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Enhancement of immunogenicity by incorporation of lipid A into liposomal model membranes and its application to membrane-associated antigens

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Summary. Incorporation of lipid A into liposomes markedly enhances the plaque-forming cell (PFC responses against model membranes that are sensitized with synthetic amphipathic antigens 2,4-dinitrophenyl-6-*N*-aminocaproyldipalmytoylphosphatidylethanolamine (DNP-Cap-DPPE) and fluorescein thiocarbamyldipalmytoylphosphatidylethanolamine (Fl-DPPE). The enhancing effect was pronounced only when lipid A and haptenic determinants were presented on the same liposome.

Lipid A was incorporated into heterologous erythrocytes and syngeneic tumour cells. Significant and selective increase of *in-vitro* PFC responses was observed against heterologous erythrocytes, and immunization of C57BL/6 mice with lipid A treated X-irradiated tumour cells (EL-4) markedly prolonged the survival of mice after challenge with homologous viable tumour cells. These data suggest that passively incorporated lipid A will be an effective method for selectively augmenting antibody formation against membrane-associated antigens.

INTRODUCTION

The components of the cell membrane play a critical role for cell-to-cell interactions or the communication between outside and inside of the membrane. Antibodies are widely used as tools for investigation of these membrane components. However, this strategy is frequently hampered by the fact that their immunogenicity is often too weak to provoke sufficient immune responses. This is particularly serious for attempts to immunize against tumour antigens.

Liposomes are artificial model membranes and have been exploited as experimental tools in a number of biological subjects including immunological phenomena. As immunogens, liposomes have the advantages that such variables as density, number or type of determinants can be regulated simply by altering the composition of the lipid mixture used to generate the model membranes. With these model membranes, we have investigated several parameters relevant to the immunogenicity of membrane-associated antigens

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Abbreviations: Chol, cholesterol; DNP-Cap-DPPE, 2,4-dinitrophenyl-6-*N*-aminocaproyldipalmitoylphosphatidylethanolamine; DPPA, dipalmitoylphosphatidic acid; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; Fl-DPPE, fluoresceinthiocarbamyldipalmitoylphosphatidylethanolamine; HBSS, Hanks's balanced salt solution; HRBC, horse erythrocytes; L, liposomes; PBS, phosphate-buffered saline; PFC, plaque-forming cells; SRBC, sheep erythrocytes.

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(Kinsky, Yasuda & Tadakuma, 1982). We have also demonstrated that lipid A can be inserted into lipid bilayers and produce a significant increase in immunogenicity of liposome antigens (Dancey, Yasuda & Kinsky, 1977).

In this communication, we describe first that the enhancing effect by lipid A was selectively expressed when lipid A and haptenic determinants are presented on the same liposome. We then obtained a similar and selective enhancing effect on the immune responses against membrane-associated antigens when lipid A was passively incorporated into them.

MATERIALS AND METHODS

Mice

Male C57BL/6 mice were supplied from the Laboratory of Experimental Animals, Institute of Medical Science, University of Tokyo, Tokyo, Japan. The animals were maintained on laboratory chow and acidified-chlorinated water and used when 2–4 months old.

Lipids

Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidic acid (DPPA) and cholesterol (Chol) were purchased from Sigma Chemical Co., St Louis, MO. Dipalmitoylphosphatidylethanolamine (DPPE) was purchased from Calbiochem-Bearing, La Jolla, CA. Chloroform-soluble lipid A was isolated as described previously (Dancey *et al.*, 1977) from *Salmonella paratyphi* B lipopolysaccharide which was generously provided by Dr N. Kochibe, Gunma University, Maebashi, Japan. 2,4-Dinitrophenyl-6-*N*-aminocaproyldipalmitoylphosphatidylethanolamine (DNP-Cap-DPPE) and fluoresceinthiocarbamyldipalmitoylphosphatidylethanolamine (Fl-DPPE) were synthesized as described previously (Six, Uemura & Kinsky, 1973; Uemura *et al.*, 1974).

