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Studies on Pharmaceutical Modification of Anticancer Agents. I. Enhancement of Lymphatic Transport of Mitomycin C by Parenteral Emulsions

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To prevent metastasis along the lymph pathways, it is greatly necessary to create a selectively high concentration of anticancer agent in the lymphatic system. The enhancement of lymphatic transport of Mitomycin C (MMC) was accomplished by the injection of fat emulsion to tissue spaces. A comparison of concentration of MMC in thoracic duct lymph and plasma after intraperitoneal and intramuscular injection showed that W/O emulsion was the most effective and followed by O/W emulsion and aqueous solution. In O/W emulsion, the one emulsified by gelatin observed to facillitate the lymphatic transport whereas the one emulsified by polysorbate 80 had little effect. This difference appears to depend upon the binding to oil droplets, because MMC was bound to oil only in the emulsion by gelatin. A measuring of oil in thoracic duct lymph and lymph nodes indicated that MMC did not always accompany with oil in the lymphatic system.

For the effective cancer chemotherapy, it is necessary to deliver a sufficiently high concentration of anticancer agent into the tumor tissues for a required period, and to minimize their concentration in the other tissue compartments of the body because of their adverse reaction. From this reason the potential drug delivery systems, which can control the release of the drug to maintain their concentration particularly in the tumor area or supply them selectively to tumor rich tissue, are desired from a viewpoint of chemotherapy and biopharmaceutical dosage design.

In order to prevent metastasis along the lymph pathways, it is essential to create a selectively high concentration of anticancer agent in the lymphatic system. On the basis of the accumulation or high transportation of lipids[2] and chylomicrons[3] into the lymphatic system during fat absorption, we attempted to utilize anticancer agents as various types of fat emulsions.

The effect of fat emulsion on lymphatic transport has not been much investigated with exception of intestinal absorption.[4,5] However Takahashi, *et al.*[6] have actually observed in cooperation with our laboratory that a parenteral administration of Mitomycin C (MMC) to tissue spaces by the emulsion preparations was more effective than the aqueous preparation for lymph node metastasis. Thus the lymphatic transport of MMC from oil in water (O/W) and water in oil (W/O) types of emulsion was studied by intraperitoneal and intramuscular injections into rat. This paper describes the fundamental aspect on the enhancement of the lymphatic transport by emulsion formulation.

1) Location: *a) Kawai-cho, Matsubara, Osaka; b) Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto.*
2) M.J. Yoffey, C.F. Courtice, "Lymphatics, Lymph and Lymphomyeloid Complex," Academic Press Inc., London and New York, 1970.
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Experimental

Materials——MMC was supplied from Sankyo Co. Ltd. Sesame oil, gelatin, polysorbate 80 and egg albumin were obtained from Nakarai Chemical Co. Ltd., and HCO-60 and SO-15 were obtained from Nikko Chemical Co. Ltd.

Preparation of Emulsions——O/W Emulsion: An oil phase was sesame oil and an aqueous phase was distilled water containing MMC, 4% of gelatin or 1% of polysorbate 80. Oil content was 20% (v/v). This mixture was sonicated for 2—5 min by Ohtake sonicator (20 kc). The mean diameter of oil droplets emulsified by gelatin was 2—3 μ, being relative uniformity, and that of oil droplets containing polysorbate 80 was 5—6 μ according to microscopic observation.

W/O Emulsion: An aqueous phase was distilled water containing MMC and oil phase was sesame oil incorporating SO-15 and HCO-60, 7.0 and 1.7% respectively. Both phases were heated separately in a water bath at 60°, and the mixture composed of 60% (v/v) of oil phase was sonicated at 60° for 2—5 min.

Procedure of Lymphatic Transport Experiments——Male Wistar rats (200—250 g) were allowed free access to food and water until approximately 30 min before surgery. The animals were anesthetized by the intraperitoneal injection of sodium pentobarbital and the thoracic duct was cannulated according to the method of Bollman, *et al.*[7] A heparin filled flexible catheter (i.d. 0.5 mm, o.d. 0.8 mm, Dural Plastics and Eng. Pty. Ltd., Australia) was inserted into the duct, and fixed with the aid of a drop of tissue cement, Aron Alpha A (Sankyo Co. Ltd.). A 1 ml aliquot of the parenteral preparation under study was administered with a syringe immediately after completion of the cannulation. For intraperitoneal studies 1 ml of the preparation was injected into the center of the peritoneal cavity, and for intramuscular studies each 0.5 ml of the preparation was injected into the centers of the thigh muscles of both rat legs. After administration, saline was supplied by the duodenal administration of 2 ml per hour to obtain constant lymph flow. The total volume of lymph collected and the concentration of drug in the lymph were determined at the end of timed intervals. The concentration of drug in whole blood was also determined at the end of each time interval.

Analytical Methods——The concentrations of MMC in lymph and plasma were determined by the cylinder plate method using *Escherichia coli B* as the test organism. Plasma protein and fat emulsion did not interfere the assay of MMC.

