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Remarkable immunostimulation effects of hybrid liposomes on human peripheral blood mononuclear cells in vitro

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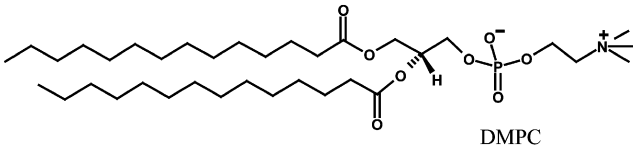
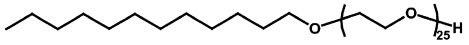
Abstract—Immunostimulation effects of hybrid liposomes (HL25) composed of 90 mol% L-α-dimyristoylphosphatidylcholine (DMPC) and 10 mol% polyoxyethylene(25)dodecyl ether ($C_{12}(EO)_{25}$) on the normal human peripheral blood mononuclear cells (hPBMCs) were investigated in vitro. The concentrations of cytokines (IFN-γ, IL-12) were significantly increased in the presence of HL25 ([DMPC] = 100 μM, [$C_{12}(EO)_{25}$] = 10 μM) and the maximum values attained were 13–14 times higher compared with those of control, though the viability and proliferation of hPBMCs were decreased under the same conditions. Such a remarkably high cytokine-production induced by liposomes without any stimulators was obtained for the first time. Furthermore, fluorescence microscopic and flow cytometric analyses indicated that the HL25 could be incorporated into hPBMCs by the membrane fusion and/or the endocytosis. These results suggest that HL25 should induce the effective immunopotentiation by stimulating the cytokine productivity of hPBMCs.

The immune system plays an important role in the maintenance of the homeostasis in vivo. It is well known that immunocytes are stimulated by bacterial cellular constituents, lipopolysaccharides, polypeptides, oligonucleotides, and other factors, which may activate the immune response by enhancing the proliferation of the cells and/or the production of cytokines.[1] On the other hand, the effects of these substances encapsulated into liposomes on the immune response have been studied and the availability of liposomes as a carrier was reported.[2–5] However, little is known about the possible immunopotentiation by liposomes without any stimulators.

Hybrid liposomes (HL), first developed by Ueoka et al.,[6] can be prepared by simply ultrasonicating a mixture of vesicular and micellar molecules in buffer solutions. It has been reported that HL composed of L-α-dimyristoylphosphatidylcholine (DMPC) and polyoxyethylenealkyl ether demonstrated remarkable inhibitory effects on the growth of various tumor cells in vitro[7–9] and in vivo.[10–12] Recently, we elucidated that HL fused and accumulated into the tumor cell membrane, activated the caspase cascades, and induced the apoptosis in tumor cells.[13–15]

In this study, we investigated the immunostimulation effects of hybrid liposomes composed of DMPC and polyoxyethylene(25)dodecyl ether ($C_{12}(EO)_{25}$) on normal human peripheral blood mononuclear cells (hPBMCs) in vitro.


DMPC


$C_{12}(EO)_{25}$

The hybrid liposomes (HL25) were prepared by sonication of a mixture containing DMPC (1.0×10^{-2} M, NOF, Japan) and $C_{12}(EO)_{25}$ (1.1×10^{-3} M, Nikko Chemicals, Japan) in 5% glucose solution as described previously.[14] The sample solutions were sterilized using membrane filter with 0.20 μm pore size. Dynamic light-scattering measurements with Otsuka Electronics ELS-8000 apparatus (Japan) showed that the size of

Keywords: Hybrid liposome; Peripheral blood mononuclear cell; IFN-γ; IL-12; Immunopotentiation.
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HL25 was less than 100 nm in diameter and stable for more than 1 month.

