1. Targeting of Liposomes Modified with Cetylmannoside to Mononuclear Phagocyte System[1,2)]

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1. Introduction

Liposomes have been expected as a potential candidate of drug carriers to deliver drugs into specific tissue or organ after intravenous injection. However, it may be actuality that satisfactory results have not been obtained because of their uptake by the mononuclear phagocyte system (MPS), instability in the blood and other obstacles for clinical use. I will present a possibility of liposomes as a potential drug carrier in this meeting.

It has been well known that liposomes are phagocytosed after intravenous injection by macrophages and monocytes which form the MPS, and that activated these cells show nonspecific cytotoxicity against tumor ceils.[3] Recently, much attention has been paid to an attempt to activate these cells with liposomes incorporating activating agents such as v-interferon, muramyl dipeptide (MDP) and other biological response modifier (BRM) for eradication of tumor metastasis.[4] Receptor recognizing the terminal mannose residues was known on the surface of the liver macrophages (Kupffer cells),[5] therefore it is expected that surface modification of liposomes with monnose improve the effect of activation of the macrophages.[6] We investigated biological disposition of liposome modified with cetylmannoside[7] after intravenous injection in rats and interaction of the liposomes with human peripheral monocytes.

2. Characteristics of Liposomes

Liposomes used in this study are multilamellar vesicales (MLV) sized by extrusion technique[8] with polycarbonate membrane (0.8 μm) and small unilamellar vesicles (SUV) prepared by ultrasonication. Liposomal size was determined by a laser light scattering instrument (LPA-3000, Otsuka Electronics, Osaka). Lipid composition of the liposomes is cetylmannoside (Man), hydrogenated egg phosphatidylcholine (PC), dicetylphosphate (DCP) and cholesterol (CH) in a molar ratio of 3 : 2 : 1 : 4 (Man-MLV and Man-SUV). Control liposomes are composed with PC/DCP/CH in a molar ratio of 5 : 1 : 4 (PC-MLV and PC-SUV). Characteristics of the lioposomes are shown in Table I. Size of liposomes was considered to affect their biological disposition.[9] The characteristics of both types of liposomes are very similar between modified and control liposomes as shown in this table.

Table I—Characteristics of Liposomes

Lipid compositions[a] (PC:Man:DCP:CH)	Mean diameters[b] (nm)	Encapsulation ratio of [^{3}H]inulin(%)
PC-MLV(5:0:1:4)	793± 31	20.3± 1.2
Man-MLV(2:3:1:4)	828± 53	27.1± 0.9[c]
PC-SUV(5:0:1:4)	73± 4	2.3± 0.3
Man-SUV(2:3:1:4)	68± 4	1.9± 0.3

[a] Molar ratio. [b] Liposome size was determined by dynamic laser light scattering instrument. [c] Significant difference from PC-MLV at $p<0.01$. Each value represents the mean± S.E. of three experiments.

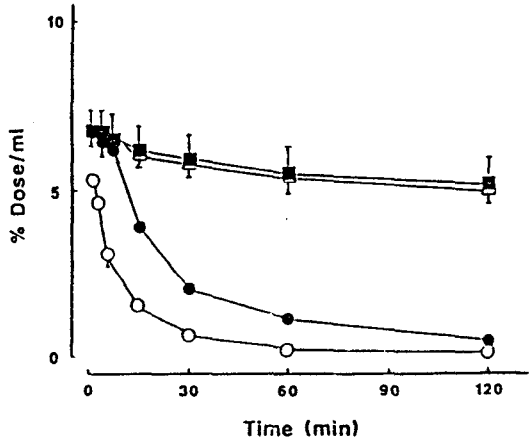

Figure 1—Time courses of blood concentrations of [^{3}H]inulin as an aqueous marker of liposomes after intravenous administration to rats. Liposomes were injected at a dose of 125 μmol/kg. Each value represents the mean± S.E. of three rats. Symbols; PC-MLV (closed circle), Man-MLV (open circle), PC-MLV (closed square), Man-SUV (open square).