Immunogens

Details for the preparation of liposomal immunogens have been published previously (Yasuda, Dancey & Kinsky, 1977a). In the current investigations, unsensitized liposomes—abbreviated as L(DPPE)—were prepared from a mixture of DPPC, Chol, DPPA, DPPE in molar ratio of 1:1:0·1:0·1, respectively. Sensitized liposomes—abbreviated as L(DNP-Cap-DPPE) or L(Fl-DPPE—were made from the same mixture except DPPE was replaced by DNP-CAP-DPPE (molar ratio 0·1 against DPPC) or Fl-DPPE (molar ratio 0·05). When lipid A was incorporated into the liposomes, lipid A was added to the mixture of lipids at a molar ratio of 0·05 as phosphate against DPPC.

The dried lipid films, which were obtained after rotary evaporation followed by vacuum desiccation, were dispersed by vortexing in phosphate-buffered saline (PBS) to yield 0·2 mM DPPC concentration as stock solution. For *in-vitro* immunization the liposomes were diluted with Hanks's balanced salt solution (HBSS) to give the desired concentration in the cell cultures. Sheep erythrocytes (SRBC) and horse erythrocytes (HRBC) were obtained from Nippon Bio-Test Laboratories Inc., Kokubunji, Tokyo, Japan. The cells were washed three times with PBS before use as indicator cells in the PFC assay or as antigen.

Treatment of erythrocytes or tumour cells with lipid A

The dried lipid A film was prepared as described above and dispersed in PBS by sonication to yield 0·1 mM lipid A concentration. One hundred microlitres of SRBC or HRBC suspension containing 4×10^8 cells was mixed with 0·5 ml of 0·1 mM lipid A solution, and incubated for 1 h at 37°. After washing three times with PBS, lipid A treated erythrocytes were further diluted for use *in vitro*. EL-4 tumour cells propagated *in vivo* in C57BL/6 mice were harvested from the peritoneal cavity, and red blood cells were removed by treatment with Tris-NH_4Cl buffer (pH 7·4). The cells were washed with PBS, and adjusted to 10^8 cells/ml. One millilitre of tumour-cell suspension was mixed with 1 ml of 0·1 mM lipid A solution and incubated at 37° for 1 h with frequent agitation. The lipid A-treated tumour cells were washed three times with PBS and used for immunization.

Spleen-cell cultures

Spleen cells were suspended at a concentration of 10^7/ml in completely supplemented Eagle's minimal essential medium containing 10% foetal calf serum (lot 80719 obtained from Flow Laboratories, Stanmore, N.S.W., Australia) and 5 mM HEPES buffer. One millilitre aliquots (1×10^7 cells/ml) were dispersed into the wells of a Linbro tissue culture plate (Linbro, Hamden, CT) with 24 flat-bottom wells. Immunogens were added in 50 μl of HBSS, and the plates were incubated at 37° in a humidified atmosphere of 7% O_2, 10% CO_2 and 83% N_2 with gentle rocking as described previously (Tadakuma *et al.*, 1982).

Haemolytic plaque assay

After cultivation *in vitro* for 4 or 5 days, IgM plaque-forming cells (PFC) in pooled duplicate wells were enumerated as described elsewhere (Tadakuma *et al.*, 1982). Trinitrophenylated and fluoresceinated sheep erythrocyte target cells (TNP-SRBC and Fl-SRBC respectively) were prepared by methods previously employed (Yasuda *et al.*, 1977a). The hapten specific responses induced by liposomes were calculated by subtraction of the number of PFC detected with non-haptenated SRBC. Data are expressed as PFC per culture (10^7 cultivated cells).

Tumour immunization and challenge

PBS (as control) or lipid A-treated EL-4 cells were dispersed in PBS and exposed to X-irradiation at a dosage of 10,000 rad. Treated, irradiated cells (10^7 cells in 0·3 ml of PBS) were injected intraperitoneally into each mouse. A second identical immunization was performed 14 days later. Intraperitoneal challenge with 1×10^5 viable cells per mouse was performed 14 days after the second immunization. The degree of immunization was assessed by survival after challenge.

