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HEPC-based liposomes trigger cytokine release from peripheral blood cells: effects of liposomal size, dose and lipid composition

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Abstract

The immune response caused by liposome stimulation was studied by assessing the level of several cytokines released from human peripheral blood cells. Liposome stimulation resulted in the release of IL-6, IL-10, IL-1β, TNF-α and IFN-γ. The size of the liposomes affected the degree of the cytokine releases with larger sized liposomes causing higher levels of cytokine induction. In addition, it appears that the lipid composition of liposomes had no effect on the degree of cytokine release. The release of cytokines occurred even in the absence of serum, suggesting that serum proteins did not contribute to liposome stimulation in peripheral blood cells. The release of cytokines induced by liposome stimulation was inhibited by the presence of either protein kinase-C (PKC) or protein tyrosine kinase (PTK) inhibitor, but not by the presence of an endocytosis inhibitor. This indicates that signal transduction via PKC or PTK is necessary, in order for human peripheral blood cells to release cytokines (IL-6, IL-10, IL-1β, TNF-α and IFN-γ) as the result of liposome stimulation. These quantitative data on the release of cytokines by liposomal stimulation provide useful information for the development of rational drug delivery systems and the safety of cytokine induction via the use of liposomes.

Keywords: Liposome; Cytokine; Human peripheral blood cell; Whole-blood induction; Immune system

Abbreviations: BSA, bovine serum albumin; CHOL, cholesterol; DCP, dicetylphosphate; ELISA, enzyme-linked immunosorbent assay; EPC, egg phosphatidylcholine; HEPC, hydrogenated egg phosphatidylcholine; HRP, horseradish peroxidase; LPS, lipopolysaccharide; mAb, monoclonal antibody; Man, cetylmannoside; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PKC, protein kinase-C; PPE, palmar-plantar erythodysesthesia; PTK, protein tyrosine kinase; Tween-PBS, PBS containing 0.05% Tween-20.

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1. Introduction

Studies of liposomes have shown that they have considerable potential for use as carriers for the in vivo delivery of drugs. Liposomal or lipid-based formulations have been approved for clinical use in the USA, Europe and a number of other countries around the world (Seaman et al., 1995; Uziely et al., 1995; Muggia, 1997). Although extensive information are available on the pharmacokinetics and therapeutic index of liposomal drugs, a complete understanding of the side effects caused by liposome has not yet been achieved. In fact, it has been reported that sterically stabilized liposomes which contain encapsulated doxorubicin, termed Doxil/Caelyx, lead to the development of palmar-plantar erythodysesthesia (PPE), which occurs in approximately 20% of patients who receive doses of Doxil/Caelyx that exceed 10–12 mg/kg per week (Gordon et al., 2000; Lyass et al., 2000). The precise underlying mechanism for this action is not available. In an earlier study, we demonstrated that intravenously administrated liposomes activated the complement system, which is one of first line humoral host defense systems (Funato et al., 1992). It is a well-known fact that small peptides, C3a and C5a, which are released as a consequence of complement activation, serve as highly potent mediators of inflammation, causing chemotactic migration, cell adhesion, the release of hydrolytic enzymes, and the formation of arachidonic acid metabolites and active oxygen species (Van den Berg et al., 1998). From these reports, it can be concluded that the administered liposomes stimulate the immune system, resulting in inflammation-like side effects.

Cytokines are released from activated immune cells and have characteristic multifunctions, which are involved in the regulation of immune response, hematopoiesis and inflammation. Based on the results described above, the administered liposomes are predicted to activate the immune system and then induce the release of cytokines. The released cytokines may contribute to the inflammation-like side effects, which are caused by the administration of liposomes. However, data on determining if the release of cytokines is induced by liposome stimulation is very limited. On the other hand, if the induction of cytokines could be regulated, it might be possible to use liposomes as a safe and potent adjuvant to the immune system of a host. Our objective in this study was to quantitatively determine the amount of released cytokines from human peripheral blood cells following incubation with liposomes. The ex vivo human whole blood cytokine induction method was used to examine the effect of liposomes on the human immune system. This ex vivo method has proved to be a useful system for the detection of cytokine induction in stimulated peripheral blood without the need for purification of the lymphocytes (Barnes et al., 1993; Van Crevel et al., 1999).