3. Disposition of Liposomes[1]

Elimination of [^{3}H]inulin as an aqueous marker of liposomes from blood and urinary excretion after iv injection are shown in Fig. 1 and Fig. 2, respectively. The elimination indicates tissue distribution and degradation in the blood, and the urinary excretion indicates instability of liposomes

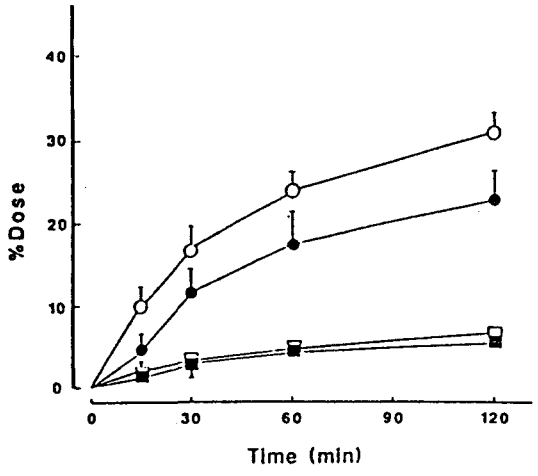

Figure 2—Time courses of cumulative urinary excretions of [^{3}H]inulin as an aqueous marker of liposome after intravenous administration to rats. Liposomes were injected at a dose of 125 μmol/kg. Each value represents the mean± S.E. of three rats. Symbols are the same as those in Fig. 1.

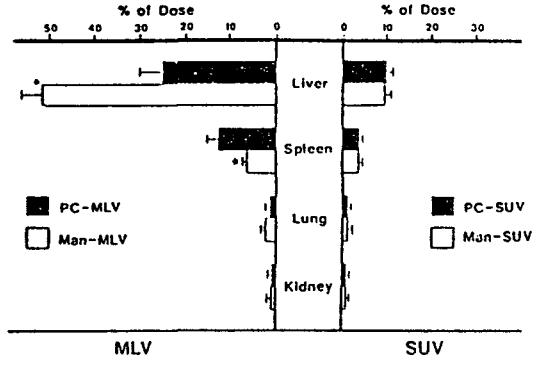

Figure 3—Organ distribution of [^{3}H]inulin encapsulated in Man-MLV and SUV 2h after intravenous administration to rats. Liposomes were injected at a dose of 125 μmol/kg. Each value represents the mean± S.E. of three rats. Significant differences from PC-MLV; *, $p<0.05$, **, $p<0.01$. Symbols; PC liposomes (closed column), Man liposomes (open column).

in the body.[9] Tissue distribution 2hr after injection are shown in Fig. 3. These results demonstrate that Man-MLV accumulate in the liver more than PC-MLV, nevertheless they are less stable in the body. Uptake clearances of liposomes by the organs are shown in Table II. About 4-fold higher affinity to the liver of Man-MLV was observed comparing with PC-MLV, however the modification did not increase the affinity to the spleen. The effect of mannose modification was not observed on the disposition of SUV, and it seems to be limitted in the phagocytosis by macrophages.

Table II—Pharmacokinetic Parameters of PC-MLV and Man-MLV

Parameters		PC-MLV	Man-MLV
AUC_{0-2h}	(%dose · min/ml)	218.34 ± 23.49	83.69± 4.84[a]
$AUC_{0-\infty}$	(%dose · min/ml)	239.91 ± 30.48	90.32± 4.62[a]
MRT	(min)	43.58 ± 4.31	34.24± 2.65
Vd_{ss}	(ml)	18.30 ± 0.68	38.22± 4.21[a]
CL_{total}	(ml/min)	0.429± 0.005	1.113± 0.057[a]
$CL_{hepatic}$	(ml/min)	0.135± 0.008	0.619± 0.080[a]
CL_{spleen}	(ml/min)	0.068± 0.010	0.068± 0.007
CL_{renal}	(ml/min)	0.104± 0.002	0.367± 0.053[a]
CL_{other}	(ml/min)	0.123± 0.042	0.059± 0.082

[a]Significant difference from PC-MLV at $p<0.01$. Each value represents the mean± S.E. of three rats.

4. Intercellular Distribution of Liposomes in the Liver[1]

The liver is composed with cells of mainly three types, those are parenchymal cells, Kupffer cells and endothelial cells, and latter two types are called as non-parenchymal cells. To investigate accurate cellular distribution, complete separation and high recovery of these cells are needed, however, it may be difficult to satisfy these requirements with present technique. In this study, the cellular distribution was estimated by the radioactivity in the four fractions separated by differential centrifugation 2hr after injection of the MLV. Distributions of the cells in the four fractions are shown in Fig. 4 and Fig. 5 shows liposomal uptake in each fraction. The highest radioactivity was found in the Kupffer cell rich fraction. Since the large liposomes can not go through the liver sinusoidal fenestrations, and endothelial cells are not able to phagocytose large particles, the radioactivity found in these fractions seems to be due to the uptake of the liposomes by the Kupffer cells.

