ELSEVIER

International Journal of Pharmaceutics 111 (1994) 15–24

international journal of pharmaceutics

Structure and tumor necrosis factor-inducing activity of dispersed particles of a lipid A analogue, GLA-60, and phosphatidylcholine mixture

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Received 10 September 1993; modified version received 27 November 1993; accepted 4 March 1994

Abstract

A synthetic monosaccharide-analogue of lipid A, GLA-60, was co-dispersed with egg PC in aqueous solution. The size distribution and structure of the dispersed particles were investigated by the dynamic light scattering (DLS) and transmission electron microscopic (TEM) methods. The particles were of liposomal structure with homogeneous size. The leakage of CF out of the liposomes in rat plasma was suppressed by augmenting GLA-60. Fluorescence anisotropy measurements showed that GLA-60 in lipid bilayers led to a rigid structure of the membranes. The activity of tumor necrosis factor (TNF) induction in mice increased with the content of GLA-60 in sonicated liposomes. The results showed that these liposomes were useful as GLA-60 formulations.

Key words: Lipid A subunit analog; GLA-60; Liposome; Size distribution; Zeta-potential; Carboxyfluorescein; Anisotropy; TNF induction

1. Introduction

Lipid A (Fig. 1), the partial structure of bacterial lipopolysaccharide (LPS) on the surface of the outer membrane of Gram-negative bacteria (Luderitz et al., 1982), is a potent biologically active site (Galanos et al., 1977; Morrison et al., 1979). Lipid A induces prostaglandins, cytokines like interferon (Homma et al., 1985), interleukin 1 (Koide et al., 1987) and tumor necrosis factor (TNF) (Beutler et al., 1986; Kumazawa et al., 1987) in mammalian cells such as macrophages and lymphocytes. This compound also possesses undesirable toxic effects such as fevering or the Shwartzmann bleeding reaction (Vogel et al., 1984; Galanos et al., 1985). Many investigations have been carried out to synthesize lipid A analogues with low toxicity. Some synthetic monosaccharide analogues of lipid A have low toxicity and retain various useful biological activities as lipid A (Ikeda et al., 1988; Kumazawa et al., 1988). These compounds are expected to be used as

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SSDI 0378-5173(94)00091-I

pharmaceutical drugs for immunomodulatory or anti-tumor therapies.

Injectable formulations of lipid A analogues have been developed in the pharmaceutical field. The major problem here is the limited solubility of lipid A analogues in aqueous solution. While lipid A analogues with short acyl chains or a succinyl group dissolve in aqueous solution, these hydrophilic analogues have diminished biological activities (Tanamoto et al., 1984; Kiso et al., 1987). Lipid A analogues have been dissolved in organic base or macromolecular like bovine serum albumin solutions (Ramsey et al., 1980; Matsuura et al., 1983; Kumazawa et al., 1984), however, these additives are toxic or immune-reactive in human body. Although the feasibility of liposomes as injectables of lipid A analogues has been claimed (Dijkstra et al., 1987, 1988, 1989; Daemen et al., 1989), no useful pharmacological effect of lipid A analogues has been detected when they are incorporated into liposomes. The decrease in pharmacological activities may be due to the reduced interaction between lipid A analogues in lipid bilayers and the plasma membranes of macrophage. These liposomes are prepared with high molar ratios of phospholipid to lipid A analogue of above 100 : 1. In this work, we prepared the liposomes with higher contents of lipid A analogue by using phospholipid and a synthetic monosaccharide analogue of lipid A,