Radioactivity of triolein-^{131}I transferred in thoracic duct lymph or lymph nodes was measured by liquid scintillation counter. Aliquots of the injection solution (50 μl), lymph (50 μl) and lymph nodes weighed were added to 15 ml of the scintillation medium and 1 ml of 1 N HCl. This medium was composed of 4 g of 2,5-diphenyloxazole, 300 ml of nonylphenolpolyethoxyethanol and 700 ml of toluene. The radioactivity was measured with a liquid scintillation spectrometer (Backman LS-232).

Measurement of Binding of MMC to Oil Droplets——The binding was measured by utilizing equilibrium dialysis method.[8] A 10 ml aliquot of O/W emulsion containing MMC or 8 ml of aqueous solution containing the same amount of MMC was respectively put into each Visking cellulose bag, and was equilibrated against 20 ml distilled water shaking overnight at room temperature. The concentration of MMC in outer phase were measured by spectrophotometrically at 214 mμ after equilibrium. The binding percent was calculated from the following equation, $\frac{3.5(M-M')}{3.5M-2.5M'}\times 100$,[9] where M is the outer concentration in the aqueous solution's case and M' is the outer concentration in the emulsion's case after equilibrium.

Results

Lymphatic and Blood Transport by Intraperitoneal Administration

Samples of thoracic duct lymph and cardiac blood were taken at 30 or 60 min intervals and were assayed for their content of biologically active drug after intraperitoneal injection of 0.4 mg per 250 g rat of MMC.

7) J.L. Bollman, J.C. Cain, and J.H. Grindlay, *J. Lab. Clin. Med.*, **33**, 1349 (1948).
8) K. Kakemi, T. Arita, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **13**, 976 (1965).
9) The apparent aqueous phase concentration of MMC (involving MMC bound to oil) in emulsion inside the bag after equilibrium is

$$M + 2.5(M-M') = 3.5M - 2.5M'$$

where 2.5 is the correction factor being devided 20 ml by 8 ml. Let M_b be the concentration of drug bound to oil droplets,

$$M_b = 3.5(M-M')$$

The binding percent is given by

$$\% \text{ bound} = \frac{M_b}{M'+M_b}\times 100 = \frac{3.5(M-M')}{3.5M-2.5M'}\times 100$$

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As shown in Fig. 1, when the aqueous solution was injected, there was no much difference between the drug concentration in thoracic duct lymph and that in plasma at any time, and the peak in lymph was at 30 min after injection. Administration of O/W emulsion prepared by polysorbate 80 as emulsifer were also nearly equal with the aqueous solution. On the other hand, when O/W emulsion containing gelatin was administered, the blood concentration was markedly reduced while the lymph concentration was kept relatively high.

Furthermore at the administration of W/O emulsion the blood concentration was markedly reduced, and the maximum lymph concentration was increased about 3 times of the aqueous solution as shown in Fig. 2.

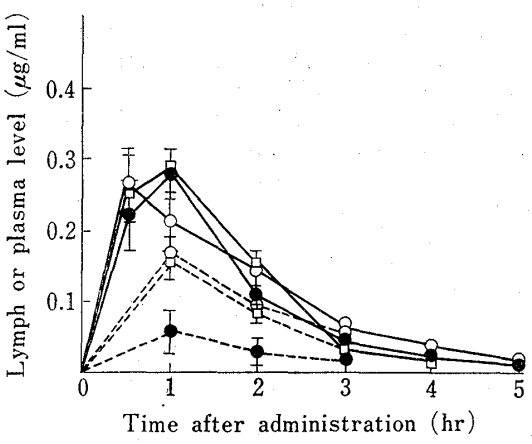

Fig. 1. Effect of O/W Emulsion on the Lymph and Plasma Levels of MMC by Intraperitoneal Administration of 0.4 mg per 250 g Rat

○ : solution, ● : O/W emulsion (Gelatin), □ : O/W emulsion (Polysorbate 80) —— : lymph, ------ : plasma
Results are expressed as the mean of at least 5 animals. The vertical bar indicates ±S.D.

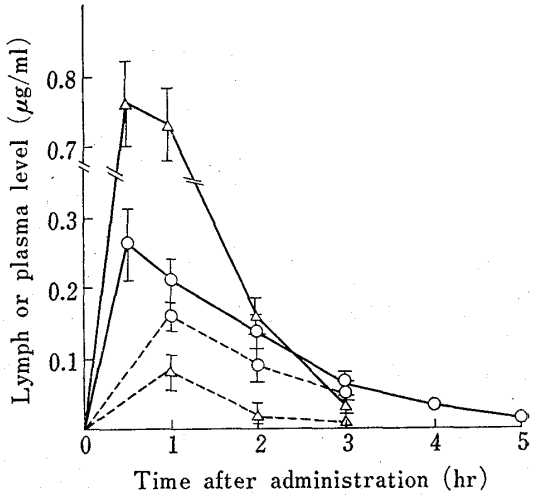

Fig. 2. Effect of W/O Emulsion on the Lymph and Plasma Levels of MMC by Intraperitoneal Administration of 0.4 mg per 250 g Rat

○ : solution, △ : W/O Emulsion
—— : Lymph, ------ : Plasma
Results are expressed as the mean of at least 5 animals. The vertical bar indicates ± S.D.

