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Lymph node targeting and pharmacokinetics of [^{3}H]methotrexate-encapsulated neutral large unilamellar vesicles and immunoliposomes

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Summary

The lymph node targeting ability and the pharmacokinetics of [^{3}H]methotrexate (MTX)-bearing neutral large unilamellar vesicles (NLUV) and immunoliposomes (IL; anti-rat immunoglobulin G-conjugated liposomes) were compared with those of free [^{3}H]MTX after intravenous (i.v.) or intramuscular (i.m.) injection to rats. The plasma radioactivity declined slowly after i.v. injection of the NLUV or IL when compared with that after [^{3}H]MTX, and might be due to the slow release of radioactivity from the NLUV or IL which are present in plasma and/or taken up into tissues. The values of $AUC_{0-24\,h}$ in the regional lymph nodes were 6.60- and 6.66-fold increased after i.m. injection of NLUV and IL, respectively, when compared with the value after free [^{3}H]MTX, and the corresponding values for nonregional lymph nodes were 5.35- and 4.78-fold. It strongly suggested that the NLUV or IL could have a better lymph targeting ability than that of free [^{3}H]MTX. The encapsulation efficiency of [^{3}H]MTX in the NLUV increased with increasing cholesterol contents up to 44 mol% and decreased thereafter as prepared by the reverse phase evaporation method. The in vitro release of radioactivity was reduced when the NLUV or IL was incubated in plasma kept at 37°C and at a rate of 50 oscillations/min (opm) when compared with the value from [^{3}H]MTX using a dialysis bag.

Introduction

It is desirable to prevent metastasis in cancer chemotherapy and the lymphatic system is an important pathway of metastasis in the most neoplastic diseases (Poste and Fidler, 1980). The quantitative entry of anticancer drugs into the lymphatic systems after local administration (Kaledin et al., 1981; Parker et al., 1981; Khato et al., 1982; Patel et al., 1984; Hirano and Hunt, 1985) might be of particular interest for the directing of anticancer agents to the metastasis within the lymphatics as well as for the visualization of lymph nodes. For this purpose, liposomes bearing anticancer drugs could be a good candi-

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date to increase the localization of the liposomes and prolong their retention time in lymph nodes, thus enabling the entrapped anticancer drug to be released into the environment slowly and at a desired rate. The lymphatic uptake of liposome-entrapped [^{14}C]inulin (Jackson, 1981), [^{14}C]sucrose (Hirano and Hunt, 1985; Hirano et al., 1985), [^{111}In]bleomycin (Tumer et al., 1983), [^{14}C]melphalan (Khato et al., 1982), [^{125}I]PVP (Patel et al., 1984) and [^{14}C]adriamycin (Parker et al., 1981) was reported after subcutaneous (s.c.), intramuscular (i.m.) or intraperitoneal (i.p.) injection to mice or rats. Moreover, various investigators have reported that liposomes can be targeted to various cells by attaching antibodies such as $F(ab')_2$ prepared from rabbit γ-globulin (Heath et al., 1980), anti-rat erythrocyte $F(ab')_2$ (Singhal and Gupta, 1986), IgG anti-DNP (Leserman et al., 1980b), antibody B1.1G6 (Leserman et al., 1980a), anti-H2K^k antibody (Heath et al., 1983) and Dal K29 (Singh et al., 1989).

The purpose of this paper is to report the lymph node targeting ability and the pharmacokinetics of [^{3}H]methotrexate (MTX)-bearing neutral large unilamellar vesicles (NLUV) and immunoliposomes (IL: anti-rat immunoglobulin G-conjugated liposomes) after intravenous (i.v.) and i.m. injection to rats. The preparation and stability of the NLUV and IL are also reported.

Materials and Methods

Apparatus

A rotary evaporator (BÜCHI 461, Büchi Co., Flawil, Switzerland), vortex type mixer (Thermolyne Maxi Mix II, type 37600, Thermolyne Corp., Dubuque, IA, U.S.A.), ultracentrifuge (L-80 Ultracentrifuge, Beckman Instrument Inc., Palo Alto, CA, U.S.A.), UV spectrophotometer (UV 2100, Shimadzu, Kyoto, Japan), microcentrifuge (Sarstedt MH 2, Sarstedt Co., Germany) and liquid scintillation counter (Rack Beta, LKB-Wallac Co., Turku, Finland) were employed.

