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Inhibitory Effects of Negatively Charged Liposomes on Nitric Oxide Production from Macrophages Stimulated by LPS

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The effects of liposomes on nitric oxide (NO) productions induced by lipopolysaccharide were investigated using thioglycollate-induced mouse peritoneal macrophages. Negatively charged liposomes composed of phosphatidic acid (PA-liposomes) and phosphatidylserine (PS-liposomes) showed inhibitory effect on NO production in a dose dependent manner, but not in liposomes composed of phosphatidylcholine (PC-liposomes). Pretreatment of macrophages with liposomes was required in order to observe the inhibitory effect on NO production. NO production induced by IFN-γ was also inhibited by negatively charged liposomes. To clarify the mechanism of inhibitory effect of liposomes, immune blotting was performed using anti mouse inducible nitric oxide synthase (iNOS) antibody. An immunoreactive band at 130 kDa was observed in the extract of control and PC-liposome-treated macrophages, whereas a faint or no band was observed in PS- and PA-liposome-treated ones. These findings revealed that the inhibition of NO production by negatively charged liposomes could be a result in the inhibition of iNOS induction, but not enzyme activity.

Nitric oxide (NO), a reactive free-radical gas, was found to be generated enzymatically from L-arginine and molecular oxygen by constitutive or inducible NO synthase (NOS) in a variety of cells, such as neuronal cells, endothelium, adrenalglands, neutrophils, Kupffer cells, and activated macrophages[1,2]. NO plays several physiological roles in mammalian systems: (i) mediator of endothelial-derived relaxation of smooth-muscles in the artery; (ii) synaptic neuronal messenger; (iii) cytotoxic agent released by macrophages; and (iv) signaling molecule that acts by binding the haem iron at the active site of soluble guanylate cyclase, stimulating the enzyme to generate cyclic GMP [1, 2]. In macrophages, NO is generated by inducible NOS (iNOS), and interferon-γ (IFN-γ) is an important factor for the priming of macrophages, and tumor necrosis factor α (TNF-α) or some other cytokines or lipopolysaccharide (LPS) is necessary for full induction of NO from activated macrophage [3–6].

It is well known that negatively charged liposomes composed of phosphatidylserine (PS) or phosphatidic acid (PA) are preferentially taken up by phagocytic cells such as macrophages [7–9]. There are numerous reports about liposomes having a potential value as carriers of antigens to macrophages or immuno adjuvants [10, 11]. However, the considerable attention has not been paid to the effects of liposomes on the NO production in macrophages. In this report, we evaluated the effects of liposomes on NO production from mouse peritoneal macrophages *in vitro,* and negatively charged liposomes composed of PS or PA effectively suppress the NO production induced by LPS, and immunoblotting analysis showed that these inhibitory effects could be a result of the decrease in iNOS induction.

METHODS

Mice. C3H/HeN mice (6–8 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan), and kept under specific pathogen-free conditions with food and water *ad labium.*

Chemicals and reagents. Phosphatidylcholine (PC) from egg yolk was kindly given from Nippon Oil and Fat Co., Ltd. (Tokyo, Japan). Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0111:B4), PS from calf brain, and PA were

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purchased from Sigma Co., Ltd. (St. Louis, MS). Polyclonal anti mouse inducible nitric oxide synthase (iNOS) antibody was obtained from Affinity BioReagent Inc. (Neshanic Station, NJ).

Preparation of Liposomes. Multilamellar liposomes composed of various phospholipids were prepared by a voltexing method and were passed through a membrane filter (0.45 mm; Corning Glassworks, Corning, NY) before use. Lipid compositions of used liposomes were PC:cholesterol = 2:1 (PC-liposomes), PC:PS:cholesterol = 1:1:1 (PS-liposomes), PC:PA:cholesterol = 1:1:1 (PA-liposomes). LPS contamination in the liposome preparation and culture media was estimated using a Limulus amoebocyte lysate assay as a routine laboratory practice (Wako Pure Chemicals Co., Ltd., Osaka, Japan), and was less than 18 pg/ml.

Preparation of macrophages. C3H/HeN mice were injected with 1.0 ml of 3% thioglycollate (Difco Laboratory, Detroit, MI) intraperitonealy. On day 4, the peritoneal exudate cells (PEC) were obtained by peritoneal lavage with 10 ml of ice-cold Hanks balanced salt solution (HBSS, Ca^{2+} and Mg^{2+} free) supplemented with 10 U/ml of heparin. PEC were washed twice and resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum, and overlaid on plastic dish (96-well culture plate, Corning). The plates were incubated in a humidified 5% CO_2 at 37°C for 2 hr to allow macrophage adherence. Each plate was washed with gentle agitation with warmed RPMI-1640 to dislodge non-adherent cells, and macrophage monolayer was obtained. About 95% of the adherent cells were judged to be macrophages according to latex particle ingestion and Giemsa staining.