2. Materials and methods

2.1. Materials

Hydrogenated egg phosphatidylcholine (HEPC), egg phosphatidylcholine (EPC) and cetylmannoside (Man) were kindly donated by the Nippon Fine Chem. Co. (Osaka, Japan). Cholesterol (CHOL) and dicetylphosphate (DCP) were purchased from Wako Pure Chem. (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. All other chemicals used were of analytical grade.

2.2. Antibodies

The following monoclonal antibodies (mAbs) were used in the enzyme-linked immunosorbent assay (ELISA) study. Purified mouse anti human IL-4 mAb, purified rat anti-mouse/human IL-5 mAb and purified rat anti-human IL-10 mAb were obtained from PharMingen Co. (CA, USA). Anti human IL-6 mAb was obtained from R&D Systems, Inc. (MN, USA). Anti human IFN-γ mAb, anti human TNF-α mAb, anti human IL-1β mAb and IL-2 were kindly supplied by Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). Biotinylated rat anti human IL-4 mAb, biotinylated rat anti human IL-5 mAb and biotinylated rat anti human IL-10 mAb were obtained from

PharMingen Co. (CA, USA). Biotinylated anti human IL-6 polyclonal antibody (poly-Ab) was obtained from R&D Systems, Inc. (MN, USA). Anti human IFN-γ poly-Ab, anti human IL-1β poly-Ab, anti human IL-2 poly-Ab and horseradish peroxidase (HRP)-conjugated anti human TNF-α mAb were kindly supplied by Otsuka Pharmaceutical Co., Ltd. HRP-conjugated goat anti rabbit IgG was obtained from Large Scale Biology Co. (CA, USA). HRP- conjugated streptavidin was obtained from Vector Co. (Burlingame, CA)

2.3. Preparation of liposomes

Liposomes were prepared under sterile conditions using previously described methods (Funato et al., 1992). A chloroform solution of HEPC, CHOL, DCP in a molar ratio of 5:4:1 (HEPC–MLV), EPC, CHOL, and DCP in a molar ratio of 5:4:1 (EPC–MLV) and Man, HEPC, CHOL, and DCP in a molar ratio of 3:2:4:1 (Man–MLV) were evaporated to dryness in a round-bottomed flask on a rotary evaporator. The remaining solvent was completely removed by reducing the pressure with a vacuum pump. The dried film in the flask was hydrated with phosphate buffered saline (PBS) by shaking with a mechanical shaker. The liposomes were extruded through a polycarbonate membrane (Nuclepore Co., CA, USA) with a pore size of 800 or 200 nm. Small unilamellar vesicles (SUV) were prepared by using an ultrasonication technique with a probe-type sonicator (UR-200P, Tomy, Tokyo, Japan) until the lipid suspension became transparent for about 2 h on ice. The total lipid concentration of liposomes in the preparation was 20 μmol/ml. The liposomal diameter was determined by NICOMP 370 (Particle Sizing Systems, CA, USA).

2.4. Whole blood-induction method

Blood samples were obtained from human healthy volunteers. One hundred μl aliquots of heparinized blood were cultured in 24-well plates containing 850 μl RPMI-1640 medium and 50 μl liposomes (20 μmol/ml) or 50 μl lipopolysaccharide (LPS) (20 μg/ml) at 37 °C for 24 h. After incubation, the cultured samples were centrifuged at 1000 rpm for 5 min to remove the cells. Cytokine levels in the culture supernatant were determined by ELISA as described below.