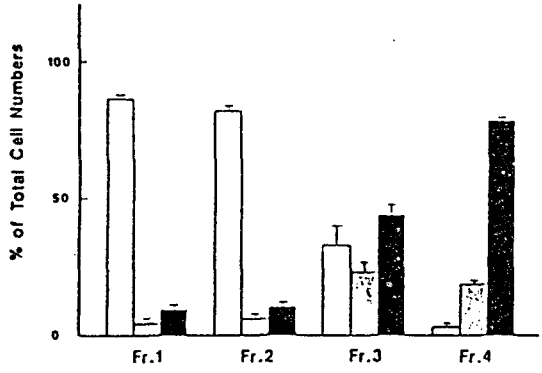

Figure 4—Cell population of four subfractions by differential centrifugation of liver cells. Each value represents the means± S.E. of six rats. Symbols; hepatocytes (open column). Kupffer cells (hatched column), endothelial cells (closed column).

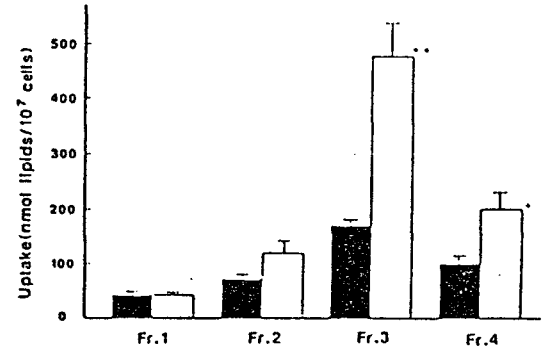

Figure 5—Effect of Man-modification on cellular distribution in the liver of MLV 2h after intravenous administration to rats. Liposomes were injected at a dose of 125 μmol/kg. Fraction 1 (Fr. 1), fraction 2 (Fr. 2), fraction 3 (Fr. 3) and fraction 4 (Fr. 4) were centrifuged at 40×g, 70×g, 140×g and 850×g, respectively. Liposomes were injected at a dose of 125 μmol/kg. Each value represents the mean± S.E.. of three rats. Significant difference from PC-MLV; *, $p<0.05$, **, $p<0.01$. Symbols; are the same as those in Fig.3.

5. Uptake Mechanism of Man-MLV by Non-Parenchymal Cells

To investigate the uptake mechanism of Man-MLV, suspended non-parenchymal cells were used. Fig. 6 shows the effects of pre-treatments of plasma on the uptake. Man-MLV showed about

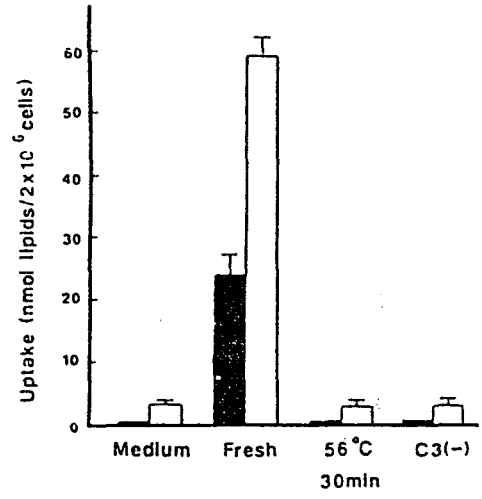

Figure 6—Involvement of complement on uptake of PC-MLV and Man-MLV by non-parenchymal cells. Non-Parenchymal cells (2×10^6 cells/vial) were incubated for 2h in medium with PC-MLV (0.5 μmol/ml, closed column) or Man-MLV (0.5 μmol/ml. open column) containing [^{3}H]inulin in the presence or absence of 5% fresh plasma. 5% pre-heated plasma or 5% C3 minus serum. Each value represents the mean± S.E. of three separate experiments.

2 times higher uptake than PC-MLV in fresh plasma. These results suggest that plasma components are indispensable for the uptake of both types of MLV in vivo. C3 deficient plasma and plasma pre-heated at 56℃ for 30 min were unable to increase the uptake. Some contributions of complement system on the uptake in vivo were suspected from these results. Uptake of liposomes pre-incubated with plasma (opsonized liposomes) was inhibited by addition of EDTA as shown in Fig. 7. The Kupffer cells have two complement receptors for C3b, i.e. CR1 and CR3, the latter demand Ca^{2+} and former does not for phagocytosis.[10] It is suggested, Therefore, that liposomes used in this experiment were opsonized with C3 and then taken up by the Kupffer cells via CR3 (not mannose receptor) in vivo. Although liposomes containing Man-MLV were expected to deliver BRM to Kupffer cells via mannose receptor as other mannose modified liposomes,[6,11] at the first stage of this work. It has become apparent in this study that