Materials

Dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylcholine (DPPC), cholesterol (CH), *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS), 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (SPDP), dithiothreitol (DTT), Sephadex G-50 (medium), anti-rat IgG (Ab), 2,5-diphenyloxazole (PPO) and 1,4-bis(5-phenyl-2-oxazolyl)benzene (POPOP) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). MTX was kindly supplied by the Choog-Wae Pharmaceutical Co. (Suwon, South Korea). Sodium [3′,5′,7-^{3}H]MTX ([^{3}H]MTX) was a product of Amersham International Plc. (Buckinghamshire, U.K.). The stock solution of [^{3}H]MTX dissolved in distilled water was used without further purification in combination with unlabeled MTX. Soluene-350® (0.5 N quaternary ammonium in toluene; $C_{25}H_{55}NO$) and Scinti-A XF® were purchased from Packard Instrument Co., Inc. (Downers Grove, IL, U.S.A.). All other chemicals were of reagent grade and used without further purification.

Preparation of NLUV and IL

The activated phospholipid derivative, maleimidobenzoylphosphatidylethanolamine (MBPE) was synthesized from DPPE and MBS by a slight modification of the reported method (Martin and Papahadjopoulos, 1982). Briefly, DPPE (80 mg) was dried by rotary evaporation in a round-bottomed flask and redissolved in 32 ml of dry chloroform (dried over a molecular sieve type 4A). MBS (48 mg) in 4 ml of dry methanol and triethylamine (200 μmol) were added to the flask. The mixture was allowed to react under nitrogen gas at room temperature until the reaction was complete. The progress of the reaction was checked by thin-layer chromatography (chloroform/methanol/water 65:25:4) using silica gel (Merck, D-6100, Darmstadt, Germany). MBPE moved further ($R_f = 0.8$) than free DPPE ($R_f = 0.6$). The organic solvent was then removed under reduced pressure and the product was redissolved in chloroform. The products were immediately applied to the silica gel column equilibrated with chloroform. The column was washed with 10 ml of chloroform followed by 12 ml of each of the following chloroform/methanol mixtures, 16:1, 16:2, 16:3 and finally 16:4. The fractions containing the desired product were pooled and con-

centrated by evaporation under reduced pressure in a rotary evaporator. The product dissolved in chloroform was stored at −4°C under nitrogen gas.

NLUV were prepared according to the reported evaporation (REV) method (Szoka and Papahadjopoulos, 1978; Kim and Lee, 1987; Kim et al., 1991) with minor modifications. Briefly, the lipid mixture composed of DPPC, CH and MBPE (molar ratio 12:11:2) was dissolved in chloroform at 50°C. The chloroform was removed from the rotary evaporator under reduced pressure and the resultant dried lipid film was again dissolved in 4 ml of a 1:2 mixture of chloroform/isopropyl ether. 1 ml of TES buffer containing 500 μg of MTX and trace amounts of [^{3}H]MTX (10 μCi) were added and the two phases were emulsified by vortex mixing. The emulsion was incubated for 30 min at room temperature. The organic phase was removed under reduced pressure at room temperature. To remove the unencapsulated drugs, the resulting dispersed liposomes were centrifuged at 45 000 rpm (180 000 × g) for 15 min, the pelleted vesicles were resuspended in TES buffer and appropriate dilutions were made.

The thiolated antibody (Ab-SH) was synthesized from SPDP, anti-rat IgG and DTT following the reported method (Martin et al., 1990). IL was prepared by mixing Ab-SH with NLUV prepared by the REV method (Szoka and Papahadjopoulos, 1978). The amounts of protein (Ab) coupled to liposomes were determined according to Lowry et al. (1951) in the presence of sodium deoxycholate. To determine the entrappment of [^{3}H]MTX in the NLUV or IL, as expressed in terms of radioactivity, a 50 μl aliquot of NLUV or IL suspension was counted in a liquid scintillation counter. The NLUV and IL were stored at 4°C and used within 12 h after preparation.

