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Effects of sialic acid derivative on long circulation time and tumor concentration of liposomes

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Abstract

The effects of a sialic acid derivative, Neu5Acβ-PA, on the blood circulation and tissue distribution of liposomes composed of dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol) and Neu5Acβ-PA were investigated compared with liposomes composed of DPPC, Chol and monosialoganglioside GM_1 in mice and rats. When liposomes containing Neu5Acβ-PA were intravenously administered into mice, the plasma concentration of liposomes containing Neu5Acβ-PA was increased, and the liver and spleen uptakes were decreased; there was no significant difference in tissue distribution between liposomes containing Neu5Acβ-PA (DPPC/Chol/Neu5Acβ-PA = 10:10:3) and those containing GM_1 (DPPC/Chol/GM_1 = 10:10:1). On the other hand, the plasma concentration of liposomes containing Neu5Acβ-PA was significantly greater than that of liposomes containing GM_1 at all times determined in rats, and was about 30.7- and 10.3-fold that of liposomes containing GM_1 at 6 and 24 h, respectively. The liver and spleen uptakes of liposomes containing Neu5Acβ-PA at 6 h were significantly reduced compared with those of liposomes containing GM_1 in rats. The tumor accumulation of liposomes was also examined. The liver/tumor ratio of liposomes containing Neu5Acβ-PA was similar to that of liposomes containing GM_1 in mice and lower than that of liposomes containing GM_1 in rats.

Keywords: Liposome; Sialic acid derivative; Reticuloendothelial system; Tumor; Drug delivery system

1. Introduction

A great number of studies have been performed on the medical applications of liposomes as drug carriers for delivery into cells of biologically active substances (Gregoriadis, 1984; Kikuchi and Inoue, 1985; Ostro, 1987). Generally speaking, when particulate carriers such as liposomes and emulsions are administered intravenously, they are readily taken up, as is well known, by the cells of the reticuloendothelial system (RES), for example, Kupffer cells of the liver and macrophages of the spleen. This is a considerable problem, however, when such carriers are used as a controlled release system or as a targeting system that allows delivery of drugs to the desired tissues other than the RES.

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Recently, a number of investigations have been carried out on the incorporation of glycolipids or glycoproteins into the liposomal membrane for prolonging the circulation time of liposomes and reducing RES uptake, e.g., sialoglycoprotein of human erythrocytes (Utsumi et al., 1983), monosialoganglioside GM_1 (Allen and Chonn, 1987), sialoglycopeptide derived from fetuin (Saito et al., 1988), hydrogenated phosphatidylinositol (Gabizon and Papahadjopoulos, 1988), glucuronic acid derivative (Namba et al., 1990), glycophorin and ganglioside GM_3 (Yamauchi et al., 1993), etc.

In this study, we used a novel synthetic sialic acid derivative (sialoglycolipid) as a mimic of GM_1, having a sialic acid group (Neu5Ac) at the terminal position of the glycolipid, for incorporation into the liposomal membrane. Sialic acid has been thought to play an important role in prolonging the circulation time of serum protein (Morell et al., 1968). We examined the biodistribution of liposomes containing the novel synthetic sialic acid derivative in tumor-bearing rats and mice and investigated its effects in prolonging the circulation time of liposomes and accumulation of these liposomes in tumors compared with liposomes containing GM_1.

2. Materials and methods

2.1. Materials

L-α-Dipalmitoylphosphatidylcholine (DPPC), cholesterol (Chol) and inulin (Mol. Wt 5000) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and GM_1 was obtained from Funakoshi Co. (Tokyo, Japan). They were used as received without further purification. [^{3}H]Inulin was purchased from New England Nuclear Research (Boston, MA). A sialic acid derivative, [2-(2-palmitoylamido-1-ethyl)-5-acetoamide-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulipyranoside]onate (Neu5Acβ-PA), shown in Fig. 1 was synthesized as described previously (Nakabayashi et al., 1993). All other chemicals were commercial products of reagent grade.

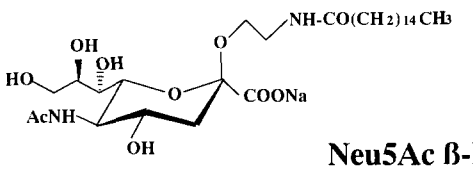

Fig. 1. Chemical structure of sialic acid derivative (Neu5Ac β-PA).