Nitrite determination. Macrophage (1×10^5 /well) were preincubated with various liposomes at indicated concentrations for 24 hr. After this time, macrophages were further incubated for 48 hr without or with various concentrations of LPS to elicit the production of NO. Macrophages were also incubated with LPS (10 ng/ml) in combination with IFN-γ to elicit the NO production. NO production was estimated by the measurement of nitrite in the culture supernatant using Griess reagent as described by Stuehr and Nathan [12]. In brief, 100 μl of culture supernatants were mixed with 100 μl of Griess reagent (1% sulfanilamide, 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 2.5% H_3PO_4) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured with a microplate reader (Corona MTP-32, Tokyo, Japan). Nitrite levels were determined using $NaNO_2$ as a standard.

Western blotting. After macrophages (9×10^6) were treated with liposomes in the presence or absence of LPS, cells were scraped and lysed with lysis buffer (10 mM Tris-buffer (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl-fluoride, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupepsin) at 4°C for 1 hr. Samples (170 μg as protein) were electrophoresed on 7.5% SDS-PAGE, and transferred onto the Immobilon P membrane (Nihon Millipore, Tokyo, Japan). The membrane was blocked in 2% BSA in PBS containing 0.1% Tween 20, and incubated with rabbit anti-inducible nitric oxide antibody for 2 hr. After washing, the membrane was incubated with peroxidase-conjugated goat anti-rabbit antibody, washed, and specific bands were detected with 0.025% of 3-amino-9-ethylcarbazole solution.

RESULTS

Effect of liposomes on NO production. The effects of liposomes composed of PC (PC-liposomes), PS (PS-liposomes), and PA (PA-liposomes) on NO production from thioglycollate elicited mouse peritoneal macrophages were studied *in vitro.* As illustrated in Fig. 1, in the absence of LPS, liposomes themselves irrespective of their charge did not affect NO productions. On the other hand, NO production elicited by LPS (10 μg/ml) was inhibited when macrophages were co-cultured with PS- or PA-liposomes. PA-liposomes showed dose-dependent inhibitory effects on NO production, and a complete inhibition was observed at 500 μg/ml (Fig. 2). PC-liposomes did not affect the levels of nitrite production by macrophages even though LPS was added.

The effects of liposomes on NO production was examined by exposing macrophages to various concentrations of LPS. NO production was observed over 100 ng/ml of LPS, and these productions were also inhibited by the pretreatment of negatively charged liposomes, PS- and PA-liposomes, but not PC-liposomes (Fig. 3).

Kinetics of inhibitory activity of liposomes. The kinetics of the NO inhibitory activity of PA-liposomes on macrophages is depicted, and at least 21 hr of PA-liposomes treatment are needed to decrease NO production by LPS-activated macrophages (Fig. 4). Preincubation of macrophages with PA-liposomes for 3, 6, or 9 hr resulted in no inhibition of their potential to produce NO. Addition of PA-liposomes to macrophage cultures at the time of LPS stimulation did not interfere with their normal production of NO. Therefore, a pretreatment appears to be required in order to observe the inhibitory activity of PA-liposomes in the NO production induced by LPS.

Effects of liposomes on NO production induced by IFN-γ. IFN-γ is also essential for the priming of macrophage to produce NO [13]. Consequently, the effects of negatively charged liposomes on

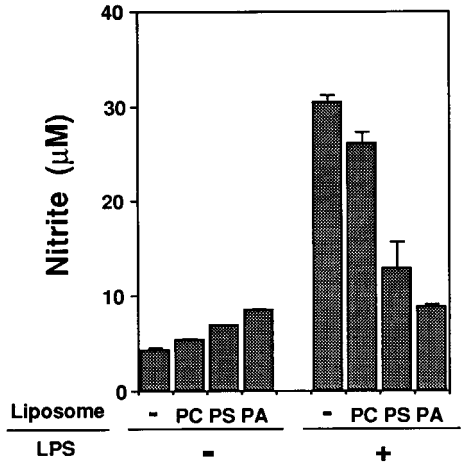

FIG. 1. Effect of various liposomes on the LPS-induced macrophage production of NO_2-. Peritoneal macrophages were incubated for 24 hr with PC-, PS- or PA-liposomes (500 μg phospholipid/ml), and further incubated for 48 hr without or with LPS (10 μg/ml). The supernatants of macrophage cultures were collected and NO_2- was measured as described under Methods. The values are the mean ± SD of triplicate cultures from three independent experiments.