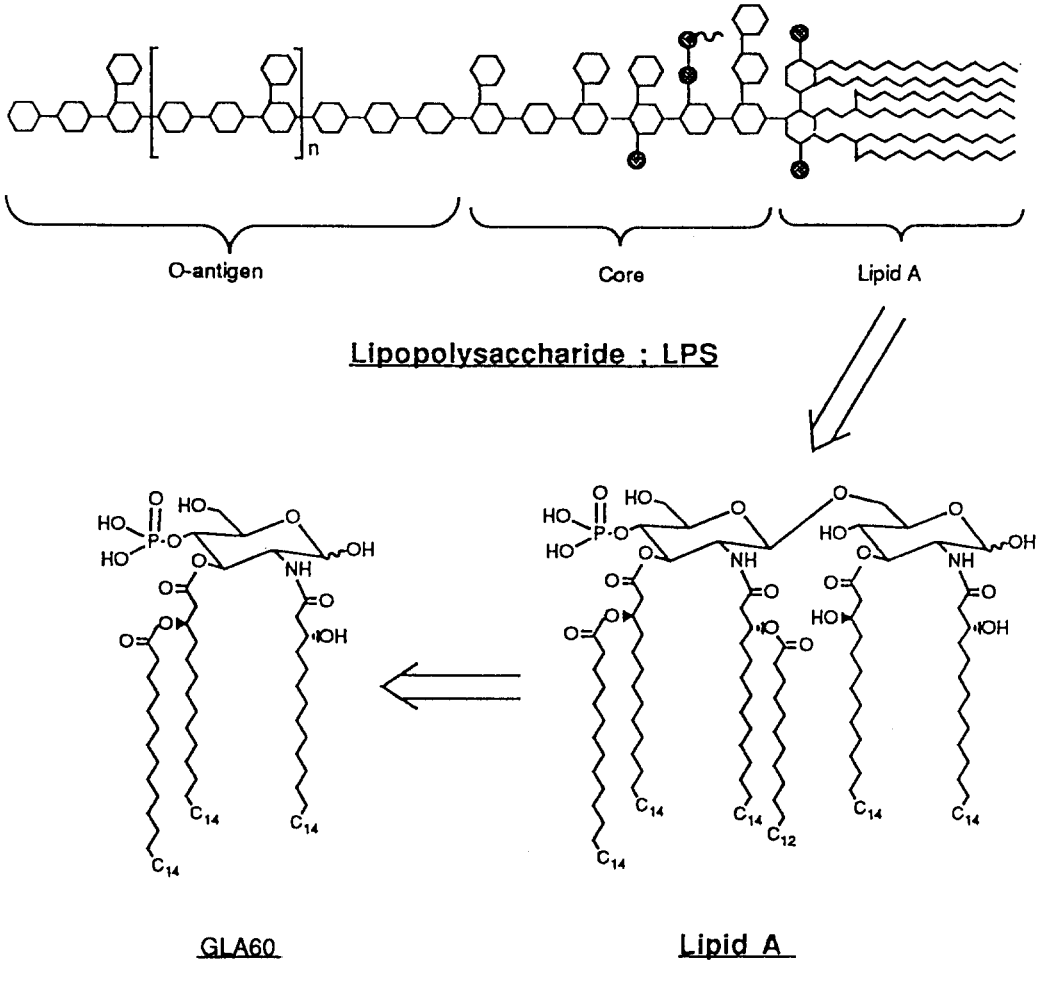

Fig. 1. Chemical structures of lipopolysaccharide(LPS), complete *E. coli* lipid A and synthetic GLA-60.

GLA-60. GLA-60 exhibits many immunopharmacological activities of lipid A with little toxicity (Kiso et al., 1987). The physicochemical properties and effect on in vivo induction of TNF in ICR mice of these lipid particles were evaluated.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (egg PC) was obtained from Asahi Kasei Kogyo Co., Ltd. (Tokyo, Japan). The monosaccharide lipid A analogue, GLA-60, was kindly supplied from the organic synthesis research laboratories at Japan Tobacco Inc. 5(6)-Carboxyfluorescein(CF) as an indicator for evaluation of the stability of liposome and 1,6-diphenyl-1,3,5-hexatriene(DPH) for investigation of the membrane fluidity of liposome were purchased from Sigma Co., Ltd (MO, U.S.A.). Sepharose CL-4B gel was obtained from Pharmacia Co., Ltd (Sweden).

2.2. Methods

2.2.1. Preparation of lipid particles containing GLA-60

Lipid particles with or without GLA-60 were prepared according to the method of Dijkstra et al. (1987). Egg PC and GLA-60 were mixed in a chloroform/methanol solvent(2:1 v/v). The solvents were evaporated under a stream of nitrogen gas and the residue was dried in vacuo for a day. The lipid film was hydrated with 150 mM NaCl aqueous solution. Then, the lipid suspension was passed through a 0.2 μm membrane filter(extrusion process) to prepare particles of uniform size. Smaller particles were prepared by sonication with a probe-type sonicator (Branson Co., Ltd; U.S.A.) at 50°C for 15 min and then centrifuged briefly to remove titanium dust. GLA-60 was insoluble and was co-dispersed with egg PC in aqueous solution.

2.2.2. Measurement of particle size

The size distribution of lipid particles was measured at 25°C by the dynamic light scattering technique using a laser particle analyzer(model LPA-3000/3100) purchased from Ohtsuka Denshi Kogyo Co., Ltd (Osaka, Japan).