2.2. Preparation of liposomes

The liposomes were composed of DPPC, Chol, Neu5Acβ-PA or GM_1 at a molar ratio of 10:10:1. Multilamellar vesicles (MLV) were prepared by the conventional method introduced by Bangham et al. (1965). Namely, the lipids were dissolved in chloroform-methanol (9:1 by vol.) in a test tube with a screw cap. A thin lipid film was produced on the wall of the test tube which was warmed in a water bath. The residual solvent was removed in a desiccator under reduced pressure for 12 h. Phosphate-buffered saline (PBS, pH 7.4) containing 1 mM of inulin and 925 kBq/ml of [^{3}H]inulin as an aqueous marker was added and the lipid film was hydrated. The test tube was then agitated on a vortex mixer for more than 5 min at a temperature above the gel-liquid crystalline phase transition temperature (T_c) of the lipid materials. To attain a more homogeneous size distribution of the liposomes, the liposomal dispersion was then sequentially passed through a polycarbonate membrane filter with 0.2, 0.1 and 0.08 μm pore size above the T_c as described by Olson et al. (1979). After extrusion, the liposomal dispersion was centrifuged three times at 150000 $\times g$ for 12, 3 and 2 h to remove inulin and [^{3}H]inulin which were not encapsulated in the liposomes. The pellet was resuspended in PBS. The DPPC concentration in the liposomes was determined by enzymatic assay using a Phospholipids B-test Wako (Wako Pure Chemical Industries, Ltd, Osaka, Japan), and the liposomal dispersion was diluted with PBS to make the final concentration of DPPC equal to its initial value.

2.3. Zeta-potential measurements

The electrophoretic mobility of the liposomes was determined with a Delsa 440 (Coulter Elec-

tronics, Inc., Hialeah, FL) after dilution of the liposomal dispersion with PBS. The light source was a 5 mW He-Ne laser at a wavelength of 632.8 nm. The zeta-potential was calculated from the electrophoretic mobility based on the Smoluchowski formula.

2.4. Particle size distribution

The mean particle size of the liposomes was determined by quasi-elastic laser light scattering (QELS) measurement with a submicron particle analyzer (Nicomp Model 370 HPL, Particle Sizing Systems). The light source was a 35 mW He-Ne laser at a wavelength of 632.8 nm.

2.5. Animal experiments

2.5.1. Normal animals

Male Sprague-Dawley (SD) strain rats weighing 180–220 g were used. Rats (three per group) were anesthetized with ether, fixed on a board and then injected in a hind limb vein with 5 μmol of total lipids in 0.25 ml of liposomal dispersion per 100 g of body weight. Blood samples (approx. 200 μl) were obtained from the jugular vein at 0.5, 1, 2, 4, 6 and 24 h after injection. Then the rats were killed at 24 h and blood samples were obtained from the inferior vena cava. The lung, spleen, kidney, and liver were then excised, rinsed with saline, and weighed, while the bone marrow was excised and weighed.

2.5.2. Tumor-bearing animal

4-week-old ICR male mice (three per group) were subcutaneously inoculated in the hind leg with 2×10^6 S180 tumor cells/0.2 ml saline. 10 days after tumor inoculation, when the local tumor weight had reached 2.5–3.0 g, the mice were used. The mice (three per group) were anesthetized with ether, fixed on a board and then injected in the tail vein vein with 0.5 μmol of total lipids in 0.1 ml liposomal dispersion per 10 g of body weight. The mice were killed at 6 h and blood samples were obtained from the heart. The lung, spleen, kidney, and liver were then excised, rinsed with saline, and weighed.

6-week-old Wistar female rats (three per group) were subcutaneously inoculated in the inguinal region with 10^7 Walker 256 tumor cells/0.33 ml. 6 days after tumor inoculation, when the local tumor weight had reached 4.0–5.0 g, the rats were given the same treatment as the normal rats and killed at 6 h.

2.6. Determination of radioactivity

Radioactivity in the plasma and tissues after intravenous injection of liposomes encapsulating [^{3}H]inulin as an aqueous marker was determined as follows. A 100 μl sample of plasma or approx. 100 mg of tissue was put on the combustion cone (Packard Instrument Co., Inc., IL, U.S.A.) and then dried at room temperature. Next, the samples were prepared by the combustion method (Automatic Sample Combustion System, Aloka ASC-113, Tokyo, Japan) using a liquid scintillation cocktail (Aquasol-II, New England Nuclear Research, Boston, MA). The radioactivity of [^{3}H]inulin of the blood and tissue samples was counted with a liquid scintillation counter (Aloka LSC-3500, Tokyo, Japan). Correction factors for the blood content in various organs were determined by examining the distribution of ^{51}Cr-labeled erythrocytes 30 min after intravenous administration as described by Liu et al. (1991). The correction factors for various organs were negligible small. The results are presented as % injected dose in each organ or blood.