Stability in plasma

The stability of [^{3}H]MTX-bearing NLUV or IL in plasma was determined using the dialysis method (Mayhew et al., 1984). 2.5 ml of plasma was pipetted into a new, clean dialysis bag (Mol. Wt cutoff 12 000–14 000, 15.9 mm/D, Medical Industries, Inc., Los Angeles, CA, U.S.A.) which had been boiled and equilibrated in PBS, and then NLUV or IL, equivalent to 0.26 μCi, was added. The bag was secured with two knots at each end and air spaces minimized as much as possible. Mixing of the contents was performed by squeezing gently and inverting the bag a few times. The bag was placed immediately into dialysate containing 80 ml of PBS kept at 37°C and a rate of 50 oscillations per min (opm). At designated intervals, 0.5 ml samples were removed from the dialysate and the radioactivity was counted. Radioactivity in the dialysate, which was measured at certain time intervals, and initial radioactivity in the dialysis tubing were expressed as C_t and C_0, respectively, and the rate of release of [^{3}H]MTX was calculated as $(C_t/C_0) \times 100$.

Animals

Male Wistar Albino rats (200–250 g) were obtained from the Laboratory Animal Center, Seoul National University (Seoul, South Korea). The rats were fed with tap water and food (Cheil Food and Chemical Co., I-Cheon, South Korea) ad libitum.

Intravenous studies

The carotid artery and jugular vein were cannulated with polyethylene tubing (Clay Adams, Parsippany, NJ, U.S.A.) under light ether anesthesia. Both cannulas were exteriorized on the dosal side of neck where each cannula terminated with long silastic tubing (Dow Corning, Midland, MI, U.S.A.). The silastic tubing was covered with wire to allow free movement of the rat. The exposed areas were closed using a surgical suture. Each animal was kept for 4–5 h in a metabolic cage to recover from anesthesia. [^{3}H]MTX (treatment I), and freshly prepared [^{3}H]MTX-bearing NLUV (treatment II) or IL (treatment III) were administered at a dose of 200 μg MTX per kg body weight via the jugular vein (injection volume approx. 1.2 ml per kg body weight). Approx. 0.3 ml of heparinized normal saline (100 U ml^{-1}) was used for flushing the cannula just after each blood sampling. Approx. 0.12 ml of blood was collected via the carotid artery at 0 (to serve as a control), 1, 5, 15, 30, 45, 60, 90, 120, 180, 240, 360 and 480 min after drug administration. The blood

samples were centrifuged immediately to minimize the possible 'blood storage effect' in the plasma concentrations of radioactivity (Lee et al., 1984, 1986), and the radioactivity in the plasma was measured immediately. At the end of 24 h, the metabolic cage was rinsed with 30 ml of distilled water and the rinsings were combined with the 24 h urine. After measuring the exact volume of the combined urine, the radioactivity in the combined urine was determined immediately.

Intramuscular studies

[^{3}H]MTX-bearing NLUV or IL was administered intramusculary according to the reported method (Arakawa et al., 1975). Before experiments, each rat was fasted for 16–20 h. [^{3}H]MTX (treatment IV), and freshly prepared [^{3}H]MTX-bearing NLUV (treatment V) or IL (treatment VI) were injected (injection volume approx. 1.2 ml per kg body weight) into the center of the right thigh muscle (musculus rectus of the rat) of the rat at a dose of 200 μg MTX per kg body weight. At scheduled time intervals, each rat was exsanguinated and blood was collected. Blood samples were centrifuged immediately, and 150 μl of plasma was collected for the determination of radioactivity. The injected right thigh muscle, right (RLN) and left (LLN) ipsilateral iliac lymph nodes, liver, spleen, kidney, lung, heart, stomach, and small and large intestines were removed, rinsed with PBS, blotted with a paper towel and weighed.