NO production induced by IFN-γ were investigated. Macrophages were activated with 100 U/ml of IFN-γ in combination with LPS (10 ng/ml) whose concentration did not induce NO production. As shown in Fig. 5, NO production was also inhibited by treatment with negatively charged liposomes, but not with PC-liposomes.

Immunoblotting. Fig. 6 shows the results of Western blot analysis for extracts of macrophages treated with various liposomes. Immunoreactive band at 130 kDa was observed in the extract of control and PC-liposome treated macrophages, whereas faint or no band was observed in PS- and PA-liposome treated ones (Fig. 6(A)). The intensity of immunoreactive band was inversely proportional to PA concentrations, and the band was completely diminished when 500 μg/ml PA were added (Fig. 6(B)). These results indicate that PS- and PA- liposomes inhibit the iNOS induction in macrophages following LPS stimulation, but not result in the inhibition of iNOS activity.

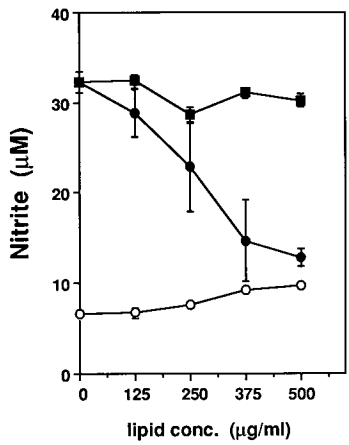

FIG. 2. Effect of liposomal dose on the LPS-induced macrophage production of NO_2-. Peritoneal macrophages were incubated with PC-liposomes (■) or PA-liposomes (●) for 24 hr, and further incubated for 48 hr in the presence of LPS (10 μg/ml). Macrophages were treated with HBSS (◯) instead of LPS. The values are the mean ± SD of triplicate cultures from three independent experiments.

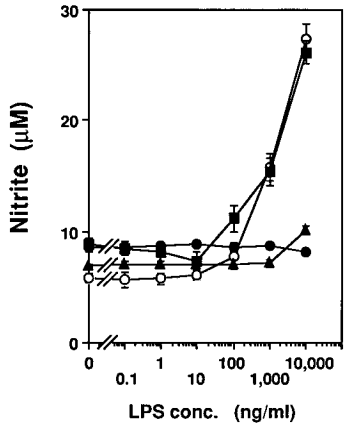

FIG. 3. Effect of LPS dose on NO_2- production and inhibitory effects of liposomes. Peritoneal macrophages were incubated with liposomes for 24 hr, and further incubated for 48 hr with various concentrations of LPS. The values are the mean ± SD of triplicate cultures from three independent experiments. No liposomes (◯), PC-liposomes (■), PA-liposomes (▲), PS-liposomes (●).

DISCUSSION

In this report, we examined the effects of liposomes on NO production from thioglycollate-induced mouse peritoneal macrophages *in vitro.* By treatment of macrophages with PA- and PS-liposomes, NO production induced by LPS was inhibited in dose dependent manner, and complete inhibition was observed at 500 μg/ml for PA-liposomes (Fig. 1 and 2). Macrophage viability after PA- and PS-liposomes treatment was estimated by Trypan blue dye exclution test, and no change in viability was observed (data not shown).

There are many reports that CD14, which is a 55 kDa protein presented on the surface of mononuclear phagocytes, is considered to be a receptor for LPS and closely related to a signal transduction of LPS for macrophage activation [14]. In addition to CD14, CD11c/CD18 is also considered to be a transmembrane signaling receptor for LPS, and have the capacity to activate cells after binding of LPS [15]. Consequently, it was suggested that inhibitory effect of liposomes

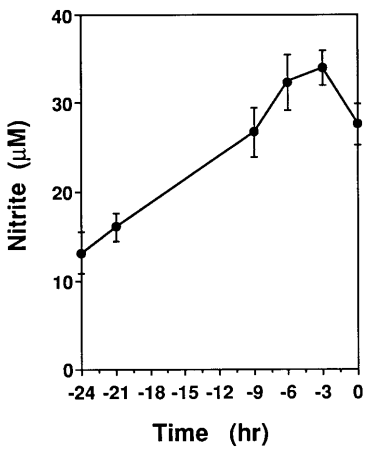

FIG. 4. Effect of time of PA-liposomes addition on the LPS activation of macrophages to produce NO_2-. PA-liposomes (500 μg phospholipid/ml) were added to the macrophage culture at indicated time before activation with LPS (10 μg/ml). After 48 hr of activation with LPS, NO_2- was measured as described under Methods. The values are the mean ± SD of triplicate cultures from three independent experiments.