RESULTS

Enhancement of PFC responses by incorporation of lipid A into liposomes

DNP-Cap-DPPE alone or DNP-Cap-DPPE plus lipid A were incorporated into DPPC/Chol/DPPA liposomes, and their immunogenic activity was assessed by adding serial (10-fold) dilutions of the liposomes to the spleen-cell cultures. As shown in Fig. 1, the marked increase of anti DNP-PFC responses was observed by incorporating lipid A into liposomes, especially at the dose of 10^{-8} and 10^{-9} M as DNP-Cap-DPPE. In contrast, the combination of equal amounts of DNP-Cap-DPPE-sensitized liposomes and unsensitized liposomes with lipid A could not change the profiles of PFC responses against DNP-sensitized liposomes. Furthermore, constant dose of DNP-sensitized liposome (10^{-8} M as DNP-Cap-DPPE) plus serial dilutions of unsensitized liposomes with lipid A did not enhance PFC responses except a slight increase at highest dose. These results strongly indicated that the enhancing effect of lipid A was most clearly seen when lipid A and haptenic determinants were presented on the same liposome.

In Fig. 2, various ratios of lipid A to DPPC were incorporated into liposomes, and their enhancing

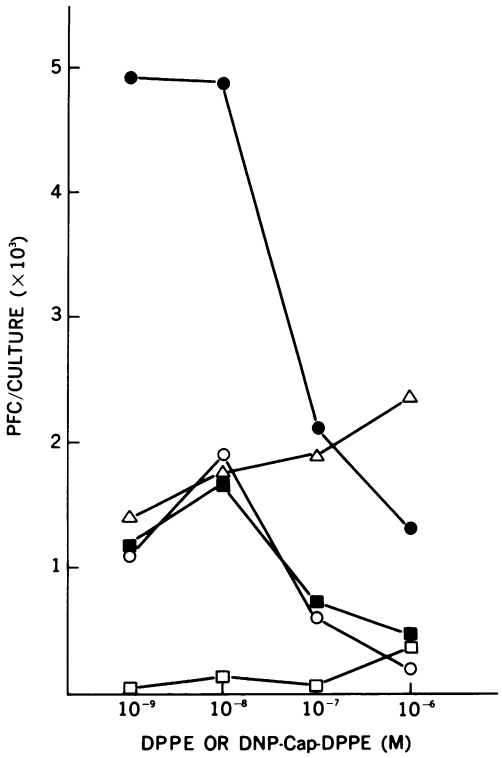

Figure 1. Effect of lipid A incorporation on the immunogenicity of DNP-Cap-PE-sensitized liposomes. Spleen cells were stimulated with serial (10-fold) dilutions of unsensitized liposomes (L) with lipid A (□), DNP-Cap-PE-sensitized liposomes without lipid A (○), or of DNP-Cap-PE-sensitized liposomes with lipid A (●). The experiments also included the cultures stimulated with equal amounts of serial dilutions of DNP-Cap-PE-sensitized liposomes without lipid A plus unsensitized liposomes with lipid A (■) and with constant dose (10^{-8} M) of DNP-Cap-PE-sensitized liposomes plus serial dilutions of unsensitized liposomes with lipid A (△). On day 4, the frequency of IgM PFC was determined with TNP-SRBC. Although the data were not shown here, the number of PFC obtained by stimulation with unsensitized liposomes without lipid A remained less than 100 PFC/culture.

effect was enumerated. Incorporation of only 0·04% lipid A against DPPC significantly increased the PFC responses and the maximum enhancement was obtained with the 1% incorporation. Higher ratio of lipid A (5%) incorporation did not increase any more at optimal dose of antigen, but seemed to increase the PFC responses markedly at suboptimal dose (see also Fig. 1).