2.2.3. Electron microscopic pictures of lipid particles

Lipid particles were rapidly frozen by using liquid nitrogen and then fractured in vacuo at 2×10^{-6} Torr. After etching treatment, the specimen was shadowed with platinum and coated with carbon. Replicas were obtained by dissolving lipids with the solvent of ethanol and chloroform. The replicas thus obtained were mounted on copper grids and examined using a JEOL (Tokyo, Japan) model JEM-1200EX transmission electron microscope (TEM). The average particle diameter was determined for 50 particles on the replica.

In the negative staining method, the dispersed lipid particles were diluted as much as 50-fold in 2% phosphotungstate solution and then applied to collodion-coated, carbon-stabilized copper grids for TEM. The micrographs were obtained using TEM. The average diameter of particle was evaluated in the same manner as for the freeze-fracture method.

2.2.4. Measurement of zeta-potential

Zeta-potentials of lipid particles were measured in 10 mM NaCl aqueous solution at 25°C by using a Ohtsuka Denshi (Osaka, Japan) model ELS-700 zeta-potential analyzer. These data were represented as mean values of duplicate measurements.

2.2.5. Leakage out of liposomes in rat plasma

Leakage of 5(6)-carboxyfluorescein(CF) out of lipid particles containing GLA-60 in rat plasma was evaluated according to the method of Senior et al. (1985). The liposomes containing 50 mM CF were eluted at the void fraction in the Sepharose CL-4B gel chromatograph, and were added to rat plasma. The stability of liposomes were evaluated by monitoring the leakage of CF fluorometrically during incubation with rat plasma at 25°C. The percent leakage of CF was calculated according to the following equation.

$$\text{leakage}(\%) = (F - F_0)/(F_\infty - F_0) \times 100$$

where F_0 represents the initial fluorescence intensity just after the addition of the liposomes to rat plasma, F is the fluorescence intensity monitored during the incubation at 25°C, and F_∞ denotes the maximum fluorescence intensity in rat plasma after completely breaking the liposomes by addition of 10 mM Triton X-100.

2.2.6. Membrane fluidity of liposomes

The membrane fluidity of lipid particles containing GLA-60 was evaluated based on the anisotropy of the fluorescent probe, DPH, dissolved in lipid bilayers as reported by Shinitzky (1984). In this experiment, DPH was added at 0.5 mol% of total lipids. The degree of polarization (P) was defined by the following equation;

$$P = (I_1 - I_2)/(I_1 + I_2)$$

where I_1 and I_2 are the fluorescence intensities parallel and perpendicular to the polarized exciting light, respectively. The value for I_2 was corrected for unequal detection of the light of two polarizations by the fluorometer.

2.2.7. In vivo TNF inducing experiments

The secretion of endogeneous tumor necrosis factor (TNF) requires at least two stimuli: the sensitization by *Mycobacterium bovis* strain *Bacillus* Calmette-Guerin(BCG) or *C. parvum* as a priming agent followed by the triggering of lipid A analogues. In this experiment, *C. parvum* was injected to female ICR mice (7 weeks) by the intravenous route at a dose of 1 mg/kg as the first step for induction of TNF. After 9 days, lipid particles with various molar ratios of egg PC to GLA-60 from 1:1 to 100:1 were injected via the same administration route. The dose of GLA-60 was 10 μg/body. Then, 90 min later, these mice were killed to collect their whole serum (TNS) containing TNF induced by GLA-60. The collected TNS was stored by freezing at −20°C. The diluted TNS (dilution ratio, $1/10$–$1/10^8$) was added to TNF-sensitive L929 cells, a transformed cell line originally derived from the C3H strain (6×10^4 cells per well). After incubation at 37°C for 48 h, the cytotoxic activity was estimated by measuring the amount of crystal violet taken up into living L929 cells. The TNF titer was calculated from the following equation:

TNF titer (U/ml)
= 1/dilution ratio of TNS for
50% cytotoxicity to L929 cells

3. Results and discussion

3.1. Size and structure of lipid particles prepared with egg PC and GLA-60

Fig. 2 and 3 show the size distributions of lipid particles of egg PC and GLA-60 mixtures evaluated by DLS measurements. Mean diameters were