2.7. Statistical analysis

Values are expressed as % injected dose and mean $\pm$ SE. Student's t-test was used for the tests comparing the two groups. p values of 0.05 or less were considered significant.

3. Results

3.1. Zeta-potential and particle size of liposomes

The zeta-potentials of liposomes containing Neu5Acβ-PA (DPPC/Chol/Neu5Acβ-PA = 10:10:1 in molar ratio) and GM_1 (DPPC/Chol/

GM_1 = 10:10:1 in molar ratio) were −11.3 and −9.3 mV, respectively, and their particle sizes were in the range of 80–90 nm (volume average diameter). These results show that the zeta-potential and particle size distribution of these liposomes are of similar value. Also, the results for the zeta-potential indicate that Neu5Acβ-PA is incorporated into the liposomal membrane.

3.2. Tissue distribution of liposomes in tumor-bearing mice

Allen and Chonn (1987) have reported that the inclusion of GM_1 significantly reduced the RES uptake of liposomes and resulted in prolonged circulation time of liposomes in normal mice. We first examined the effect of GM_1 on the tissue distribution of liposomes after intravenous administration in tumor-bearing mice at 0.5, 1, 2, 4 and 6 h (Fig. 2). As shown in Fig. 2, the tumor, liver and spleen concentrations of liposomes containing GM_1 were increased, while the plasma concentration was decreased as a function of time. The plasma/RES (liver and spleen) and liver/tumor ratios of liposomes containing GM_1 at 6 h after intravenous administration were 1.00 and 2.19, respectively. Fig. 3 demonstrates the tissue distribution of liposomes containing various molar ratios of Neu5Acβ-PA at 6 h after intravenous administration in tumor-bearing mice. As can be seen in Fig. 3, the plasma concentration of liposomes containing Neu5Acβ-PA was increased, and the liver and spleen uptakes were decreased with increasing molar ratio of Neu5Acβ-PA. The tumor concentration was also increased with increasing molar ratio of Neu5Acβ-PA. In the case of liposomes containing Neu5Acβ-PA (DPPC/Chol/Neu5Acβ-PA = 10:10:2), the tumor concentrations of liposomes containing Neu5Acβ-PA and GM_1 at 6 h were comparable. The plasma/RES and liver/tumor ratios of liposomes containing Neu5Acβ-PA (DPPC/Chol/Neu5Acβ-PA = 10:10:3) at 6 h after intravenous administration were 0.73 and 2.37, respectively. It was suggested that there were no significant differences in tissue distribution between liposomes containing Neu5Acβ-PA (DPPC/Chol/Neu5Acβ-PA = 10:10:3) and li-

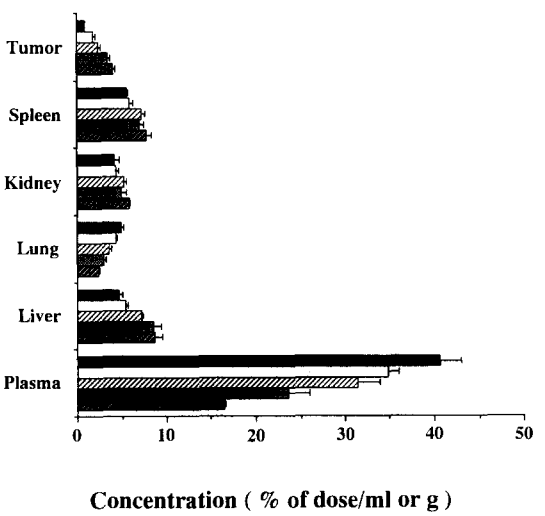

Fig. 2. Tissue distribution of liposomes containing GM_1 (DPPC/Chol/GM_1 = 10:10:1) in tumor-bearing mice. Each bar represents the mean ± S.E. (■) 0.5 h after intravenous administration, (□) 1 h after intravenous administration, (▨) 2 h after intravenous administration, (▩) 4 h after intravenous administration, (▨) 6 h after intravenous administration.

