Chem. Pharm. Bull. 30(4)1504—1507(1982)

An Immunochemical Study with Potassium Ion-Loaded Liposomes

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(Received September 24, 1981)

Potassium ion-loaded liposomes and a potassium ion-selective electrode have been utilized for monitoring the complement-mediated immune lysis reaction; the excellent reproducibility suggests that analytical utilization of such liposomes in immunoassay procedures may be advantageous.

Keywords——liposome; ion-selective electrode; potassium ion; marker ion; Forssman antigen; complement-mediated immune lysis; immunoassay

It is well known that liposomes containing lipid antigen or haptens can be damaged by the complement-mediated immune lysis reaction, and the extent of the lysis has been determined by following the release of an entrapped marker such as glucose,[1,2] fluorophor,[3] or spin label,[4] by the use of various spectrometric detection methods. In addition to those techniques, potentiometric measurements of marker ions using ion-selective electrodes (ISE) have been developed in recent years. Potentiometric measurements have some inherent advantages over the previous methods, *i.e.*, simplified procedure, low cost, and ease of continuous monitoring of the immune lysis process.[5–8]

D'Orazio and Rechnitz prepared sheep erythrocyte ghosts loaded with trimethylphenylammonium ions ($TMPA^+$), and the release of the $TMPA^+$ marker upon immune lysis was monitored by a $TMPA^+$ ISE.[5,6] They succeeded in the quantification of selective immune response with cell ghosts, but not with liposomes because of high leakage of the markers which they employed.[5] Quite recently, Shiba *et al.* found that tetrapentylammonium ions (TPA^+) could be trapped in liposomes.[7,8] Using such liposomes containing an appropriate lipid antigen, they succeeded in trace analysis of the immune lysis reaction in a microliter sample. However, their liposomes had to be freshly prepared for each experiment, because longer storage (*ca.*$>$12 h) of liposomes resulted in scattered data.[8]

For the practical application of liposomes to immunoassay procedures (*e.g.* complement fixation test[5,6]), it is desirable to find a marker ion which can be stored for a longer period. In the present study, the potassium ion (K^+) was chosen as the marker for the reasons described below. First, the self-diffusion rate of K^+ through the liposomal membrane used in this study was expected to be slow, because the membrane was almost impermeable to Na^+.[9] Second, K^+ ISE is commercially available, and can also be prepared easily by using a polyvinyl chloride (PVC) membrane incorporating valinomycin.[10] Third, K^+-loaded liposomes and K^+ ISE can readily be applied to the previous potentiometric analyses of immunoassays (*e.g.* thin layer potentiometric detection of immunoreagents[7,8]).

This note presents an application of K^+-loaded liposomes and K^+ ISE to the study of an immunochemical reaction; attention has primarily been directed to the reproducibility obtainable in immune response analysis with K^+-loaded liposomes.

Materials and Methods

Liposome Preparation——Forssman antigen incorporated into liposomes was used as a lipid antigen. A lipid mixture containing Forssman antigen was isolated from sheep erythrocytes according to the method of Rose and Oklander.[11] About 50 mg of lipid was extracted from 50 ml of preserved sheep blood which was stabilized and diluted with Alsever's solution (50 v/v%). Liposomes were prepared as described

previously[8] using 20 μmol of dipalmitoylphosphatidylcholine (14.7 mg), 15 μmol of cholesterol (5.8 mg), 2 μmol of dicetylphosphate (1.1 mg), and the above lipid extract (1.0 mg). The dried thin film of the lipid mixture was swollen in 0.15 M KCl at 55°C and centrifuged to remove untrapped marker. The resulting pellet of the lipid mixture was dispersed in 2 ml of 0.15 M NaCl solution to yield a 10 mM phospholipid suspension.

Reagents——Preserved sheep blood was purchased from Nippon Bio-Test Laboratories Inc. Rabbit anti-sheep hemolysin and guinea pig complement (lyophilized) were obtained from Kyokuto Pharmaceutical Industry Co., Ltd. The titer of this complement was 270 CH_{50} units/ml. The antiserum was decomplemented by heating at 56°C for 30 min. The level of K^+ in the antiserum and complement was reduced by dialysis at 4°C; the sera were kept at −20°C (for antiserum) or −80°C (for complement) in small aliquots until needed. Chemicals used were all of analytical reagent grade. Water was distilled and deionized.