2.5. Peripheral blood mononuclear cell (PBMC)-induction method

Heparinized venous blood (20 ml), obtained from healthy donors, was diluted with RPMI 1640 containing 10 U/ml of heparin (20 ml). The mononuclear cells were then separated by Ficoll–Hypaque^{R} PLUS (Amersham Pharmacia Biotech AB, Uppsala, Sweden) density gradient centrifugation. The cells at the interface were collected and washed twice in RPMI 1640 containing 10 U/ml of heparin. The washed cells were placed into 24-well plates at a concentration of 1×10^6 per ml in RPMI 1640 supplemented with 10% fetal bovine serum, and incubated with 50 μl of the liposome preparation (20 μmol/ml) or 50 μl of LPS (20 μg/ml) at 37 °C for 24 h. After incubation, the cultured samples were centrifuged at 1000 rpm for 5 min to remove the cells. Cytokine levels in culture supernatant were determined by ELISA as described below.

2.6. Determination of released cytokines

Plates were coated with anti-IL-1β mAb (6.7 μg/ml), anti-IL-2 mAb (13.3 μg/ml) or anti-IFN-γ mAb (5 μg/ml) in 0.1 M $NaHCO_3$, pH 8.2, or anti-TNF-α mAb (6.7 μg/ml), anti-IL-4 mAb (2 μg/ml), anti-IL-5 mAb (2 μg/ml), anti-IL-6 mAb (0.5 μg/ml) or anti-IL-10 mAb (2 μg/ml) in PBS, followed by an overnight incubation. The wells were immediately washed with PBS containing 0.05% Tween-20 (Tween-PBS) and blocked with 0.1% bovine serum albumin (BSA) in PBS for 2 h. Samples or standard cytokines in 0.1% BSA in PBS were added to the wells, followed by an overnight incubation. The plates were then washed three times with Tween-PBS. For determining TNF-α, 100 μl of HRP-conjugated anti human TNF-α mAb was added. For determining IL-1β, IL-2 or IFN-γ, 100 μl of anti human IL-1b poly-Ab, anti human IL-2 poly-Ab or anti human IFN-γ poly-Ab was added to each well, followed

by a 2 h incubation. The plates were washed with Tween-PBS, supplemented with HRP-conjugated goat anti rabbit IgG and incubated for 2 h. For determining IL-4, IL-5, IL-6 or IL-10, 100 μl of biotinylated rat anti human IL-4 mAb, IL-5 mAb, IL-6 poly-Ab or IL-10 mAb was added to each well, followed by a 2 h incubation. The plates were washed with Tween-PBS, supplemented with HRP-conjugated streptavidin and incubated for 2 h. For determining TNF-α, IL-1β, IL-2 or IFN-γ, 100 μl of enzyme substrate (1 mg/ml *o*-Phenylendiamine (Sigma, MO, USA) in 0.1 M sodium citrate buffer, pH 5.0) was added to each well, followed by incubation. The reaction was stopped by adding 100 μl of 2 N H_2SO_4, and adsorption at 492 nm was read in microplate reader. TMB kit (SCYTEK lab., UT, USA) was used for determining IL-4, IL-5, IL-6 or IL-10, 100 μl of TMB reagent was added to each well and incubated. The reaction was stopped by the TMB stop solution, and the absorption at 450 nm was read in microplate reader. All procedures were performed at room temperature. Sensitivity and range of TNF-α assay were 6.8 and 6.8–5000 pg/ml, respectively. Sensitivity and range of IL-1β, IL-2, IFN-γ, IL-4, IL-5, IL-6 or IL-10 assay were 13.7 and 13.7–10 000 pg/ml, respectively.

3. Results

3.1. Cytokine induction by liposome stimulation

The effect of liposome stimulation on the human immune system was examined by evaluating the cytokine release following the incubation of human peripheral blood with liposomes (HEPC–MLV, 800 nm in a diameter) for 24 h. As shown in Fig. 1, the release of IL-6, IL-10, IL-1β, TNF-α and IFN-γ were clearly detected, while those of IL-2, IL-4 and IL-5 were not. Indeed, the amount of released cytokine was comparable to that released by stimulation of LPS, a potent activator of cells of the monocyte/macrophage cell lineage. The effect of liposomal dose on cytokine release (IL-6, TNF-α or IL-1β) was also studied. The release of each cytokine increased in a dose-dependent manner, reaching a maximum level (Fig. 2). It appeared that the liposomes (HEPC–MLV), which do not contain any potent immune stimulator such as lipid A, are able to induce the release of cytokines (IL-6, TNF-α and IL-1β) from peripheral human blood cells.