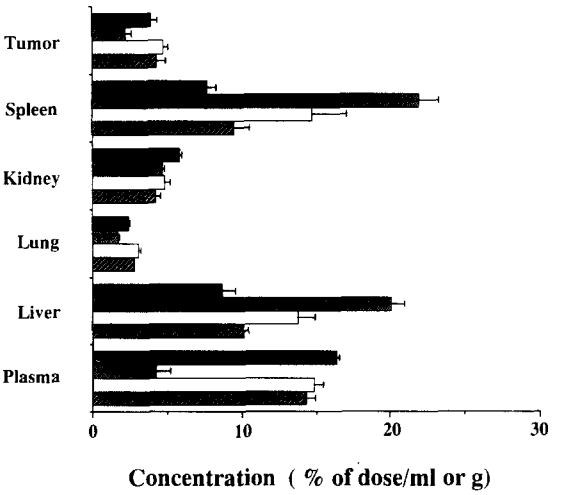

Fig. 3. Tissue distribution of liposomes containing various molar ratios of NeuAcβ-PA or GM_1 in tumor-bearing mice. The distribution was measured 6 h after intravenous administration. Each bar represents the mean ± S.E. (■) DPPC/Chol/GM_1 = 10:10:1, (▨) DPPC/Chol/Neu5Acβ-PA = 10:10:1, (□) DPPC/Chol/Neu5Acβ-PA = 10:10:2, (▩) DPPC/Chol/Neu5Acβ-PA = 10:10:3.

posomes containing GM_1 (DPPC/Chol/GM_1 = 10:10:1).

3.3. Blood circulation and tissue distribution of liposomes in normal and tumor-bearing rats

The blood circulation and tissue distribution of liposomes containing Neu5Acβ-PA (DPPC/Chol/Neu5Acβ-PA = 10:10:1) in normal and tumor-bearing rats compared with liposomes containing GM_1 (DPPC/Chol/GM_1 = 10 : 10 : 1) were subsequently determined. Because many reports of long-circulating liposomes involved examination not in rats but in mice (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen et al., 1989), we investigated the effects of Neu5Acβ-PA on the blood circulation and tissue distribution of liposomes in rats. Fig. 4 and 5 show the effect of Neu5Acβ-PA on the blood circulation and tissue distribution of liposomes after intravenous administration in normal rats. As shown in Fig. 4, the plasma concentration of liposomes containing Neu5Acβ-PA was significantly higher than that of liposomes containing GM_1 at all times determined, and was about 30.7- and 10.3-fold that of liposomes containing GM_1

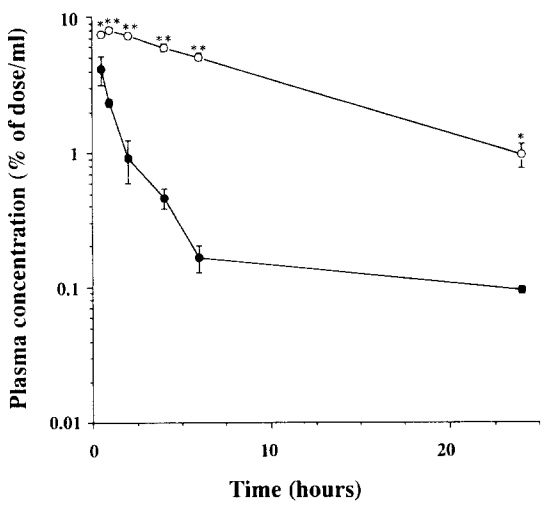

Fig. 4. Plasma clearnace of liposomes containing Neu5Acβ-PA or GM_1 from the circulation in normal rats. Each bar represents the mean ± S.E. $^*p < 0.05$, $^{**}p < 0.01$ compared to GM_1 liposomes. (○) DPPC/Chol/Neu5Acβ-PA = 10:10:1, (●) DPPC/Chol/GM_1 = 10:10:1.