Measurement of radioactivity

The tissue samples were solubilized using Soluene-350® (1 ml/0.2 g tissue) at 50°C for 24 h. After cooling to room temperature, they were decolorized by bleaching with 0.2 ml of isopropyl alcohol and 0.4 ml of 35% hydrogen peroxide. All samples treated with Soluene-350® were neutralized with 0.1 ml of 5 N HCl. 10 ml of scintillation cocktail (Scinti-A XF® (Packard Co.): 1 : 1 PPO/POPOP cocktail (a mixture of 667 ml of toluene, 333 ml of Triton X-100, 5.5 g of PPO and 0.1 g of POPOP) was then added and the samples were equilibrated for at least 24 h prior to counting. The counts obtained were corrected using the standard channel ratio method. In the present study, total radioactivity was measured, therefore, it does not represent [^{3}H]MTX only, but rather corresponds to the sum of [^{3}H]MTX, [^{3}H]MTX-bearing NLUV or IL and their possible metabolites.

Pharmacokinetic analysis

The following pharmacokinetic parameters were calculated using general methods (Chiou, 1978; Gibaldi and Perrier, 1982); the area under the plasma concentration-time curve from time zero to time infinity (AUC), area under the first moment of AUC (AUMC), mean residence time (MRT), apparent volume of distribution at steady state (V_{ss}), and time-averaged total (CL), renal (CL_R) and nonrenal (CL_{NR}) clerances. In the calculation of CL_R, the AUC obtained from time 0 to 24 h ($AUC_{0-24\ h}$; 30.8, 390 vs 158 percentages of dose min ml^{-1}) were employed because urine was collected for up to 24 h.

Statistical analysis

The data were analyzed for statistical significance ($P < 0.05$) by analysis of variance test among means for unpaired data.

Results and Discussion

Preparation of [^{3}H]MTX-bearing NLUV or IL

The encapsulation efficiency of [^{3}H]MTX (as expressed in terms of radioactivity) in NLUV, prepared by the REV method was examined at

TABLE 1

Effect of cholesterol contents on the encapsulation efficiency of MTX

Lipid composition		% of encapsulation (as MTX)
DPPC [a]:CH [b]:DPPE [c]	CH (mol%)	
12:3.5:1.5	21	10.6
12:6:1.5	31	12.5
12:11:2	44	20.0
12:15:1.5	53	15.3

[a] Dipalmitoylphosphatidylcholine.
[b] Cholesterol.
[c] Dipalmitoylphosphatidylethanolamine.

various molar ratios of CH ranging from 21 to 53 mol% (Table 1). The efficiency increased with increasing CH up to 44 mol% (percent encapsulation of MTX was 20.0%) and declined thereafter. The decrease in efficiency at 53 mol% of CH could be due to the fact that liposomes are not readily formed above 50 mol% of CH. A similar result was also reported by Umeda et al. (1986) showing that liposomes containing an equimolar ratio of DPPC and CH were stable. Therefore, liposomes containing DPPC and CH at a molar ratio of 12:11 were chosen in the present study. CH is also known to reduce the permeability of drugs from liposomes, therefore, CH ensures the stability of liposomes in serum (Allen and Cleland, 1980).

Preparations of IL at various antibody concentrations are listed in Table 2. Variations in the initial protein (antibody) concentrations in the reaction mixture (19.7–274 μg ml^{-1}) caused large variations in the protein/lipid ratio of the products; the amounts of coupled protein increased with increasing protein concentration in the reaction mixture. This allows the liposomes to be modified with theoretical amounts of protein by controlling the initial protein concentration.

In vitro release of radioactivity from [^{3}H]MTX-bearing NLUV or IL in plasma using a dialysis bag

Fig. 1 shows the percent radioactivity released from the dialysis bag containing free [^{3}H]MTX, [^{3}H]MTX-bearing NLUV or IL in plasma kept at 37°C and a rate of 50 opm as a function of time. The release of radioactivity from free MTX was