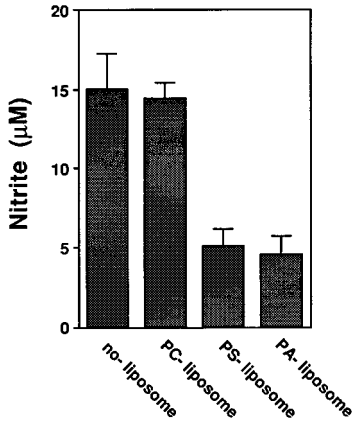

FIG. 5. Effect of liposomes on NO_2- production induced by IFN-γ. Peritoneal macrophages were incubated with liposomes (500 μg phospholipid/ml) for 24 hr, and further incubated for 48 hr with 100 U/ml of IFN-γ in combination with LPS (10 ng/ml). The values are the mean ± SD of triplicate cultures from three independent experiments.

on NO production induced by LPS is a result from the inhibition of LPS binding to these receptors, by the formation of LPS-liposome complex or liposomes act as a competitor for the receptors. However, a pretreatment appears to be required in order to observe the inhibitory activity of PA-liposomes in NO production, and no inhibitory effect was observed when macrophages were treated with liposomes and LPS at the same time (Fig. 4). Further, NO production induced by IFN-γ in combination with 10 ng/ml of LPS was also inhibited by the pretreatment of negatively charged liposomes (Fig. 5). These findings suggest that inhibitory effects of liposomes on NO productions does not result from the suppression of LPS or IFN-γ binding to the receptors.

Further, NO production by LPS was found over 100 ng/ml of LPS, and negatively charged liposomes inhibited NO production induced by these concentrations of LPS (Fig. 3). It is well known that signal transduction through CD14 is initiated by low concentrations of LPS (<10 ng/ml), and the presence of other signaling pathway for high concentrations of LPS (100 ng/ml) is suggested [16]. Recently, CD11c/CD18 was capable of initiating signal transduction in response to high concentration of LPS, as assessed by the induced translocation of nuclear factor-κB (NF-κB) [15, 17]. Thus, the contribution of CD11c/CD18 to the inhibitory effect of negatively charged liposomes on NO production by LPS is suggested.

There are numerous reports concerning the uptake of negatively charged liposomes by phagocytic cells [7, 8], and we already reported that PA- and PS-liposomes are preferentially taken up

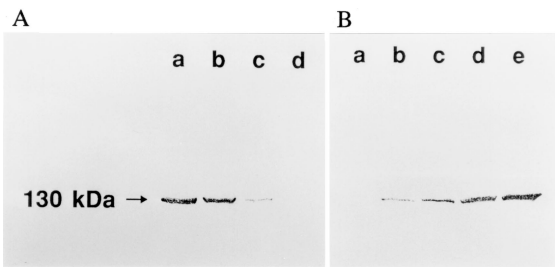

FIG. 6. Immunoblotting analysis of mouse peritoneal macrophages by the antiserum raised against iNOS. (A) Macrophages were treated with HBSS (a), PC-liposomes (b), PS-liposomes (c), or PA-liposomes (d) for 24 hr, and further incubated with LPS (10 μg/ml) for 48 hr. Macrophage lysates were subjected to Western blotting as described under Methods. (B) The effects of PA-liposomal dose on the induction of iNOS. Lipid concentrations are as follows: a, 500 μg/ml; b, 375 μg/ml; c, 250 μg/ml; d, 125 μg/ml; e, 0 μg/ml.

to the same extent by peritoneal macrophages [9]. Recently, Sambrand and Steinberg [18] reported that macrophage receptor for oxidized low density lipoprotein (OxLDL) is plasma membrane protein of 94-97 kDa, and PS-liposomes show strong binding to the protein and this binding clearly inhibited OxLDL binding to macrophages. Endocytosis of OxLDL through this receptor suppressed LPS-induced activation/binding of NF-κB to its cognate nucleotide [19]. NF-κB has been suggested to mediate LPS and IFN-γ induction of NOS in rat alveolar macrophages [20] and murine bone marrow-derived macrophages [21]. Western blot analysis revealed that PA-liposomes inhibited the induction of iNOS, but not enzyme activity (Fig. 6). From these findings, we would propose that PA- and PS-liposomes taken up by macrophages through OxLDL receptor could suppress LPS-induced NF-κB activation, and cause the inhibition of NO production.

In conclusion, negatively charged liposomes composed of PA and PS showed inhibitory effect on NO production. By Western blot analysis, it is revealed that the inhibition of NO production may come from the suppression of iNOS induction, but not inhibition of iNOS activity. Results from our studies may have potential biological significance. The detailed inhibitory mechanism of negatively charged liposomes for NO production from macrophages is now in progress.

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