Selective enhancement of PFC responses by lipid A incorporated liposomes was further confirmed by the

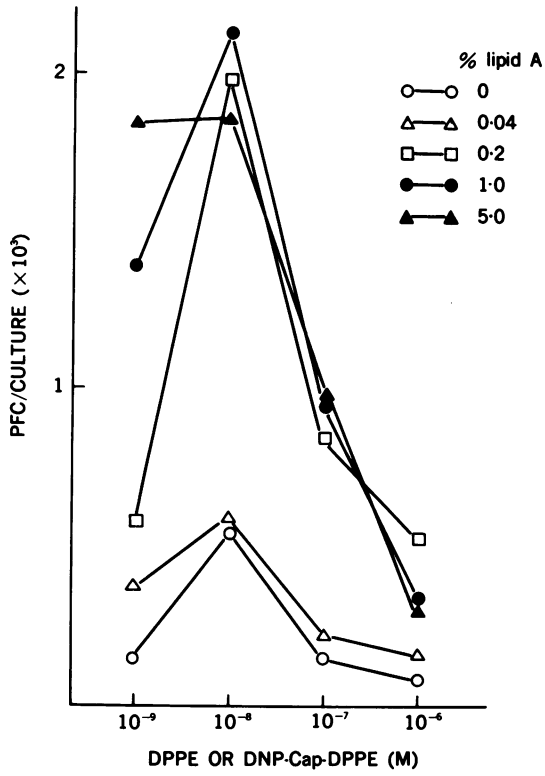

Figure 2. Effect of dose and density of lipid A incorporated in the DNP-Cap-PE-sensitized liposomes. DNP-Cap-PE-sensitized liposomes were prepared with varying amount of lipid A (0–5% against DPPC) as indicated, and the spleen cells were stimulated with serial dilutions of these liposomes. On day 4, the frequency of IgM PFC was determined.

experiments employing another hapten Fl-DPPE-sensitized liposomes (Table 1). The PFC responses obtained in the spleen-cell cultures, which were co-stimulated with DNP-sensitized and Fl plus lipid A-sensitized liposomes or vice versa, clearly demonstrated that the enhancing effect by lipid A incorporation was selectively expressed on the PFC responses against liposomes where lipid A and haptenic determinants coexisted.

Enhancement of antibody production by lipid A-treated red blood cells

Since lipid A could exert its enhancing effect without covalent attachment to antigenic determinant as shown in the liposome experiments, it was sought whether passive incorporation of lipid A into the cell membranes could augment the immunogenicity of membrane-associated antigens. Sheep red blood cells were treated with 0·1 mM lipid A solution at 37° for 1 hr. After washing, PBS treated SRBC (as control) or lipid A-treated SRBC were added to the spleen cell cultures at various dilutions as indicated in Fig. 3. A marked increase of PFC responses against SRBC was observed, with the maximum enhancement being obtained at a lower dose of lipid A-treated SRBC than of untreated SRBC. However, this shift of the optimum dose was not so obvious on day 4 of culture (data not shown). The possibility that the effect of lipid A treatment was selectively expressed on the lipid A-treated cells and not on the bystander cells was tested further (Table 2). SRBC and HRBC were treated with lipid A and the spleen cells were stimulated with these two antigens. It is obvious from the experiments summarized in Table 2 that at the doses used in these experiments the enhancing effect is selectively expressed on the PFC responses against lipid A-treated red blood cells.

Table 1. Selective enhancement of PFC responses by coexistence of lipid A and haptenic determinants on the same liposome

Immunogen*	PFC/culture TNP SRBC	PFC/culture Fl-SRBC
L(DPPE)	35	90
L(DPPE+lipid A)	160	150
L(DNP-Cap-DPPE)	610	—‡
L(DNP-Cap-DPPE+lipid A)	2720	—
L(Fl-DPPE)	—	635
L(Fl-DPPE+lipid A)	—	4030
L(DNP-Cap-DPPE)+L(Fl-DPPE)†	630	880
L(DNP-Cap-DPPE+lipid A)+L(Fl-DPPE)	3025	870
L(DNP-Cap-DPPE)+L(Fl-DPPE+lipid A)	1110	3230

* DNP-Cap-DPPE- or Fl-DPPE-sensitized liposomes (L) were prepared with or without 5% lipid A, and added to the culture at final dilution of 10^{-8} M as DPPE.