First, we performed the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) assay[16] to examine the effects of HL25 on the viability and proliferation of hPBMCs (Cambrex Bio Walersville, USA), the donor age: 27 years, sex: male, race: Caucasian. 1.0×10^6 hPBMCs in 100 μl of RPMI-1640 (Gibco RL, USA) medium containing 10% fetal calf serum (FCS, PAA Laboratories, Australia) were inoculated in a 96-well tissue culture plate (Sumilon, Japan) and incubated in a humidified 5% CO_2 incubator for 24 h at 37 °C. Ten microliters of sample solutions was added to each well and the viable cell numbers were estimated by measuring the absorbance at 450 nm with commercial cell counting kit (Dojindo Laboratories, Japan). The results are shown in Figure 1. The number of hPBMCs was slightly increased in the presence of HL25 at the lower concentrations ([DMPC] = 10–40 μM). However, the number of hPBMCs was decreased 48 h after the incubation with HL25 at the higher concentrations ([DMPC] = 80 and 100 μM). The observation indicates that HL25 induced the cell death of hPBMCs and decreased the viability under the same concentration conditions. These results show that HL25 could exert serious influences upon the growth and death of hPBMCs.

Second, the immunostimulation effects of HL25 on hPBMCs were examined on the basis of the production of cytokines (IFN-γ and IL-12) by sandwich enzyme-linked immunosorbent assay (ELISA). hPBMCs were cultured in the presence of HL25 under the same conditions of WST-8 assay. The concentrations of IFN-γ and IL-12 in the supernatants of culture media were determined with (h)IFN-γ and (h)IL-12 p40/p70 ELISA SYSTEMs (Amersham Biosciences, UK) according to the manufacturer's instruction, respectively. As shown in Figure 2a, the production of IFN-γ by hPBMCs was increased in the presence of HL25 under the higher concentration conditions ([DMPC] = 80 and 100 μM). The production of IFN-γ observed at the higher concentration of HL25 was even greater than that in the presence of additional IL-12 (1.0 ng/ml, DIACLONE Research, France) which is known as an IFN-γ inducing interleukin (data not shown). Especially, markedly high production of IFN-γ was obtained after 72 h at the concentration of [DMPC] = 100 μM. The concentration of IFN-γ attained reached 13.6 times (0.414 pg/10^4 cell) that of control medium, though further incubation reduced the concentration of IFN-γ. Similarly, the production of IL-12 by hPBMCs was also increased significantly at the same concentration of [DMPC] = 100 μM, and the maximum concentration of IL-12 (11.1 pg/10^4 cell) was observed after 72 h, which was 13.1 times that of control medium (Fig. 2b). These

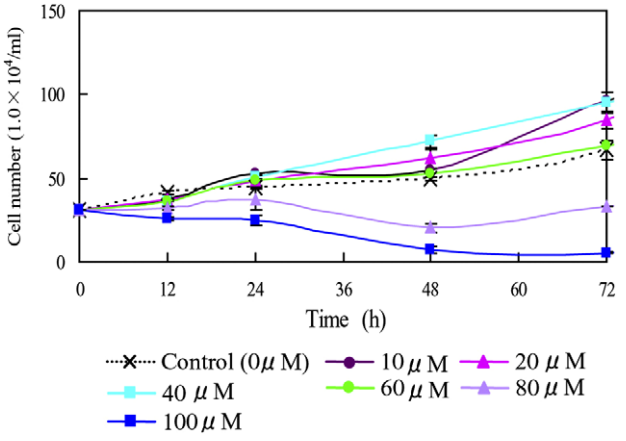

Figure 1. Cell number of hPBMCs in the presence of HL25. [DMPC] = 10–100 μM, [$C_{12}(EO)_{25}$] = 1.0–10 μM. Data presented are means (n = 3); bars, SDs.