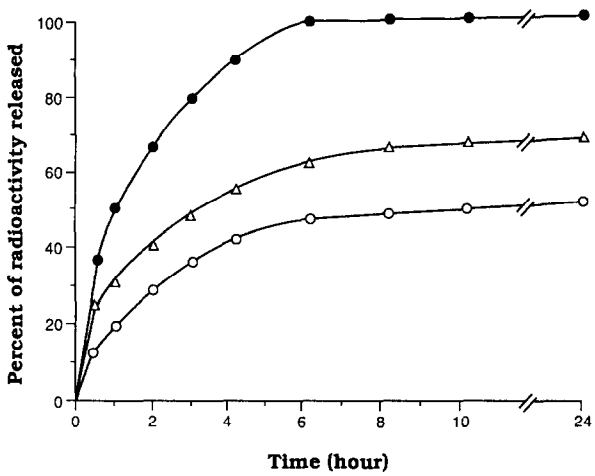

Fig. 1. Percentages of radioactivity (as expressed in terms of [^{3}H]MTX) released from the dialysis bag containing plasma spiked with free [^{3}H]MTX (●), [^{3}H]MTX-bearing neutral large unilamellar vesicles (○) or immunoliposomes (△) after incubation in PBS kept at 37°C and a rate of 50 opm.

fast; essentially complete release was achieved up to 6 h of incubation. However, the release of radioactivity from NLUV or IL displayed an initial rapid release period up to approx. 3–4 h, thereafter being very much slower. The rapid release of radioactivity up to 3–4 h of incubation could be due to the release of radioactivity which is encapsulated in the surface nearby of the NLUV or IL. The release of radioactivity from NLUV or IL seemed to be slower than that of free MTX; approx. 62, 47 and 100% of radioactivity were released from the IL, NLUV and free MTX, respectively, up to 6 h of incubation, the corresponding values up to 24 h incubation being 69, 53 and 100%. These observations appear to indicate (Allen and Cleland, 1980) that plasma components could interact with the liposomes and then destabilize the liposomal membranes to leak out the entrapped drugs. The release of radioactivity from the IL tended to be greater than that from the NLUV, and may be attributed to the presence of rat IgG in rat plasma, i.e., the membrane of the IL was more easily destabilized than that of the NLUV, since antigen (rat IgG) in rat plasma readily reacts with antibody (anti-rat IgG) on the IL surface.

TABLE 2

Conjugation ratios of anti-rat IgG antibody (Ab) to liposomes under various antiby concentrations

Reactants		Product
Ab concentration (mg ml^{-1})	Lipid concentration (μmol ml^{-1})	Ab:lipid (μg μmol^{-1})
0.0197	1.53	7.91
0.0591	1.53	14.0
0.0709	1.53	18.0
0.274	1.53	35.5

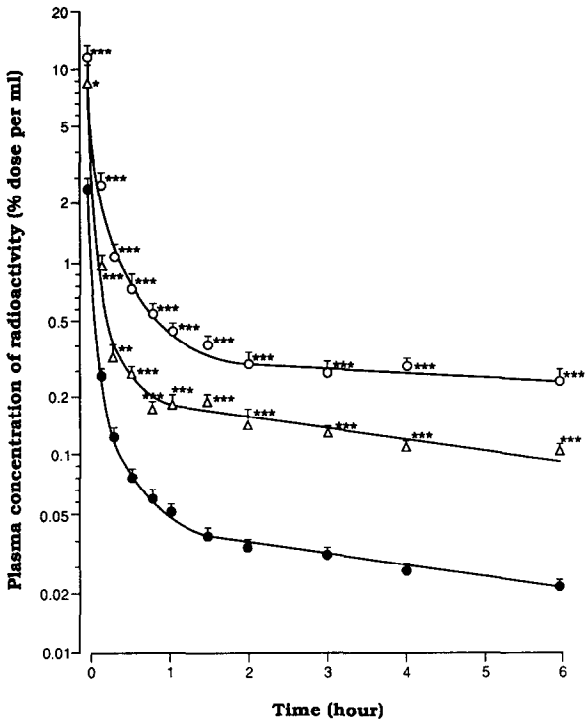

Fig. 2. Plasma concentration-time profiles of radioactivity after i.v. administration of free [^{3}H]MTX (●, treatment I), [^{3}H]MTX-bearing neutral large unilamellar vesicles (○, treatment II) and immunoliposomes (△, treatment III). Bars represent standard deviation. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with the values from treatment I.