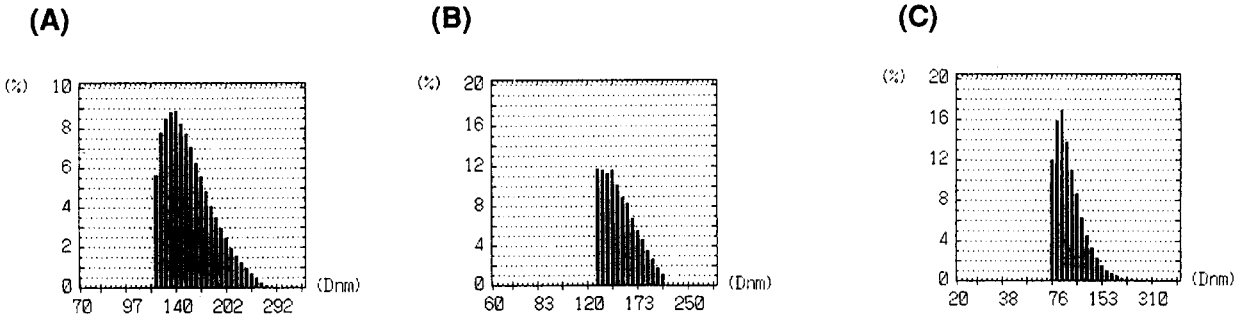

Fig. 2. Size distributions of extruded lipid particles as determined by the dynamic light scattering method: (A) egg PC; 157 ± 29 nm (mean ± S.D.); (B) egg PC:GLA-60 (4:1); 153 ± 19 nm (mean ± S.D.); (C) egg PC:GLA-60 (2:1); 100 ± 26 nm (mean ± S.D.).

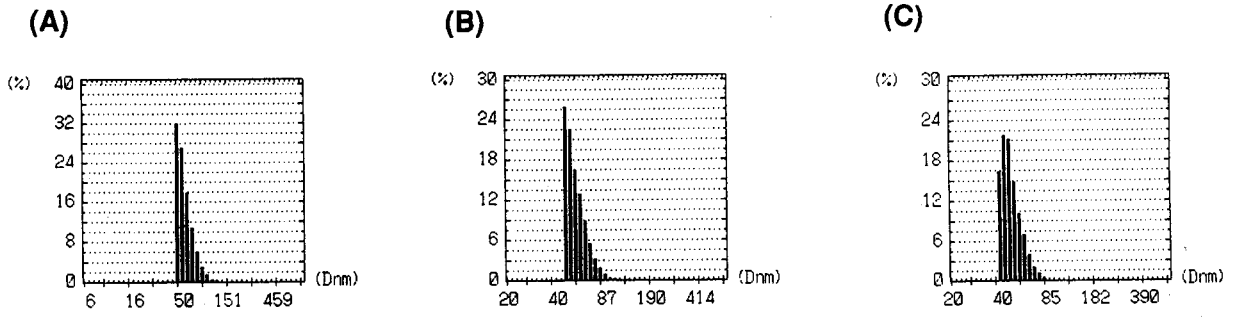

Fig. 3. Size distributions of sonicated lipid particles as determined by the dynamic light scattering method: (A) egg PC; 60.0 ± 13 nm (mean ± S.D.); (B) egg PC: GLA-60 (4:1); 60.6 ± 11 nm (mean ± S.D.); (C) egg PC: GLA-60 (2:1); 52.1 ± 9 nm (mean ± S.D.).

100–157 nm for extruded particles and 52–61 nm for sonicated particles, respectively.

The lipid particles were also investigated by TEM techniques using freeze-fracture and negative staining methods. Fig. 4 displays extruded particles observed by the freeze-fracture method. Particles were spherical and of similar size as estimated by DLS measurement. The average particle diameters were 158 nm for egg PC, 162 nm for egg PC: GLA-60 (10:1) and 135 nm for egg PC: GLA-60 (2:1) lipid particles, respectively. Fig. 5 and 6 show the TEM photographs obtained by the negative staining technique. The inside of almost all particles was stained by phosphotungustic acid, demonstrating that these lipid particles were of vesicular (liposomal) structure. Furthermore, we could achieve complete recovery of egg PC and GLA-60 in liposomal fractions