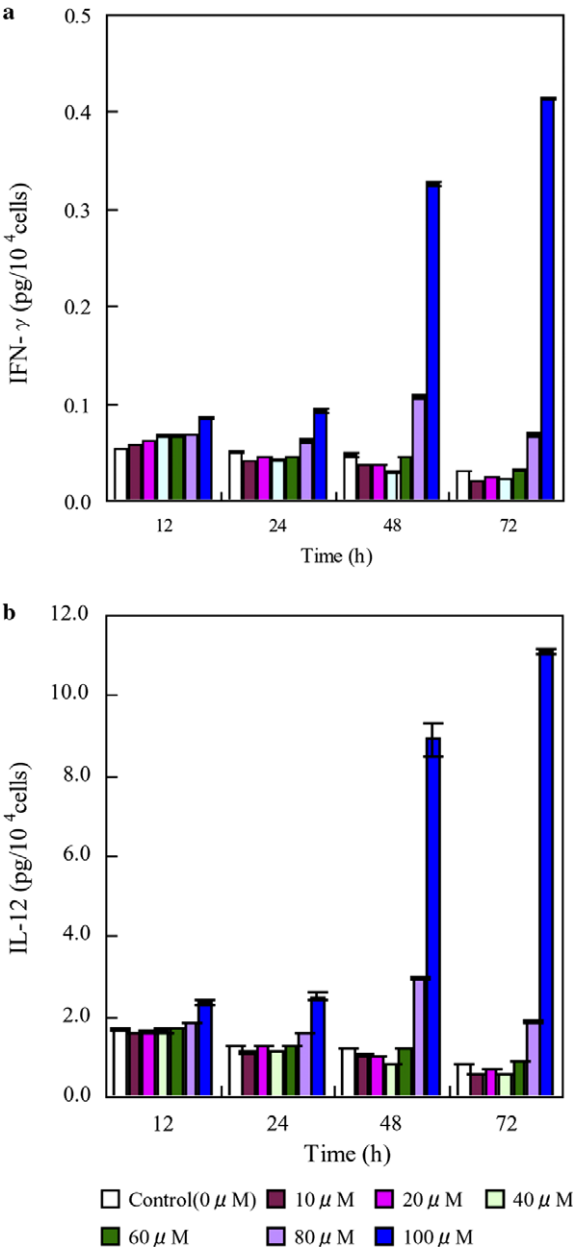

Figure 2. Time courses of the concentration of cytokines (IFN-γ (a) and IL-12 (b)) in culture media of hPBMCs in the presence of HL25. [DMPC] = 10–100 μM, [$C_{12}(EO)_{25}$] = 1.0–10 μM. Data presented are means (n = 2); bars, SDs.

observations indicate that HL25 should have remarkable induction effects on the production of cytokines IFN-γ and IL-12 secreted by hPBMCs, though the viability and proliferation were decreased under the same conditions. Recently, it was reported that IFN-γ was closely related to the activation-induced cell death (AICD) of T lymphocytes.[17–19] Although the mechanistic details are not clear, the excess amounts of IFN-γ might have induced the AICD or other feed back control to the immune response of hPBMCs at higher concentrations of HL25.

Furthermore, fluorescence microscopic analysis of hPBMCs treated with HL25 containing fluorescence-labeled lipid: 1-palmitoyl-2-[12-(7-nitro-2-1,3-benzoxadiazoi-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphocholine (NBDPC, Avanti Polar Lipids, USA) was performed using Leica TCS-SP confocal laser microscope (Germany). After the cultivation of hPBMCs (1.0×10^6 cells/ml) with HL25-NBDPC in a humidified 5% CO_2 incubator at 37 °C, the cells were treated with 0.25% trypsin (Gibco RL, USA), centrifuged at 2000 rpm for 5 min to remove the medium, and resuspended with phosphate-buffered saline (PBS (−)) in a glass-bottom culture dish. The fluorescence microscopic observations were carried out employing Ar and He–Ne lasers for excitation of NBDPC (excitation/detection = 488/505–555 nm). Figure 3 shows the fluorescence micrographs of hPBMCs treated with HL25-NBDPC. It was observed that lymphocytes and monocytes exhibited the green fluorescence after 12 h at the concentration of [DMPC] = 20 μM (Fig. 3a) and after 48 h at the concentration of [DMPC] = 60 μM (Fig. 3b), respectively. These findings support the idea that the HL25 could be incorporated into the cells in an early stage after addition of HL25 even under the lower concentration conditions. Interestingly, the plasma membrane of lymphocytes and the intracellular granules of monocytes were well stained by HL25-NBDPC. In addition, we performed the preliminary analysis of the hPBMCs treated with HL25-NBDPC using a flow cytometer (EPICS XL, Beckman Coulter, USA) with an Ar laser and observed the increase in fluorescence intensities of lymphocytes and monocytes by increasing the concentration of HL25-NBDPC in culture media after 24–72 h. Probably, HL25 could be incorporated into the plasma membrane of lymphocytes by membrane fusion and into the lysosomes of monocytes by endocytosis,[2,20] which stimulates the cytokine productivity of hPBMCs.