† Equal amounts of DNP-Cap-DPPE-sensitized liposomes and Fl-DPPE-sensitized liposomes were added in the same culture.

‡ Not done.

Effective tumour immunization by lipid A-treated cells

C57BL/6 mice were pre-immunized twice with 10^7 irradiated EL-4 tumour cells, which were treated with or without lipid A, and were challenged with 10^5 viable

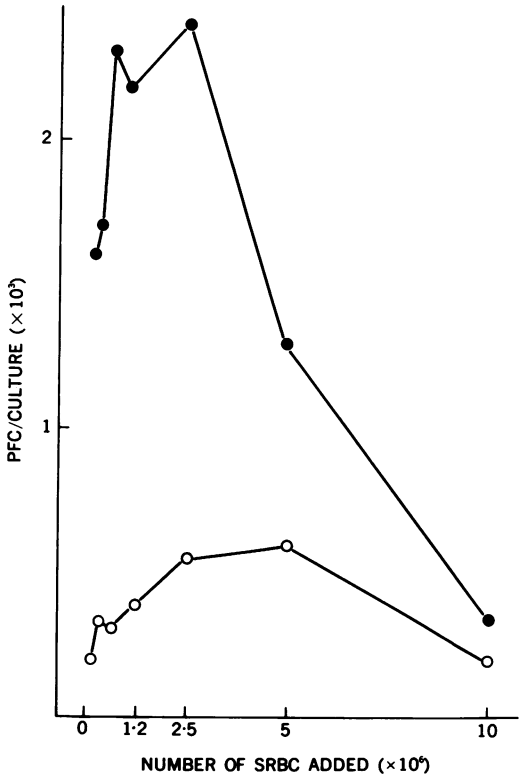

Figure 3. Enhancement of PFC responses by lipid A-treated SRBC. SRBC (4×10^8) were treated with 0·1 mM lipid A solution at 37° for 1 hr. After washing, PBS-treated SRBC as control (○) or lipid A-treated SRBC (●) were added to the culture at final doses as indicated on the abscissa. On day 5, the frequency of IgM PFC was determined.

cells. The survival after challenge in the different test groups is shown in Fig. 4. Prolongation of survival in mice immunized with untreated irradiated cells was marginal. In contrast, preimmunization with lipid A-treated cells significantly prolong the survival and one mouse is still alive at this moment (more than 10 months).

In Fig. 5, the dose effect of lipid A treatment for effective immunization was investigated. Tumour cells treated with 0·01 mM, 0·1 mM and 1 mM of lipid A suspension were irradiated, and injected twice before challenging viable tumour cells. Optimal tumour immunization was obtained with either 0·1 mM or 1 mM lipid A treatment, but even 0·01 mM had a detectable enhancing effect. However, significant difference was not observed between 0·1 mM and 1 mM lipid A treatment.

Table 2. Selective expression of enhancing effect on the PFC responses against lipid A-treated red blood cells

Immunogen*	PFC/culture	
	SRBC	HRBC
None	65	20
SRBC	310	—†
HRBC	—	375
SRBC(lipid A)	4970	—
HRBC(lipid A)	—	2290
SRBC+HRBC	290	410
SRBC(lipid A)+HRBC	5730	640
SRBC+HRBC(lipid A)	564	2165
SRBC(lipid A)+HRBC(lipid A)	4500	1565

* SRBC and HRBC were treated with PBS (as control) or lipid A as described in Fig. 3. 5×10^5 cells of each red blood cell type were added to the spleen-cell cultures and the frequency of IgM PFC was determined on day 5.

† Not done.