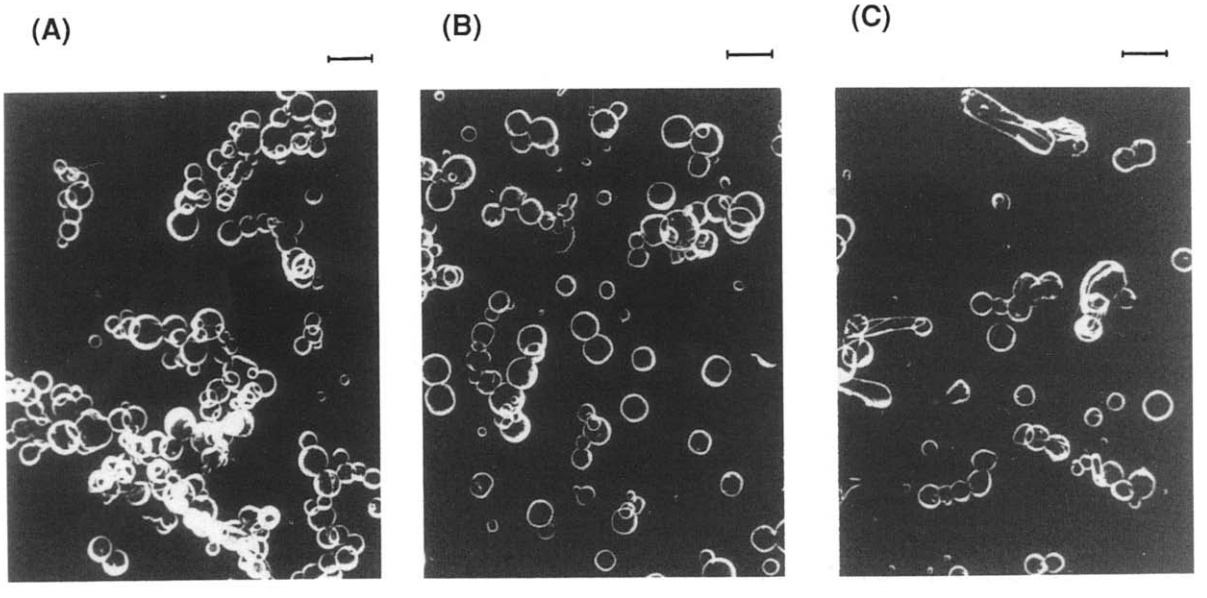

Fig. 4. TEM pictures of extruded lipid particles prepared by the freeze-fracture method (bar represents 200 nm in all photos): (A) egg PC; (B) egg PC: GLA-60 (10:1); (C) egg PC: GLA-60 (2:1).

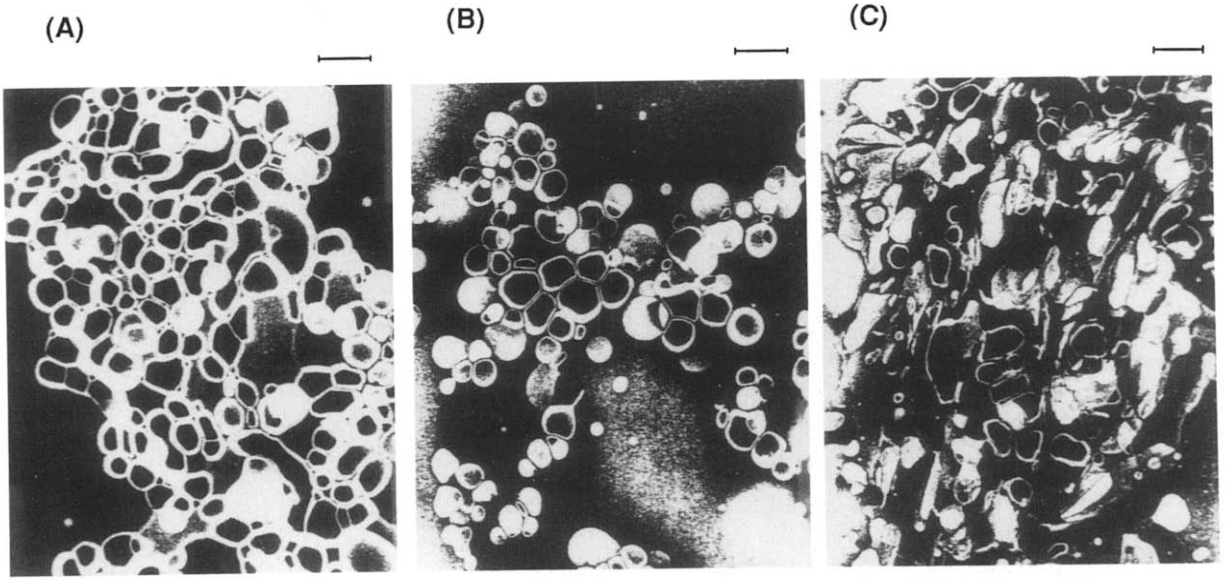

Fig. 5. TEM pictures of extruded lipid particles prepared by the negative staining method (bar represents 200 nm in all photos): (A) egg PC; (B) egg PC:GLA-60 (10:1); (C) egg PC:GLA-60 (2:1).