3.2. Effect of liposomal physicochemical properties on the release of cytokines

The effect of liposome composition on cytokine release was examined. Data for EPC–MLV and Man–MLV are shown as a percentage of release of cytokine (IL-6, TNF-α and IL-1β) with the concentration of cytokine release induced by HEPC–MLV as 100% (Fig. 3). Significant differences in the cytokine release were not observed. We examined the effect of the lipid component of the liposomes on the release of cytokines. Human peripheral blood was incubated in a well of a

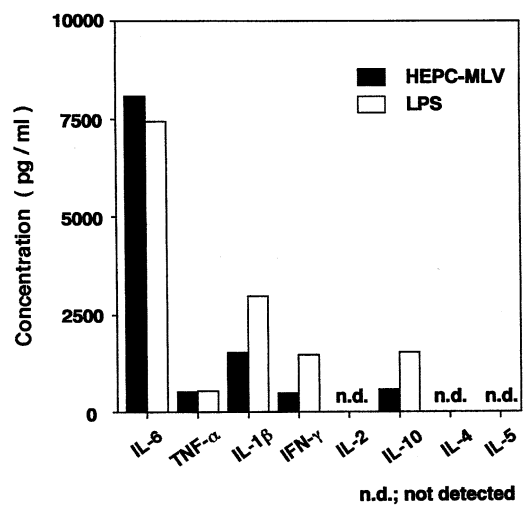

Fig. 1. Cytokine induction by liposome stimulation. One hundred μl of human peripheral blood, 850 μl of RPMI-1640 medium and 50 μl of liposome suspension (20 μmol/ml) or LPS (20 μg/ml) were incubated in 24-well plates at 37 °C for 24 h. The liposomes used in this assay were HEPC–MLV (HEPC:CHOL:DCP = 5:4:1 at a molar ratio, Mean diameter, 800 nm). After incubation, the cytokine concentrations in the culture supernatants were determined according to methods described in Section 2. Closed column represents the cytokine concentrations induced by liposome stimulation. Open column represents the cytokine concentrations induced by LPS. Results are from a representative experiment, and are means of triplicate assays.

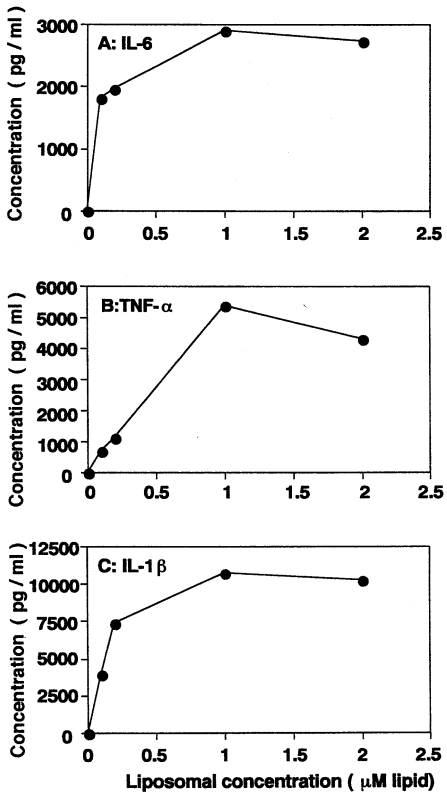

Fig. 2. Effect of the liposomal dose on cytokine induction. Fifty μl of liposomes (HEPC–MLV, Mean diameter 800 nm) having different concentrations were incubated in 24-well plates with 100 μl of human peripheral blood and 850 μl of RPMI-1640 medium at 37 °C for 24 h. After incubation, the culture supernatants were obtained and each cytokine concentration was determined according to methods described in Section 2. Results are from a representative experiment, and are means of triplicate assays.

