of theophylline. In the range of 2×10^{-8} M - 4×10^{-9} M, the rate of current decrease was proportional to the logarithmic concentration of theophylline.

DISCUSSION

The LIS described here combines the advantages of SMIA and EIS. Because of the amplification of antigen-antibody complexing reaction by liposome lysis and enzymatic reaction, high sensitivity was performed. The LIS studied here not only requires no separation procedure, but also is more sensitive than EIS. The sensitivity range of LIS ($\sim 4 \times 10^{-9}$ M) was comparable to that of SMIA ($\sim 10^{-9}$M) and RIA ($\sim 10^{-9}$ M), which were tested in our laboratory with the same anti-theophylline antiserum. The specificity of the LIS was confirmed by the lack of cross-reactivity of structurally related haptens. Another advantage of LIS over SMIA and RIA is simplicity. The new assay system eliminates both the use of expensive equipment and radioisotopes. The analytical results can be displayed directly in electric signal output. For practical use, the amount of sample is an important factor. The LIS system requires only small amounts of sample (total 400 µl), much smaller than required for EIS ($\sim$ 2 ml) The LIS method suggests the possibility for simple, rapid and sensitive measurement of serum concentrations of drugs.

During the course of this work, we became aware that similar immunoelectrode system, using tetrapentylammonium ion loaded liposomes and ion selective electrode, was independently developed by Fujiwara et al.(12).

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