In conclusion, marked immunostimulation effects of hybrid liposomes (HL25) composed of 90 mol% DMPC/10 mol% $C_{12}(EO)_{25}$ on the cytokine (IFN-γ, IL-12) productions of normal human peripheral blood mononuclear cells (hPBMCs) were obtained for the first time. Hybrid liposomes composed of phosphatidylcholine and polyoxyethylenealkyl ether inhibited the growth of various tumor cells,[7–9,11–15] while they showed no effects on the proliferation of normal cells (e.g., endothelial cells from human umbilical vein[21] and human primary hepatocytes[8]) in vitro. Recently, we reported that hybrid liposomes distinguished between tumor and normal cells, and fused and accumulated into the tumor cells having more membrane fluidity.[22] In this study, HL25 could be incorporated into the lymphocytes and monocytes, which have relatively high membrane fluidity,[23] and stimulated the cytokine productivity. It is worth noting that hybrid liposomes have not only the inhibitory effects on the growth of tumor cells but also the activation effects of immunocytes simultaneously. This study suggests that hybrid liposomes would be effective biological response modifiers (BRM) in active immunotherapy.

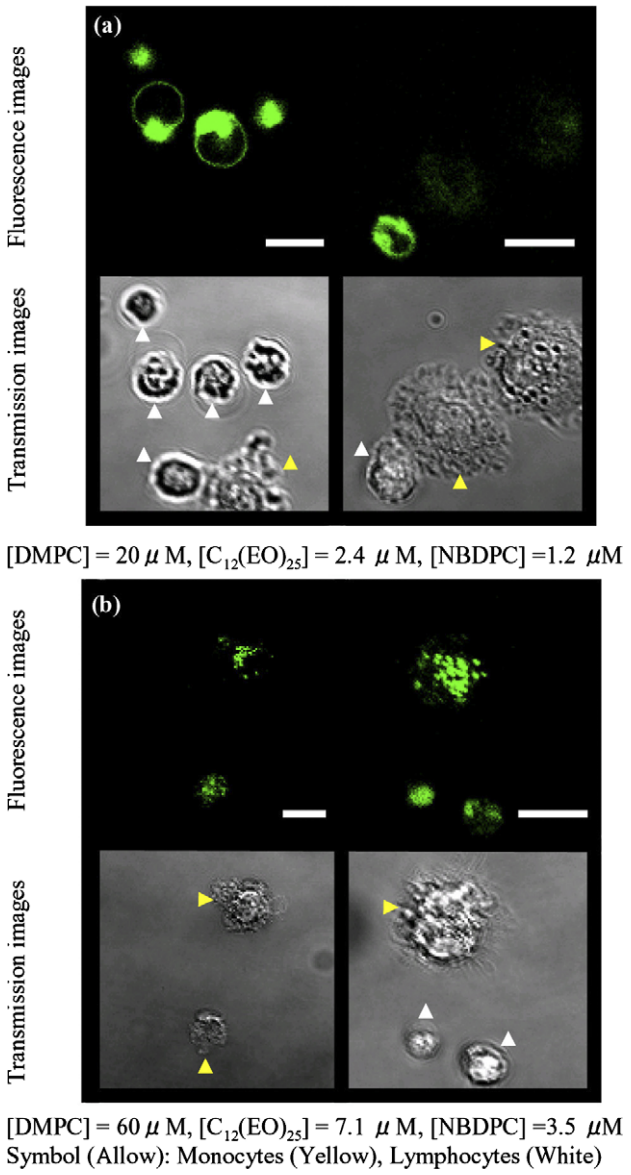

Figure 3. Fluorescence micrographs of hPBMCs treated with HL25-NBDPC after 12 h (a) and 48 h (b). Scale bars, 10 μm.

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