24-well plate which had been coated with either HEPC, DCP or CHOL (1 μmol per well). The plates coated with each lipid were prepared by adding a methanol solution of each lipid and subsequent drying at room temperature overnight. No remarkable cytokine release was observed compared with HEPC–MLV stimulation (Table 1). The effect of liposome size on cytokine release was also examined. The liposomes induced the release of IL-6, TNF-α and IL-1β in a size dependent manner, up to 800 nm in a diameter (Fig. 4). Interestingly, no detectable levels of cytokine release by SUV (SUV: 50 nm)-stimulation were found. In addition, we examined cytokine release (IL-6, TNF-α or IL-1β) from the isolated PBMC in the presence or absence of human serum. After an incubation with HEPC–MLV for 24 h, no significant difference was observed in the presence or absence of human serum (data not shown).

3.3. The mechanism of the cytokine release by liposome stimulation

In order to study the mechanism of cytokine release by liposome stimulation observed in this study, the effect of treatment with various inhibitors for endocytosis, protein kinase-C (PKC), the movement of membrane vesicles and protein tyrosine kinase (PTK) on cytokine release (IL-6, TNF-α or IL-1β) was examined (Fig. 5). Treat-

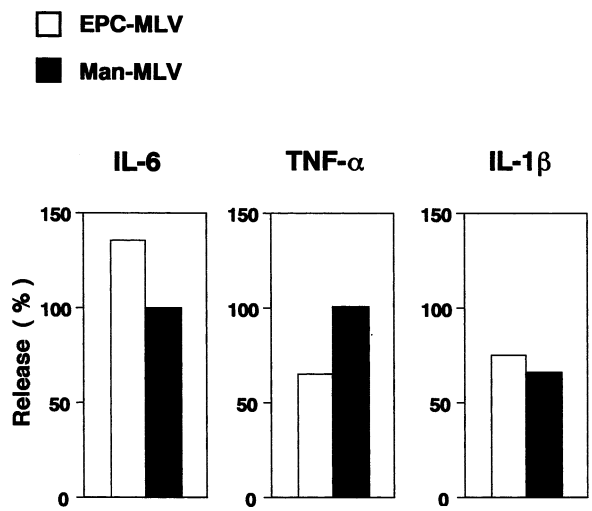

Fig. 3. Effect of liposomal lipid composition on cytokine induction. Fifty μl of HEPC–MLV (HEPC:CHOL:DCP = 5:4:1, mol/mol), EPC–MLV (EPC:CHOL:DCP = 5:4:1, mol/mol) or Man–MLV (Man:HEPC:CHOL:DCP = 3:2:4:1, mol/mol) suspension was incubated in 24-well plates with 100 μl of human peripheral blood and 850 μl of RPMI-1640 medium at 37 °C for 24 h. After incubation, the culture supernatants were obtained and cytokine concentrations were determined according to methods described in Section 2. The sizes of liposomes used in this assay were 800 nm in a diameter. The percentage of induced cytokine by either EPC–MLV or Man–MLV stimulation was calculated by dividing the amount of induced cytokine by either EPC–MLV or Man–MLV by the amount of cytokine induced by HEPC–MLV. Results are from a representative experiment, and are means of triplicate assays.

Table 1
Cytokine induction by either the lipid composed of liposomes or liposome stimulation.