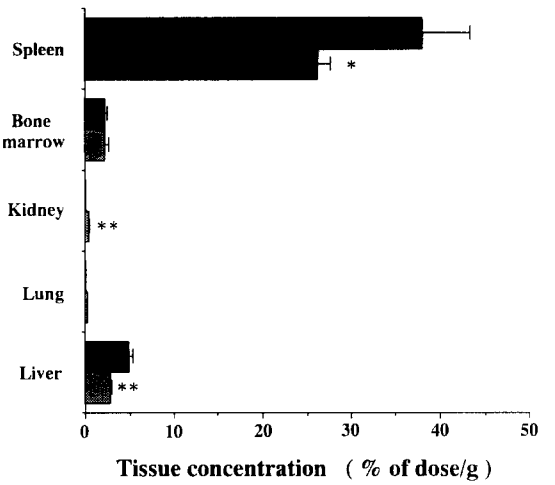

Fig. 5. Tissue distribution of liposomes containing NeuAcβ-PA or GM_1 in normal rats. The distribution was measured 24 h after intravenous administration. Each bar represents the mean ± S.E. $^*p < 0.05$, $^{**}p < 0.01$ compared to GM_1 liposomes (■) DPPC/Chol/GM_1 = 10:10:1, (▨) DPPC/Chol/Neu5Acβ-PA = 10:10:1.

at 6 and 24 h. For the liver and spleen uptakes of liposomes as shown in Fig. 5, liposomes containing Neu5Acβ-PA at 24 h were significantly reduced compared with liposomes containing GM_1. The plasma/RES ratio of liposomes containing Neu5Acβ-PA or GM_1 at 24 h after intravenous administration was 0.033 or 0.0022. The most striking aspects of these results were that the activity of GM_1 in prolonging circulation time and reducing uptake by the RES was scarcely effective in rats, while that of Neu5Acβ-PA was effective not only in mice but also in rats. Similar results were also obtained for the effect of Neu5Acβ-PA on the blood circulation and tissue distribution of liposomes after intravenous administration in tumor-bearing rats (data not shown). The plasma concentration of liposomes containing Neu5Acβ-PA was about 50.3-fold that of liposomes containing GM_1 at 6 h. The plasma/RES ratios of liposomes containing Neu5Acβ-PA or GM_1 at 6 h after intravenous administration in tumor-bearing rats were 0.27 or 0.0017, and the liver/tumor ratios of these liposomes at 6 h were 0.90 (Neu5Acβ-PA) or 8.50 (GM_1). Fig. 6 shows the tissue distribution of liposomes containing various molar ratios of

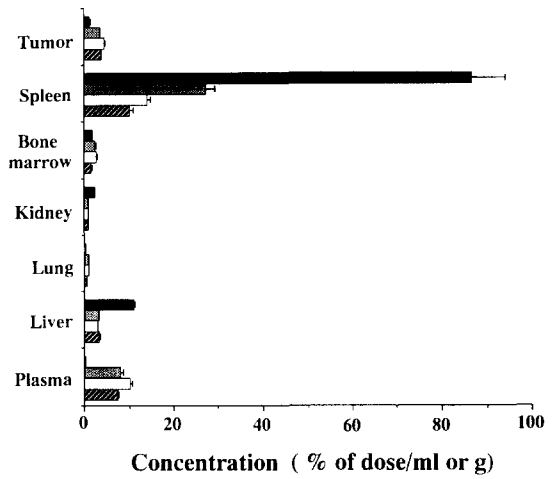

Fig. 6. Tissue distribution of liposomes containing various molar ratio of Neu5Acβ-PA or GM_1 in tumor-bearing rats. The distribution was measured 6 h after intravenous administration. Each bar represents the mean ± S.E. (■) DPPC/Chol/GM_1 = 10:10:1, (▨) DPPC/Chol/Neu5Acβ-PA = 10:10:1, (□) DPPC/Chol/Neu5Acβ-PA = 10:10:1, (▨) DPPC/Chol/Neu5Acβ-PA = 10:10:3.

Neu5Acβ-PA at 6 h after intravenous administration in tumor-bearing rats. As can be seen in Fig. 6, in the case of liposomes containing Neu5Acβ-PA, there were no significant differences in plasma, liver and tumor concentrations at various molar ratio of Neu5Acβ-PA, although the spleen uptake was decreased with increasing molar ratio of Neu5Acβ-PA. It was suggested that liposomes composed of DPPC, Chol and Neu5Acβ-PA at a molar ratio of 10:10:1 were effective for prolonging circulation and reducing the RES uptake.

4. Discussion

In the present study we examined the blood circulation and tissue distribution of liposomes containing Neu5Acβ-PA compared with those of liposomes containing GM_1. Neu5Acβ-PA is a novel synthetic glycolipid and is readily incorporated into the liposomal membrane; its structure is a simple one compared to those of the other glycolipids.