DISCUSSION

As model membrane antigens, hapten-sensitized liposomes have been used to examine the influence of various parameters that are relevant to the immunogenicity of membrane-associated antigens. Previous works by us and others revealed the following points are essential for exertion of immunogenic activity: moderate membrane fluidity (Yasuda, Dancey & Kinsky, 1977b; Houte *et al.*, 1981; Hashimoto *et al.*, 1982), appropriate epitope density of antigenic determinants (Tadakuma *et al.*, 1980) and some protrusion of antigenic determinants from the membrane surface (Dancey, Isakson & Kinsky, 1979).

Those observations provide us the hints on how to augment the immunogenic activity of membrane-associated antigens. Since it is hard to regulate the density or distribution of actual membrane-associated antigens, the manipulation of membrane fluidity will be very practicable in increasing the immunogenicity. We and other have already reported that the incorporation of cholesterol, which works as a modulator of membrane fluidity, greatly increased the PFC responses especially when the cholesterol was incroporated into liposomes with high fluidity or into solid liposomes (Demel & Kruyff, 1976; Houte *et al.*, 1981; Hashimoto *et al.*, 1982). Furthermore, Shinitzky, Skornick & Haran-Ghera (1979) demonstrated that

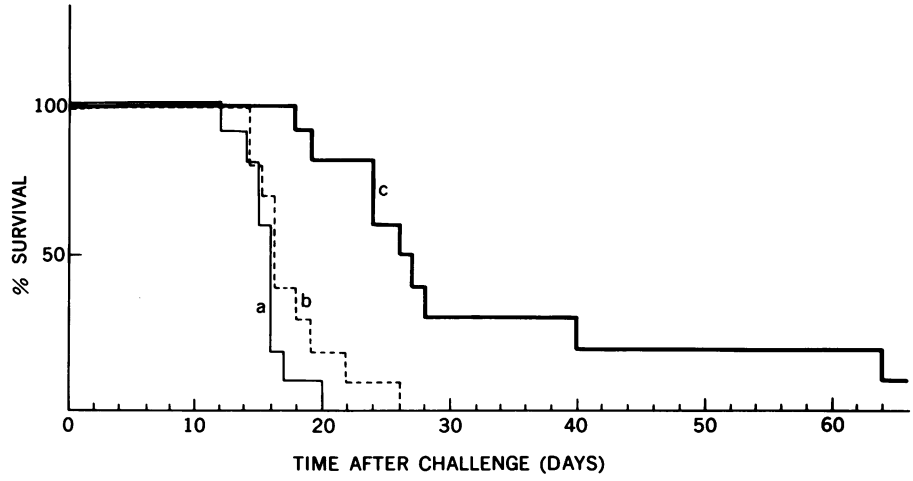

Figure 4. Effect of lipid A-treated tumour cells for immunization on survival profiles after challenge with 1×10^5 viable tumour cells. C57BL/6 mice were challenged with EL-4 tumour cells after no treatment (a), or after two immunizations with 10^7 irradiated cells (b) or 10^7 irradiated lipid A-treated cells (c). Each group contained 10 mice.