Electrode System——A K^+ ISE was constructed as reported previously[10] with some modifications. PVC (25 mg), valinomycin (1 mg), and *n*-dibutylphthalate (50 μl) were dissolved in tetrahydrofuran (2—3 ml). The mixed solution was poured into a flat Petri dish of 30 mm diameter, and then the solvent was evaporated off slowly at room temperature. The resulting PVC membrane was cut out, and stuck on an acrylic tube (outer diameter 10 mm, inner diameter 5 mm) with adhesive (Aron Alpha, Toa Synthetic Chemical Industry, Co., Ltd.). The electrochemical cell used in this study is as follows: Ag, AgCl/0.01 M KCl/PVC membrane/sample solution/1 M NH_4NO_3 (salt bridge)/0.01 M KCl/Ag, AgCl. Electromotive force (emf) between a pair of Ag/AgCl electrodes was measured with an electrometer (Hokuto Denko Ltd., HE-101), and recorded on a Yokogawa Technicorder F (type 3052). Figure 1 shows the calibration plots of K^+ ISE in a modified veronal saline (3.12 mM barbital, 1.82 mM barbital sodium, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.145 M NaCl). The electrode exhibited a Nernstian response from 0.1 M to 10^{-5} M K^+. The selectivity coefficients of the electrode against Na^+, Ca^{2+} and Mg^{2+} were 7×10^{-5}, 2×10^{-5}, 2×10^{-5}, respectively. These were measured in mixed solutions containing a constant amount (0.1 M) of interfering ion and various amounts of K^+.[12]

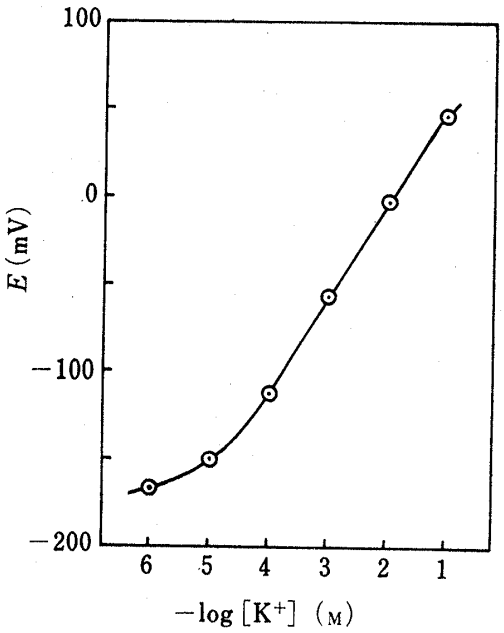

Fig. 1. Response of K^+ ISE in Modified Veronal Saline

Results and Discussion

In order to confirm K^+ release upon immune lysis, the time response of the immunoreaction of the K^+-loaded liposomes was measured. A typical result is shown in Fig. 2. The immune

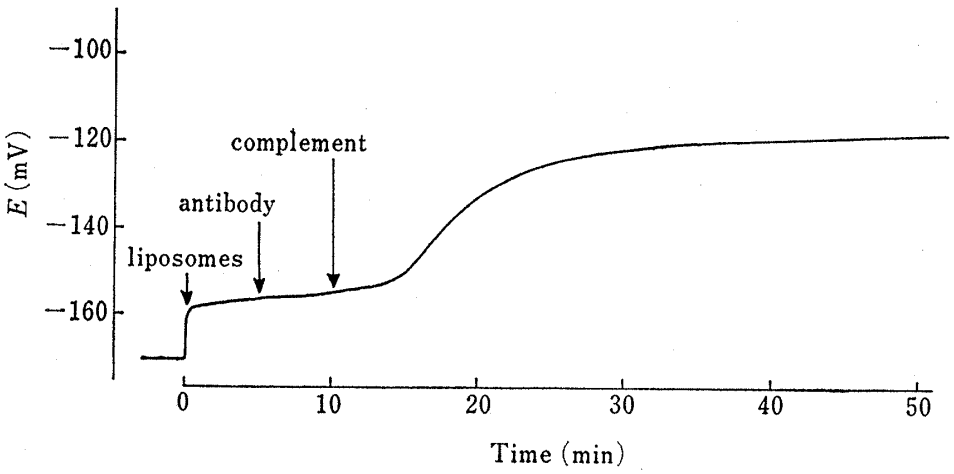

Fig. 2. Potential Change due to K^+ Release upon Immune Lysis

reaction was carried out at 37°C in a beaker (10 ml) containing 2 ml of modified veronal saline. Addition of an aliquot of liposomes (0.2 ml) produced an increase of the K^+ concentration which was attributed to the untrapped K^+ unremoved by centrifugation. Subsequent additions of 1 : 50 dilution of antiserum (0.1 ml) and 1 : 10 dilution of guinea pig complement (0.1 ml) caused a release of K^+ from the liposomes. Of course, the rate of K^+ release was dependent on the dilution of antiserum and of complement; in the present system, the immune response could be measured up to 1 : 1000 dilution of antiserum and 1 : 50 dilution of complement (Figs. 3 and 4). These observations indicate that the combination of K^+-loaded liposomes and the K^+ ISE are suitable for direct monitoring of the immune lysis response.