	Lipids			Liposomes
	HEPC	DCP	CHOL	HEPC-MLV
IL-6 (pg/ml)	n.d.	n.d.	n.d.	264.7
TNF-α (pg/ml)	n.d.	5.7	6.8	454.5
IL-1β (pg/ml)	n.d.	2.7	14.0	271.1

n.d.; not detected
24-well plates were coated with each lipid (HEPC, DCP or CHOL (1 μmol/well)). Hundred μl of human peripheral blood and 850 μl of RPMI-1640 medium were cultured in that plates at 37°C for 24 hours. On the other hand, 50 μl of liposome suspensions were incubated in 24-well plates with 100 μl of human peripheral blood and 850 μl of RPMI-1640 medium at 37°C for 24 hours. After incubation, cytokine concentrations in culture supernatants were determined according to the method described in Materials and Methods. Results are from a representative experiment, and are means of triplicate analyses.

ment with Cytochalasin B (an inhibitor for endocytosis) had no effect on the release of cytokines up to 40 μM. Brefeldin A (an inhibitor of the movement of membrane vesicles between the rough endoplasmic reticulum and Golgi) slightly inhibited the releases of TNF-α and IL-1β, but when IL-6, up to 90 μM. H-7 (a PKC inhibitor) and Herbimycin A (a PTK inhibitor) were examined, neither completely inhibited the release of cytokines. These results demonstrate that both PKC and PTK, but not the endocytosis pathway, contribute to the release of IL-6, TNF-α or IL-1β, and the movement of membrane vesicles between the rough endoplasmic reticulum and Golgi involves, in part, the release of TNF-α and IL-1β.

4. Discussion

The release of cytokines (IL-2, IL-4, IL-6, IL-10, IL-1β, TNF-α and IFN-γ) from human peripheral blood cells following incubation with liposomes was determined. Interestingly, following the incubation, monocyte-derived cytokines (IL-6, IL-10, IL-1β, IFN-γ and TNF-α) were detected, while lymphocyte-derived cytokines (IL-2, IL-4 and IL-5) were not (Fig. 1). It appears that the liposomes used in this experiment have the ability to stimulate monocytes in human peripheral blood, but not in lymphocytes. The level of each cytokine induced by the liposomes was comparable to that induced by LPS (1 μg/ml) (Fig. 1). It is well-known that LPS has the ability to stimulate human monocytes and macrophages, resulting in inducing the release of IL-1β and TNF-α, which represent major mediators in the pathogenesis of septic shock and bacterially mediated local

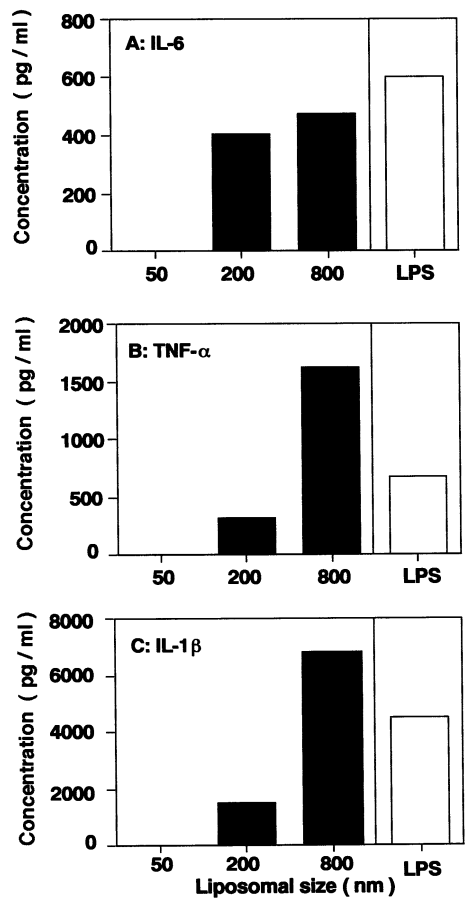

Fig. 4. Effect of liposome size on cytokine induction. Fifty μl of liposome suspensions (20 μmol/ml) having different sizes (50, 200, 800 nm) was mixed with 100 μl of human peripheral blood and 850 μl of RPMI-1640 medium. The mixture was incubated in 24-well plates at 37 °C for 24 h. After incubation, cytokine concentrations in culture supernatants were determined according to methods described in Section 2. LPS (20 μg/ml) were used as positive control. Results are from a representative experiment, and are means of triplicate assays.