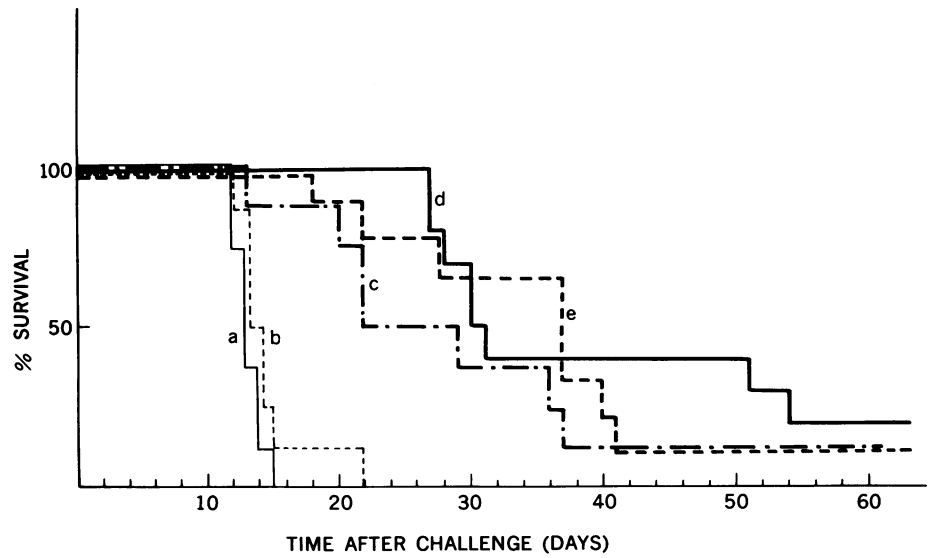

Figure 5. Effect of lipid A dose in the treatment of tumour cells for immunization. EL-4 tumour cell suspensions were treated with equal volume of 0·01 mM lipid A solution (c), 0·1 mM lipid A solution (d) or 1 mM lipid A solution (e) at 37° for 1 hr. After a wash with PBS and irradiation, they were used for immunization. Control groups received (a) no treatment and (b) untreated irradiated EL-4 tumour cells. Each group consists of (a) eight mice, (b) eight mice, (c) eight mice, (d) 10 mice and (e) nine mice.

significant prolongation of survival in mice after challenge with viable tumour cells was achieved by immunization with irradiated, cholesterol- or cholesterol hemisuccinate-treated cells.

Besides the modulation of membrane fludity, we have developed the incorporation of lipid A into hapten-sensitized model membranes. As shown in this paper and previous papers (Dancey *et al.*, 1977; Dancey, Yasuda & Kinsky, 1978; Tadakuma *et al.*, 1982), incorporation of only a small amount of lipid A greatly enhances the immune responses. The covalent conjugation of lipid A and haptenic determinants is not necessary and the enhancing effect is selectively expressed on the PFC responses against the haptenic determinants that are presented on the same liposome with lipid A. Furthermore, we observed the incorporation of lipid A obscured the epitope density effect for induction of PFC responses and markedly increased even the PFC responses against hapten-sensitized liposomes with low density determinants (unpublished observation). In this communication, passive administration of lipid A was attempted to see whether such treatment could increase the immunogenicity of membrane-associated antigens as shown in the model membrane system. Significant enhancement of PFC responses was observed against lipid A-treated heterologous erythrocytes *in vitro*, and the experiments co-stimulated with lipid A-treated SRBC and untreated HRBC, or vice versa, clearly demonstrated that the effect was selective. The data were not shown here, but a similar enhancing effect was observed in experiments *in vivo* by administration of lipid A-treated SRBC or HRBC, especially at suboptimal doses (10^7 cells/mouse).

The effective prophylactic immunization against homologous tumour cells in mice was tested by treating the animals with lipid A-treated, irradiated cells. In the system of EL-4-C57BL/6 mice, the mean duration of survival of the mice immunized with lipid A-treated cells was singificantly longer than that of the mice immunized with untreated tumour cells. In the dose effect of lipid A treatment, a significant difference was observed between 0·01 mM and 0·1 mM lipid A treatment but we could not find much difference between 0·1 mM and 1 mM lipid A. This might be due to the fragility of tumour cells treated with 1 mM lipid A, since lipid A has some cytotoxic effect at high dose (unpublished observation).

In conclusion, we have demonstrated that the incorporation of lipid A into hapten-sensitized liposomes selectively enhances the PFC responses against the determinants that were presented on the same liposome. Similarly, significant increase of immunogenicity was observed after treatment of heterologous erythrocytes and syngeneic tumour cells with lipid A. The method described here may be a practicable method for obtaining enhanced immune responses against membrane-associated antigens, including tumour-associated antigens. Recently, we succeeded in synthesizing the lipid A analogues that have adjuvant activity when incorporated into hapten-sensitized model membranes (Yasuda *et al.*, 1982). It therefore seems that selective enhancement of immunogenicity of membrane-associated antigens can be achieved with chemically defined materials. These systems may open up ways to developing the practical application of selective immune enhancement without such side effects as the pyrogenicity or necrotizing effect of native lipid A.

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