from gel column chromatography. These observations demonstrated the complete association of GLA-60 with egg PC liposomes. When the content of GLA-60 was more than twice that of the egg PC molecule, there were several broad peaks of particle size in the DLS measurement. The

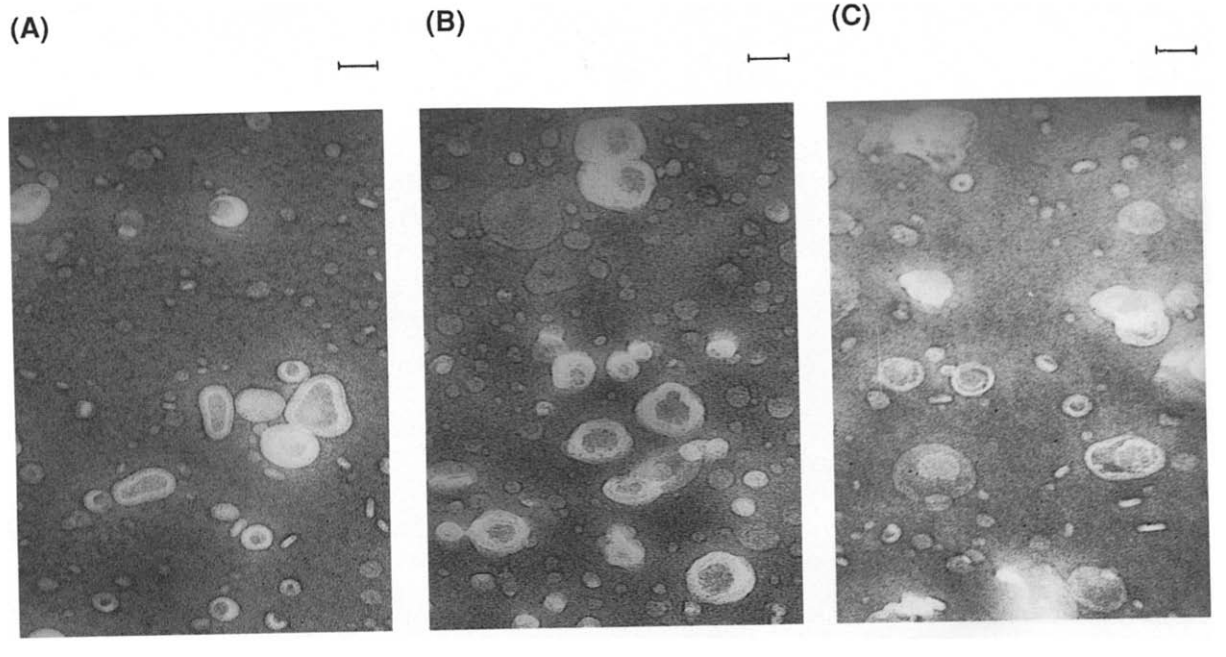

Fig. 6. TEM pictures of sonicated lipid particles prepared by the negative staining method (bar represents 50 nm in all photos): (A) egg PC; (B) egg PC:GLA-60 (4:1); (C) egg PC:GLA-60 (2:1).

50% content of GLA-60 may be the limit for the preparation of homogeneous liposome dispersions.

3.2. Surface charge, stability in rat plasma and membrane fluidity of lipid particles containing GLA-60

GLA-60 is negatively charged in neutral aqueous solution. Fig. 7 represents the zeta-potential of liposomes as a function of the GLA-60/egg PC mole ratios. The negative value of the zeta-potential increased with content of GLA-60. The phosphate group at the head sugar moiety of GLA-60 was considered to be at the surface of the lipid membrane and confered the liposomes with negative charge.

The stability of liposomes containing GLA-60 in rat plasma was evaluated on the basis of the leakage profile of 5(6)-carboxyfluorescein(CF) from the vesicles. Fig. 8 shows that the leakage rate of CF decreases increasing content of GLA-60. The lipid membrane with an egg PC/GLA-60 molar ratio of less than 2:1 had lower permeability to CF. On the other hand, liposomes of higher egg PC/GLA-60 mole ratios showed the burst-release profile of the control liposome. The egg PC membrane is in the liquid crystalline state at 25°C