Pharmacokinetics after i.v. injection

The mean arterial plasma concentration-time curves of radioactivity after i.v. administration from treatments I–III are shown in Fig. 2, and the relevant pharmacokinetic parameters are listed in Table 3. After i.v. administration, the plasma levels declined rapidly up to 1–2 h, and declined slowly thereafter with significantly higher plasma levels from NLUV and IL than those from free [^{3}H]MTX. This resulted in higher AUC values and lower CL values from treatments II and III than those from treatment I. The terminal half-lives were considerably longer from treatments II and III; the values were 336, 1460 and 810 min from treatments I–III, respectively. The plasma concentrations of radioactivity from treatment II were significantly higher than those from treatments I and III and the plasma levels declined more slowly from treatments II and III than those from treatment I. This could be due to slow release of radioactivity from NLUV (treatment II) or IL (treatment III) which are present in blood and/or taken up into tissues, and could be expected based on the in vitro release study (Fig. 1). The kidney is the main organ for eliminating MTX and its metabolite, 7-hydroxymethotrexate, however, MTX-bearing NLUV or IL itself could not be excreted via the kidney. Therefore, the amounts of radioactivity excreted in 24 h urine decreased significantly ($p < 0.001$) from treatments II and III; the mean values were 45.6 ± 7.33, 31.5 ± 1.23 and 26.3 ± 9.90% of the administered radioactivity from treatments I–III, respectively. As a result, the values of CL_R from treatments II and III decreased considerably compared with that from treatment I (1.48, 0.0807 vs 0.167 ml min^{-1} kg^{-1}). The values of MRT (359, 1920 vs 1010 min) increased considerably from treatments II and III, and might be due to

TABLE 3

Some pharmacokinetic parameters of radioactivity after intravenous administration of free [^{3}H]MTX (treatment I), [^{3}H]MTX-bearing neutral large unilamellar vesicles (treatment II) and immunoliposomes (treatment III)

	Treatment I	Treatment II	Treatment III
$t_{1/2}$ (min)	336	1460	810
AUC (% of dose min ml^{-1})	32.0	1730	212
AUMC (% of dose min^2 ml^{-1})	11500	1410000	214000
MRT (min)	359	1920	1010
CL (ml min^{-1} kg^{-1})	3.13	0.136	0.472
CL_R (ml min^{-1} kg^{-1})	1.48	0.0807	0.167
CL_{NR} (ml min^{-1} kg^{-1})	1.65	0.0553	0.305
V_{ss} (ml kg^{-1})	1120	262	478

the slow release of [^{3}H]MTX from NLUV or IL which are accumulated in tissues (or organs) and/or present in plasma.

Disappearance of radioactivity from the i.m. injection site (right thigh muscle)

The disappearance of radioactivity from the i.m. injection site (right thigh muscle) from treatments IV–VI is shown in Fig. 3a. The disappearance of free [^{3}H]MTX after i.m. injection (treatment IV) was rapid and essentially complete; 95.2% of the administered radioactivity had disappeared from the injection site after 30 min of i.m. administration and only 0.19% of the administered radioactivity remained at 2 h after injection. The rapid and almost complete disappearance of free MTX after i.m. administration has also been reported in rabbits (Yoon et al., 1990) and humans (Shen and Azarnoff, 1978). However, the disappearance of radioactivity from the injection site from treatments V and VI (not significantly different between treatments V and VI) was considerably slower than that from treatment IV; approx. 16.4 and 23.1% of the administered dose remained at the injection site even at 24 h after i.m. administration from treatments V and VI, respectively. These results suggest that radioactivity may be slowly absorbed from the injection site due to slow release of radioactivity from NLUV or IL (Arakawa et al., 1975), and/or slow drainage of NLUV or IL into lymph nodes.