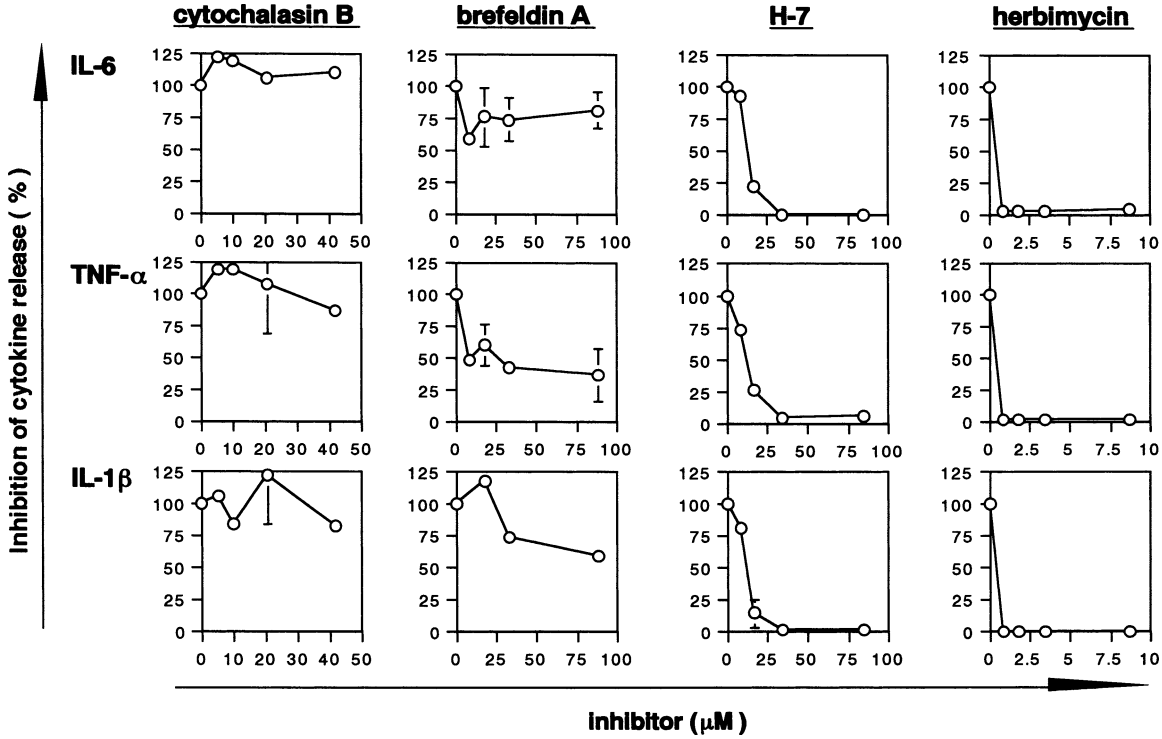

Fig. 5. Percent inhibition of liposome-stimulated cytokine induction by PBMC. PBMC (1×10^6) was preincubated in the presence or absence of various inhibitors for 1 h at 37 °C. Following preincubation, 50 μl of a HEPC–MLV suspension (20 μmol/ml, 800 nm in a diameter) was added to the PBMC and the incubations continued in the presence of inhibitors at 37 °C for 24 h. After incubation, the culture supernatants were obtained and cytokine levels were determined according to methods described in Section 2. Results represent mean ± S.D. from independent three experiments.

tissue destruction (Grau, 1990; Finch-Arietta and Cochran, 1991; Wiese et al., 1999). Therefore, we assume that liposomes, when administered into the human body, are able to stimulate the immune system, resulting in an inflammatory response.