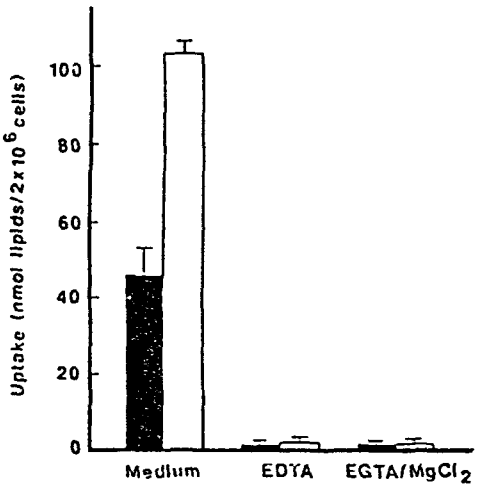

Figure 7—Effect of EDTA or EGTA/$MgCl_2$, treatment on uptake of pre-opsonized PC-MLV and Man-MLV by non-parenchymal cell. Liposomes were opsonized by incubating with fresh rat plasma 37℃ for 30 min. Non-Parenchymal cells (2×10^6 cells/vial) were incubated for 2h in medium with pre-opsonized PC-MLV (0.5 μmol/ml, closed column) or Man-MLV (0.5 μmol/ml, open column) containing [^{3}H]inulin in the presence or absence of 3 ml EDTA or EGTA/$MgCl_2$. Each value represents the mean± S.E. of three separate experiments.

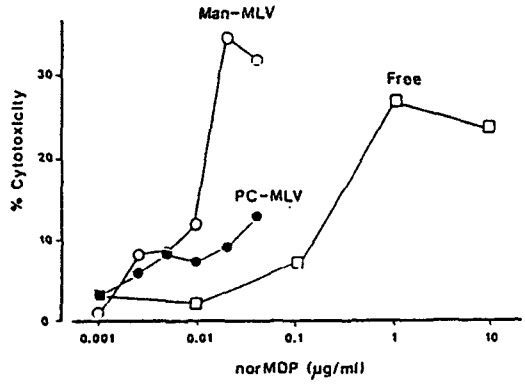

Figure 8—Enhancement of monocyte-mediated cytotoxicity by norMDP encapsulated in Man-MLV. norMDP (20 μg/ml) diluted with RPMI 1640 medium was entrapped in PC-MLV or Man-MLV. Free norMDP (open square). PC-MLV (closed cirele) or Man-MLV (open circle) encapsulated norMDP at various concentration of lipids was incubated with 1×10^3 monocytes for 24h. Then the cells were washed and their cytotoxicity against labeled A375 melanoma cells was assayed at and E/T ratio of 10: 1. Incubations were terminated 72h later. Cytotoxicity on tumor cells was determined as a percentage of that of untreated monocytes.

the liposomes are opsonized with complement component (C3) and then phagocytosed by Kupffer cells via complement receptor (CR3). It has been reported that predominant receptors for phagocytosis of Kupffer cells are complement receptor and those of spleen macrophages are Fc receptor.[12] This is considered as the reason for that Man-MLV showed the higher affinity to the liver and not to the spleen. On the other hand, contribution of other mannose specific plasma protein (e.g. mannose binding protein, MBP), which has property to activate alternative complement pathway,[13] are also considered, because of the higher uptake of Man-MLV than PC-MLV.

6. Cytotoxicity of Monocytes Treated with Man-MLV Containing MDP[2]

Although the ultimate purpose of this study is the activation of the Kupffer cells, it is difficult to use resident macrophages for in vitro expermemts, because of their spontaneous activation during isolation processes. Human peripheral monocytes purified by elutriation technique[14] were used for the investigation of the biological responses stimulated by the liposomes. Cytotoxicity of the monocytes activated with liposomes containing desmethyl-muramyl dipeptide (norMDP) against human melanoma cells (A375) was shown in Fig. 8. The monocytes were rendered tumoricidal activity by in vitro treatment with much lower concentration of norMDP in Man-MLV than of free norMDP. PC-MLV containing norMDP also activated the monocytes to the tumoricidal state, but were less effective than Man-MLV containing that. This result presents a possibility to activate the cells of MPS to tumoricidal state by treatment with Man-MLV containing BRM.