Targeting of [^{3}H]MTX-bearing NLUV or IL in regional (RLN) and non-regional (LLN) lymph nodes

The potential of MTX-bearing NLUV or IL as a lymphotropic carrier of MTX has been investigated. Fig. 3b and c shows the localization of radioactivity in primary regional (RLN) and contralateral (LLN) lymph nodes from treatments IV–VI, respectively. From treatments V and VI, the radioactivity in the right iliac lymph nodes (RLN) was generally 3–10-fold higher than that from treatment IV at each time interval sampled and similar results were also obtained from LLN, although the radioactivity in LLN was lower than that in RLN. It should be noted that there were no significant differences in lymph targeting between NLUV and IL for both RLN and LLN. However, it was reported (Mangat and Patel, 1985) that liposomes 'coated' with non-specific human IgG disappeared more rapidly from the injection site (footpad of mice or rats) than control liposomes (meaning that absorption was faster

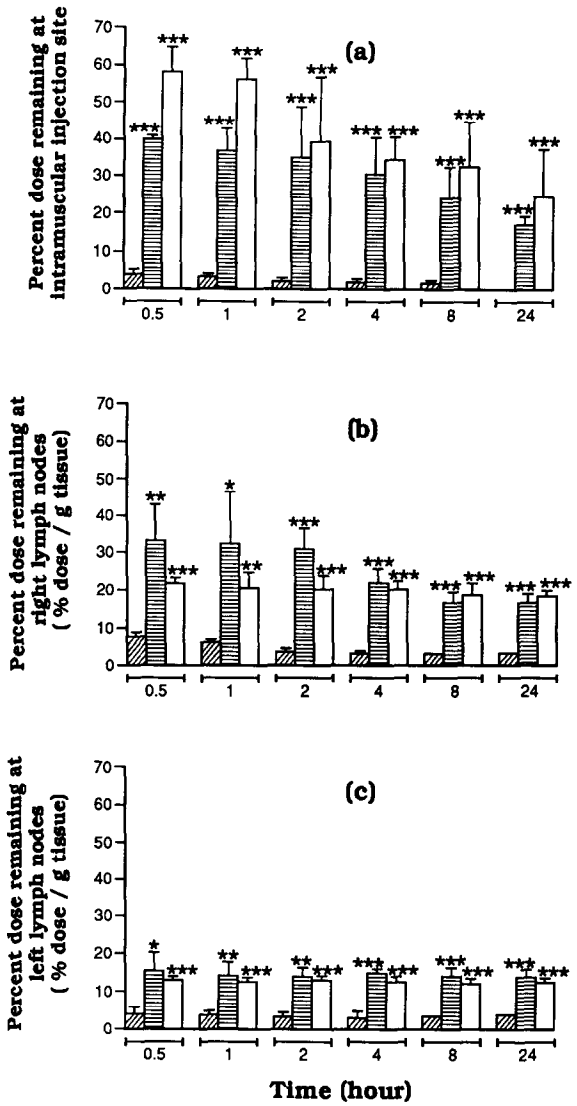

Fig. 3. The percentages of the dose remaining at i.m. injection site (a), right lymph nodes (b) and left lymph nodes (c) after i.m. injection of free [^{3}H]MTX (▨, treatment IV), [^{3}H]MTX-bearing neutral large unilamellar vesicles (▤, treatment V) and immunoliposomes (□, treatment VI). Bars represent standard deviation. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with the values from treatment IV.