It has been reported that LPS-protein complexes (endotoxins) extracted from the outer membrane of gram-negative bacteria are potent adjuvants (Gupta et al., 1993; Johnson, 1994; Gupta and Siber, 1995). Both humoral and cell-mediated immunity were found too elevated considerably by LPS stimulation. However, the profound toxicity of these endotoxins precluded their use in any human vaccines. Attempts have been made to detoxify LPS without affecting its adjuvanticity (Gupta et al., 1992; Naidu and Chander, 1999). The incorporation of LPS or lipid A into liposomes reduced their toxicity whereas the adjuvant effect was retained or even increased (Alving, 1993). In the present study, we were able to show that liposomes without any added LPS or lipid A induced the release of cytokines, the same as did LPS (Fig. 1). Liposomes, except for cationic liposomes such as lipofectin^R, could be relatively non-toxic and less pyrogenic than the LPS type, since they are composed of phospholipid and CHOL, which is found in virtually all mammalian cell membranes. The unique properties of liposomes in terms of lipid composition and diameters can easily be used to regulate the levels and type of cytokines following stimulation. Therefore, they represent good candidates for immunological adjuvants for use in human vaccines, although the precise bioactivity of the released cytokine observed in this study needs to be experimentally tested.

The possibility still exists that the immunostimulatory properties of the liposomes observed in this study might be due to contaminating LPS during liposome preparation. As shown in Fig. 4, cytokines were undetectable following stimulation by the SUV (50 nm), whereas the MLV (800 nm) which is composed of the same lipid composition as SUV significantly induced several cytokines. In addition, the LPS or the LPS–SUV mixture treated by a sonication procedure retained a remarkable activity relative to inducing cytokine release from human peripheral blood cells (data not shown), suggesting that the less-induction in the SUV did not result from the inactivation of the contaminated LPS during the sonication procedure used in preparing the SUV. Considering this collective data, we conclude that the release of cytokines observed in this study are not due to contaminants in the LPS.

It appears that the PKC and PTK are responsible for the release of IL-6, TNF-α or IL-1β, as the result of liposome stimulation (Fig. 5). In addition, no significant differences were found for cytokine release in the presence or absence of human serum (data not shown). These results lead us to hypothesize that direct interactions between liposomal membranes and the peripheral blood cells, presumably monocytes, is necessary in order to induce the release of cytokines which were observed in this study. It has been reported that LPS binds to several different molecules on the surface of phagocytes (Geng et al., 1993; Shapira et al., 1994; Detmers et al., 1996; El-Samalouti et al., 1999), resulting in an increase in the secretion of cytokines via its effect on the rate of transcription and mRNA stability (Geng et al., 1993). It has recently been demonstrated that LPS increases protein tyrosine phosphorylation and PTK activity in macrophages (Shames et al., 1999). Therefore, the direct interaction of liposomes with cells could involve signal transduction and the secretion of cytokines as occurs with LPS. Another recent study demonstrated that internalization of the LPS bound on the cells requires the induction of TNF-α in primary rat Kupffer cells (Lichtman et al., 1998). However, our current result indicates that the endocytosis of the liposomes does not require the induction of cytokine release (Fig. 5). Collectively, the signaling transduction pathways, which lead to the release of cytokines by liposome stimulation, may be different from the pathway in which LPS participates. The intracellular signaling pathway by which liposomes activate monocytes and macrophages to induce cytokine release are now being characterized in our laboratory.

The findings reported here show that liposomes stimulate human peripheral blood cells, resulting in the induction of cytokines. The induction of cytokines increased with increasing size of the liposomes: i.e. larger liposomes led to higher cytokine induction. The surface area interacted with the cells is large in large liposomes due to their low curvature compared with small liposomes. The larger liposomes may be able to bind to several different molecules on the cells that involve signal transduction and the secretion of cytokines, although the precise mechanism is not clear. All approved liposomal drugs in current use have, on average, diameters of 50–100 nm. These liposomal drugs may not perturb the human immune system when they are administered. With respect to large sized liposomes with diameters in the range of 800 nm, care should be exercised when these are used as a drug carrier, since such liposomes may overstimulate the immune system. Nevertheless, it might be possible to regulate cytokine release by changing their physical or chemical properties, such as their size and the lipid composition. This suggests that liposomes could be available for use as a safe adjuvant of the human immune system. Further experiments to test this hypothesis are now in progress in our laboratory.

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