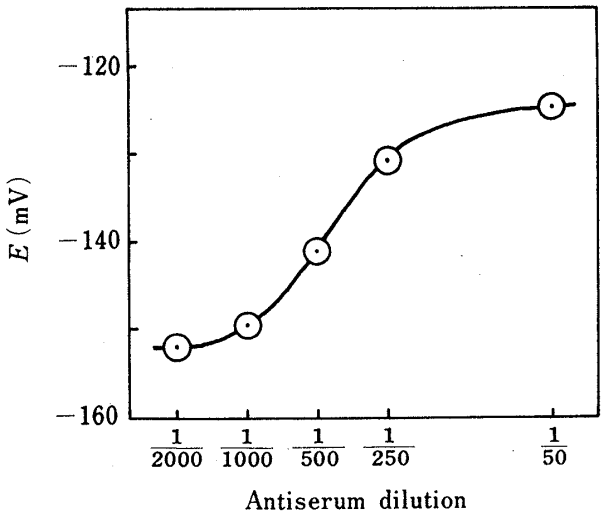

Fig. 3. Potential *vs.* Concentration of Antiserum

Liposomes, diluted antiserum, and complement (1 : 10 dilution) were added in the manner shown in Fig. 2. Potential was recorded at 15 min after complement addition.

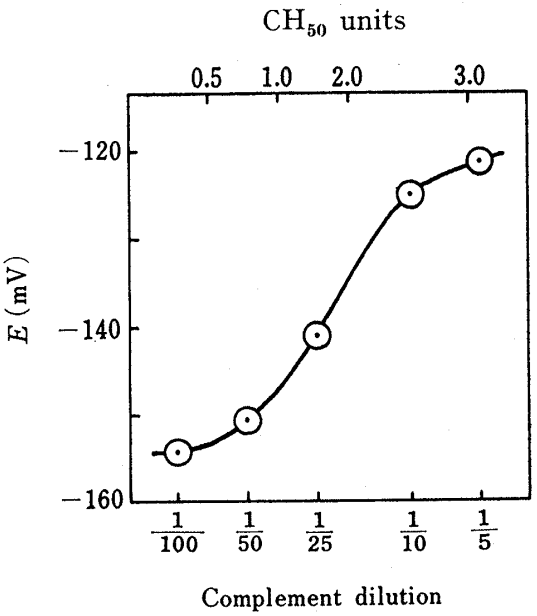

Fig. 4. Potential *vs.* Concentration of Complement

Liposomes, antiserum (1 : 50 dilution), and diluted complement were added in the manner shown in Fig. 2. Potential was recorded at 15 min after complement addition.

Figure 5 shows the reproducibility of the present liposome system. In those experiments, liposomes were stocked in a refrigerator (4°C) before the immunoreaction. Little potential change was observed upon addition of liposomes alone, indicating that the leakage of K^+ during storage was almost negligible. "After lysis" in the figure denotes the potential change due to K^+ efflux within 60 min after complement addition. A similar potential change upon immune lysis could be observed even after 1 month.

Although the above experiment showed that the liposomes containing entrapped K^+ were stable for a fairly long period, we further investigated the stability from the viewpoint of the permeability of the marker. Here, we tried to compare the self-diffusion rate of K^+ with that of TPA^+ used previously;[7,8] the measurements were performed using liposomes without antigen. Diffusion rates were expressed as the ratio of the amount of the leaked ion to that of the total marker ion captured.[9,13] The total amount of the marker was determined by dissolving the liposomes in chloroform. The amount of leaked ion was estimated from the marker efflux after incubation of the liposomes at various temperatures for 1 h. As shown in Fig. 6, the self-diffusion rate of K^+ is much slower than that of TPA^+, indicating that the stability of K^+ in the liposomes is superior to that of TPA^+. The higher permeability of TPA^+ may be due to the lipid-soluble character of TPA^+, since TPA^+ can easily be dissolved in many organic solvents (such as benzene, chloroform, and methanol).