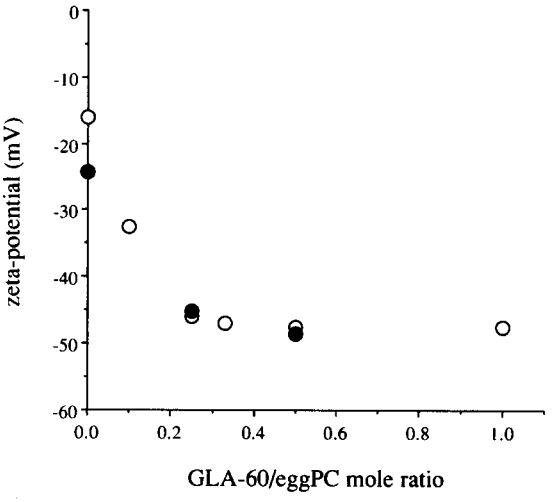

Fig. 7. Zeta-potential of liposomes as a function of the GLA-60/egg PC mole ratio (Values were estimated by duplicate measurements): (○) extruded liposomes; (●) sonicated liposomes.

(Chapman et al., 1973). GLA-60 has three saturated acyl groups and is possibly in the gel state at 25°C. The membrane fluidity or rigidity of extruded liposomes and sonicated small liposomes was evaluated by anisotropy measurement of the fluorescent probe, DPH, entrapped in the lipid bilayer. Fig. 9 shows the fluorescent

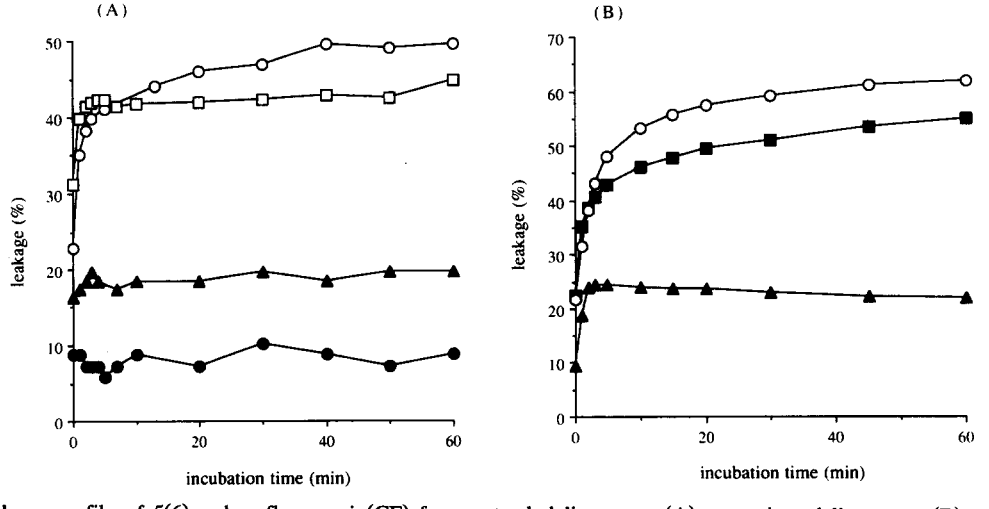

Fig. 8. Leakage profile of 5(6)-carboxyfluorescein(CF) from extruded liposomes (A) or sonicated liposomes (B) prepared with various different mole ratios of egg PC/GLA-60 in rat plasma: (■); egg PC control; (○) egg PC:GLA-60 = 4:1; (□) egg PC:GLA-60 = 3:1; (▲) egg PC:GLA-60 = 2:1; (●) egg PC:GLA-60 = 1:1.

anisotropy of DPH dissolved in the hydrophobic region of vesicles as a function of GLA-60/egg PC mole ratio at 25 or 37°C. The degree of polarization(P) increases with the content of GLA-60 in the egg PC bilayer, indicating that the membrane fluidity is lower in highly GLA-60 loading liposomes. The rigidity of lipid membrane may increase with the content of GLA-60 in the egg PC bilayer and lower the permeability to CF.

3.3. TNF-inducing activities of lipid particles containing GLA-60

It has been reported that lipid A analogues induce high levels of TNF in animal plasma through the activation of monocytes. These analogues are expected to activate the immune system and to act selectively against several tumorigenic or transformed cells cytotoxically and cytostatically, and finally to prevent tumor metastasis (Ruff et al., 1981; Old, 1985). The TNF-inducing activities were estimated after intravenous administration of liposomes entrapping GLA-60 to female ICR mice. We used sonicated small liposomes in this experiment, since these vesicles were considered to be convenient for sterilization