It has been reported that liposomes containing GM_1 exhibited a prolonged circulation time and reduced the RES uptake in mice (Allen and Chonn, 1987). It has been suggested that the negative charge of sialic acid of GM_1 is shielded from the surface by the presence of two neutral sugars, and this shielded negative charge effect may consequently prevent or decrease opsonization of the liposomes (Allen et al., 1989). In the case of phosphatidylinositol, it was considered that the negative charge was also shielded by sugar (Gabizon and Papahadjopoulos, 1988). In addition, it was suggested that the role of sialic acid of serum protein was important for prolonging circulation time (Morell et al., 1968), and the role of the membrane sialic acid of erythrocytes was also important for their survival (Durocher et al., 1975). It was also considered that the presence of sialic acid residues of glycophorin and ganglioside GM_3 on the liposomal surface played an important role in the blood circulation of the liposomes (Yamauchi et al., 1993). On the other hand, some negatively charged synthetic phospholipids or glycolipids have been investigated. In general, it has been observed that liposomes containing phosphatidylserine (PS), phosphatidylglycerol (PG) or phosphatidic acid (PA) (negatively charged liposomes) are rapidly removed from the blood circulation (Senior, 1987). In addition, it was suggested that a hydrophilic carbohydrate moiety and a sterically hindered negatively charged group are effective in retarding liposome clearance (Gabizon and Papahadjopoulos, 1992). However, *N*-glutaryl dioleoylphosphatidylethanolamine (DOPE), *N*-adipyl DOPE (Park et al., 1992) and glucuronic acid derivative (Namba et al., 1990) exhibit a prolonged circulation time of liposomes. In the case of these lipids, the negative charge of the lipids is situated at the terminal position of the molecule and are not shielded by other functional groups. Therefore, we believe that this is not important for the position of sialic acid in the glycolipid. In addition, facile methods have been developed which are applicable to large-scale preparation of the sialic acid derivative (sialoglycoside), and large quantities of it can readily be made (Higashi et al., 1992). Commercial production of liposomes containing naturally

occurring substances such as GM_1 is not feasible due to the large quantities needed. Consequently, we have synthesized Neu5Acβ-PA for prolonging circulation time and reducing the RES uptake of liposomes. As anticipated, the activity of Neu5Acβ-PA in prolonging circulation time and reducing the RES uptake of liposomes is efficient not only in mice (Fig. 3) but also in rats (Fig. 4–6). It has not been clarified why the ability of GM_1 in prolonging circulation time of liposomes was effective in mice but not in rats. It is presumed that some opsonins in the blood of rats would be affected by liposomes containing GM_1, however, further examination to clarify the results is necessary. Although the mechanism of the activity of Neu5Acβ-PA is not yet clear, it is postulated that the hydration of the liposomal surface because of the existence of sialic acid is one reason. Further study is needed to elucidate this mechanism.

In these experiments, we have examined the tumor accumulation of the liposomes. In our experiments, the particle sizes of the liposomes were in the range of 80–90 nm. It has been suggested that the liposome size affects the tumor accumulation of liposomes, and the optimal size range of liposomes has been suggested to be 70–200 nm (Liu et al., 1992). It is considered that the liposome diameter in these experiments is of sufficient value in the disposition of liposomes in tumor-bearing mice and rats. The liver/tumor ratio of liposomes containing Neu5Acβ-PA was similar compared with that of liposomes containing GM_1 in mice (Fig. 3) and lower than that of liposomes containing GM_1 in rats (Fig. 6). One of the mechanisms of accumulation of liposomes containing Neu5Acβ-PA in tumor tissue is considered to be passive targeting. Because of the enhanced permeability of tumor vasculature and little recovery from lymphatics, macromolecules can be progressively accumulated in the tumor tissues (Matsumura and Maeda, 1986). Gabizon and Papahadjopoulos (1988) and Oku et al. (1992) have shown that long-circulating liposomes are also accumulated in tumors via the above mechanism. Thus, it is considered that the accumulation of liposomes containing Neu5Acβ-PA could be attributed to the same mechanism.

5. Conclusion

Our results indicate that a synthetic sialic acid derivative, Neu5Acβ-PA, is valuable for increasing the plasma concentration and reducing the RES uptake of liposomes not only in mice but also in rats. Furthermore, liposomes containing Neu5Acβ-PA are accumulated in solid tumors. Neu5Acβ-PA can be produced in large quantities and is readily incorporated into the liposomal membrane. It is thought that this sialic acid derivative has considerable potential for the application of liposomes as a targeting and slow release system.

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