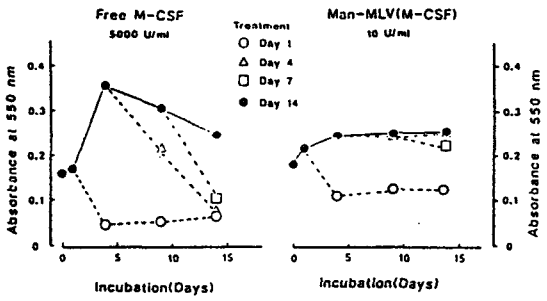

Figure 9—Prolongation of monocyte survival by M-CSF encapsulated in Man-MLV. Monocytes (1×10^3 cells/well) were incubated for the indicated periods in medium with free M-CSF (5000 U/m*l*) or M-SCF (10 U/m*l*) encapsulated in Man-MLV, and then the monocyte survival was measured with MTT assay. Each value represents the mean± S.E. of triplicate cultures. Data are representative of three separate experiments.

7. Longevity of Monocytes Treated with Man-MLV Containing M-CSF[2)]

Macrophage-colony stimulating factor (M-CSF) has been known as a cytokine mediating the differentiation of monocytes to macrophages.[15)] Effects of Man-MLV encapsulating M-CSF on the monocytes was investigated in this study. As shown in Fig. 9, free M-CSF showed growth of monocytes at 5000 U/m*l* and disappearance of the effect was observed by washing out. On the other hand, M-CSF encapsulated in Man-MLV showed survival of the monocytes at 10 U/m*l*, and the effect was maintained by the treatment for 4 days. Optical micrographic observation revealed a morphological difference of these cells. The cells treated with free M-CSF showed cylindrical shape and ones with liposomal M-CSF showed spherical shape as shown in Fig. 10. Monocyte-derived-macrophage also showed the longevity by the treatment with M-CSF in Man-MLV, and PC-MLV were less effective. It is suggested that the prolongation of survival of monocytes by M-CSF in Man-MLV is due to the delivery of M-CSF to the cytoplasm of monocytes, allowing its interaction with intracellular sites, and that intracellular disposition of Man-MLV is different from that of PC-MLV. Growth

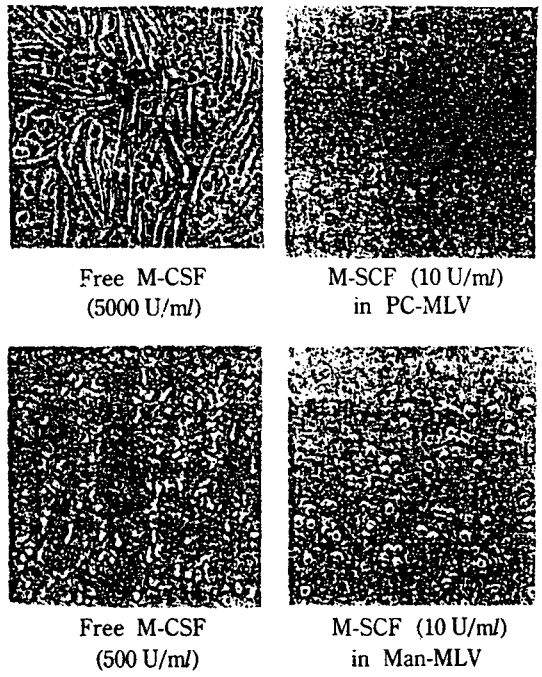

Figure 10—Differential effect of free M-SCF and M-CSF encapsulated in Man-MLV against monocytes. Monocytes were incubated for 7 days in medium with free M-CSF, PC-MLV or Man-MLV containing M-CSF.

of monocytes is considered to be mediated by signals from cell surface receptors at high concentration of free M-CSF. Results obtained in this experiment presented a possibility to control the cell functions with liposomal BRM, which is different from the functions mediated by free form.

8. Conclusions

In this work, we found that modification of multilamellar liposomes with cetylmannoside (Man-MLV) increases their uptake by the rat Kupffer cells after intravenous injection, and reveals the mechanism that the liposomes were opsonized with plasma complement component (C3) and then phagocytosed by the cells via complement receptor (CR3). The effects were not observed for SUV and it was suggested that liposomal size is important factor for the uptake.

It became apparent that norMDP encapsulated in Man-MLV were able to activate more effecti-

vely human monocytes to tumoricidal state than in PC-MLV or free form, and M-CSF in Man-MLV were able to induce different function of the cells from that by free form at lower concentration in vitro.

These findings indicate the potential values of Man-MLV as carrier vehicles of BRMs for in vivo activation of human monocytes and macrophages to enhance the host's defence system against infections and cancer.

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