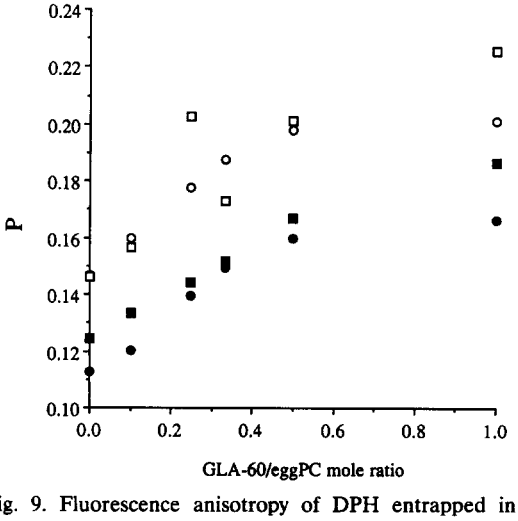

Fig. 9. Fluorescence anisotropy of DPH entrapped in extruded liposomes or sonicated liposomes prepared with various different mole ratios of egg PC/GLA-60: (○) extruded liposomes (25°C); (●) extruded liposomes (37°C); (□) sonicated liposomes (25°C); (■) sonicated liposomes (37°C).

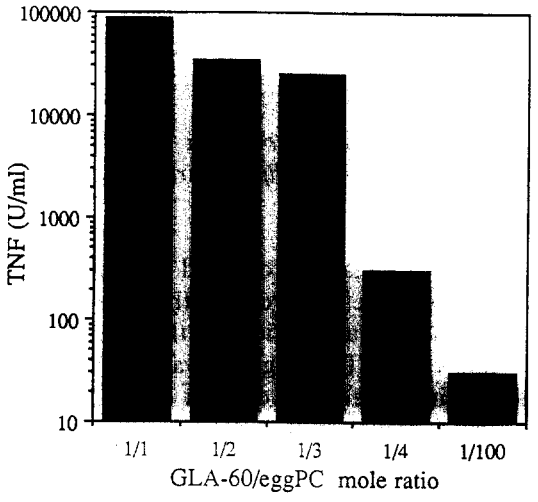

Fig. 10. In vivo TNF-inducing activities of small liposomes entrapping GLA-60.

by filtration. These liposomes are also considered to be advantageous in manufacturing using a high-pressure homogenizer. Fig. 10 shows the TNF-inducing activities of the small liposomes as a function of the mole ratios of egg PC/GLA-60. Vesicle with an egg PC/GLA-60 ratio of 100:1 gave a very low induction-activity of TNF as the control liposome. The vesicles with more than 25 mol% of GLA-60, however, showed appreciable induction activities of TNF. Many published works on the liposomes with lipid A analogues have concluded that the biological activity of lipid A analogues diminishes significantly if the compounds are incorporated into the lipid membrane due to preventing the interaction of the hydrophobic portion of lipid A with the plasma membranes of macrophages (Dijkstra et al., 1987, 1988, 1989; Daemen et al., 1989). In these works, the phospholipid/lipid A analogues mole ratios were greater than 100:1. Similarly, when the egg PC/GLA-60 mole ratio was above 4:1, we could not observe the TNF induced in plasma after intravenous injection. However, we did observe increased TNF-inducing activities with increasing composition of GLA-60 in the lipid bilayer (Fig. 10). These data indicated that GLA-60 was efficiently entrapped by macrophages, when GLA-60 was relatively rich in the liposomes. As liposomes are likely to be taken up by the reticuloendothe-

lial systems (RES) when injected into blood, it is expected that GLA-60 in liposomes will be entrapped efficiently by the liver and spleen macrophages as compared with the free form of GLA-60. The membrane fluidity of vesicles affects the efficiency of phagocytosis or endocytosis of the vesicles (Hafeman et al., 1980; Lewis et al., 1980; Munn et al. 1982). Vesicles loading the higher composition of GLA-60 are more rigid and give rise to greater biological activities of macrophages. Negatively charged liposomes are known to be taken up readily by macrophages. However, in our experiments, any pharmacological activities could not be detected when the negatively charged GLA-60 liposomes modified with phosphatidylserine in place of phophatidylcholine were injected to ICR mice (data not shown). Other factors such as phase separation of egg PC and GLA-60 in liposomes may be related to TNF induction. Phase separation is well known to induce enhanced leakage of liposomes. Incorporation of GLA-60, however, suppressed the permeability of CF (Fig. 8). Slight aggregation of GLA-60 is possible in liposomes and leads to increased receptor-mediated uptake of liposomes by macrophages. Further work on aggregation by using the interfacial monolayer technique will appear in the near future.

In conclusion, the liposomes of GLA-60 and egg PC were useful as injectable formulations of lipid A analogues and represented high TNF inducing activities.

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