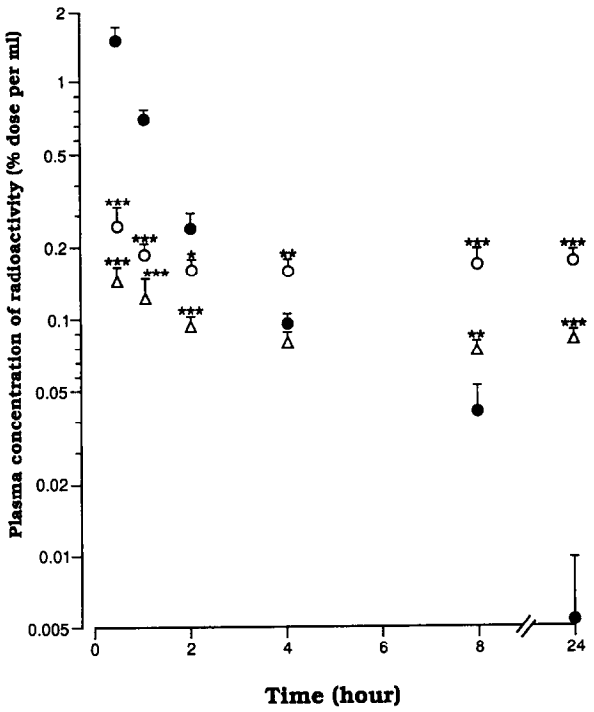

Fig. 4. Plasma concentration-time profiles of radioactivity after i.m. administration of free [^{3}H]MTX (●, treatment IV), [^{3}H]MTX-bearing neutral large unilamellar vesicles (○, treatment V) and immunoliposomes (△, treatment VI). Bars represent standard deviation. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with the values from treatment IV.

than that of control liposome), and increased their localization as well as retention time in lymph nodes. The difference could be due to the differences in affinity of the antibodies used; Fc-receptor bearing migrating cells such as lymphocytes, monocytes or neutrophils may bind to the IgG-coated liposomes and could have carried these liposomes to lymph nodes (Mangat and Patel, 1985). However, in the present study Ab is non-specific and its Ag (rat IgG) is distributed throughout the body of rats.

Plasma concentration-time profiles of radioactivity after intramuscular injection

Fig. 4 shows the plasma concentration-time profiles of radioactivity after i.m. injection from treatments IV–VI. The initial (up to 2 h) levels of plasma radioactivity from treatments V and VI were significantly lower than that from treatment IV, and might be due to the slow drainage of the vesicles into lymphatics (Fig. 3a) and considerable entrappment of NLUV or IL at lymph nodes (Fig. 3b and c). However, the plasma levels declined more slowly from 2 h after injection from treatments V and VI than those from treatment IV, and might be due to slow release of radioactivity from NLUV or IL at blood and/or lymph nodes. It was reported (Patel et al., 1984) that liposomes injected s.c. were drained from the injection site into the circulation via lymphatics: in lymphatics, some liposomes were degraded, others were retained in the lymph nodes and the rest reached the circulation. It should be noted that similar values for the terminal half-lives were obtained after i.v. (336 min) and i.m. (292 min) administration of free [^{3}H]MTX, as expected based on the fast and essentially complete absorption of free [^{3}H]MTX after i.m. administration.

Organ distribution of radioactivity after i.m. injection

Table 4 lists the values of $AUC_{0-24\,h}$ of radioactivity for each organ from treatments IV–VI. The values in RLN were 6.60- and 6.66-fold increased from treatments V and VI, respectively, as compared with that from treatment IV, and

TABLE 4

Area under the concentration-time curve of total radioactivity from time 0 to 24 h ($AUC_{0-24\,h}$) after intramuscular injection of free [^{3}H]MTX, [^{3}H]MTX-bearing neutral large unilamellar vesicles (NLUV) and immunoliposomes (IL) to rats

Tissues	$AUC_{0-24\,h}$ (% of dose h g^{-1} tissue or ml^{-1} plasma)		
	Free MTX	NLUV	IL
RLN	65.0	429	433
LLN	61.7	330	295
Liver	33.7	29.7	36.1
Spleen	9.09	4.73	5.86
Kidney	159	35.5	50.8
Lung	12.6	3.84	4.88
Heart	3.38	3.28	7.98
Stomach	15.0	8.48	6.72
Small intestine	25.2	22.7	10.4
Large intestine	18.8	16.3	8.26
Plasma	2.28	4.07	1.93

the corresponding values for LLN were 5.35 and 4.78. This strongly suggested that NLUV or IL could have good lymph targeting ability. It should be noted that the $AUC_{0-24\,h}$ for kidney, the main organ for eliminating MTX, was 4.45- and 3.13-fold lower from treatments V and VI, respectively, as compared with that from treatment IV. The value of $AUC_{0-24\,h}$ for the GI tract was 2.32-fold lower from treatment VI than that from treatment IV. This might suggest that the i.m. administration of NLUV and IL may have lesser side effects in the kidney, and IL in the GI tract than those of free [^{3}H]MTX. This hypothesis, however, remains to be validated.

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