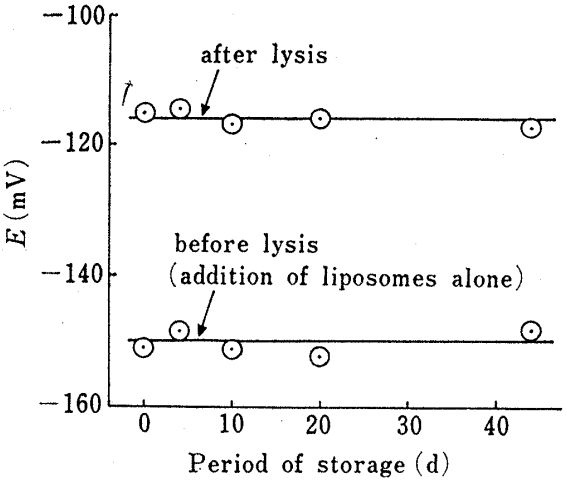

Fig. 5. Reproducibility of Reaction with the Present Liposomes

Immune reactions were performed under the same conditions as in Fig. 2. See the text for additional details.

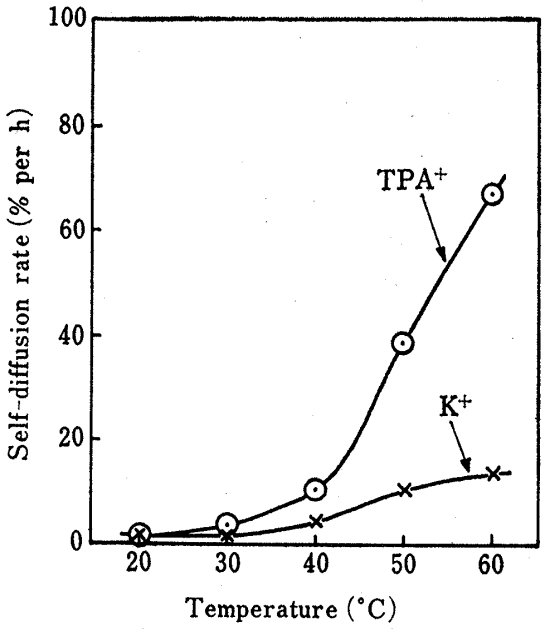

Fig. 6. Self-diffusion Rates of K^+ and TPA^+ through Liposomes

Liposomes were composed of dipalmitoylphosphatidylcholine, cholesterol, and dicetylphosphate in a molar ratio of 1: 0.75: 0.1.

While the experimental data presented here are limited, the results prove the analytical usefulness of the K^+-loaded liposomes in terms of the excellent repoducibility in the measurement of immune lysis. In addition, the use of the K^+ ISE is advantageous for practical analysis, because the electrode can easily be adapted in the laboratory. It is evident that the application of this system should provide a sensitive immunoassay,[7,8] and offer a basic technique for the study of liposome membrane immunochemistry.

References and Notes

1) S.C. Kinsky, *Biochim. Biophys. Acta*, **265**, 1 (1972).
2) J.A. Haxby, C.B. Kinsky, and S.C. Kinsky, *Proc. Natl. Acad. Scil. U.S.A.*, **61**, 300 (1968).
3) H.R. Six, W.W. Young, Jr., K. Uemura, and S.C. Kinsky, *Biochemistry*, **13**, 4050 (1974).
4) G.K. Humphries and H.M. McConnell, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 1691 (1974).
5) P. D'Orazio and G.A. Rechnitz, *Anal. Chem.*, **49**, 2083 (1977).
6) P. D'Orazio and G.A. Rechnitz, *Anal. Chim. Acta*, **109**, 25 (1979).
7) K. Shiba, T. Watanabe, Y. Umezawa, S. Fujiwara, and H. Momoi, *Chem. Lett.*, **1980**, 155.
8) K. Shiba, Y. Umezawa, T. Watanabe, S. Ogawa, and S. Fujiwara, *Anal. Chem.*, **52**, 1610 (1980).
9) D. Papahadjopoulos, K. Jacobson, S. Nir, and T. Isac, *Biochim. Biophys. Acta*, **311**, 330 (1973).
10) G.J. Moody and J.D.R. Thomas, "Ion-Selective Electrodes in Analytical Chemistry," Vol. 1, ed. by H. Freiser, Plenum, New York, 1978, Chapter 4.
11) H.G. Rose and M. Oklander, *J. Lipid Res.*, **6**, 428 (1965).
12) G.J. Moody and J.D.R. Thomas, "Selective Ion Sensitive Electrodes," Merrow, Watford, England, 1971, Chapter 2.
13) K. Inoue, *Biochim. Biophys. Acta*, **339**, 390 (1974).