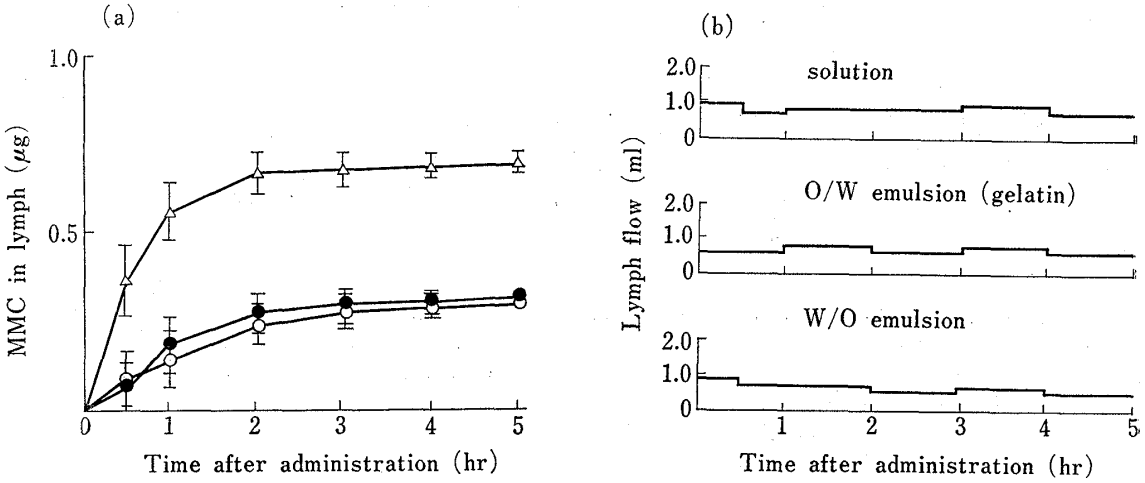

Fig. 3. Cumulative Amount of MMC transferred into Thoracic Lymph after Intraperitoneal Administration

(a) cumulative curve (b) lymph flow
○: solution, ●: O/W emulsion(gelatin), △: W/O emulsion
Results are expressed as the mean of at least 5 animals. The vertical bar indicates±S.D.

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The cumlative amounts transferred in thoracic duct lymph from the peritoneal cavity are summarized in Fig. 3. Lymph flow did not much change in any time and any of the preparations, by supplying of saline into the duodenum. Since every cumulative curves reached maximum in 3 or 4 hours after administration, it is considered that transfer of MMC into thoracic duct lymph completes within 4 hours. The cumulative amount from W/O emulsion was the highest among the four preparations, about 2.5 times of the aqueous solution. The cumulative maximum of the aqueous solution and W/O emulsion were 0.07% and 0.17% of administration dose respectively. But these values do not correspond to percent transferred into lymph due to metabolic inactivation of MMC which supposed to be occurred.

Lymphatic and Blood Transport by Intramuscular Administration

Ballard[10] mentioned in his review that the molecules or ions with low molecular weights intramuscularly injected are absorbed primarily *via* blood capillaries. The concentration of the small molecules in lymph and blood after injection, however, has not almost been investigated until recently. At the intramuscular administration of the aqueous solution the lymph concentration was always exceeding 1.5—2.5 times over the plasma concentration as shown in Fig. 4. When O/W emulsion containing gelatin was injected, the concentration in lymph and plasma was both increased. At the administration of W/O emulsion, the lymph concentrations were the highest as similar to the intraperitoneal administration.

The cumurative amounts transferred in thoracic duct lymph from the muscle are shown

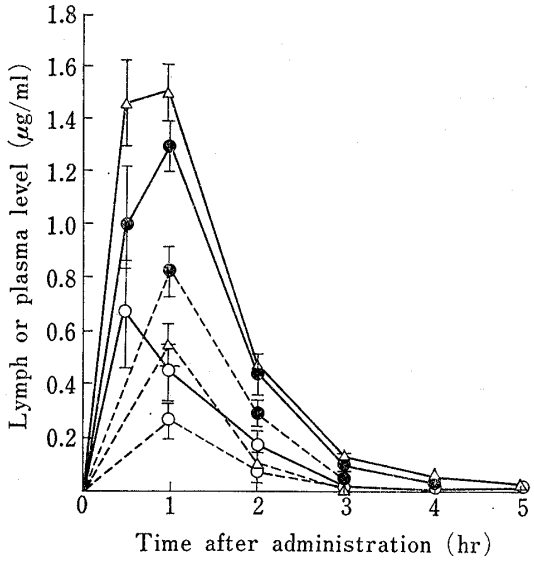

Fig. 4. Effect of O/W Emulsion and W/O Emulsion on the Lymph and Plasma Levels of MMC by Intramuscular Administration of 0.4 mg per 250 g Rat

○ : solution, ● : O/W emulsion (gelatin), △ : W/O emulsion —— : lymph, ------ : plasma
Results are expressed as the mean of at least 5 animals. The vertical indicates ± S.D.

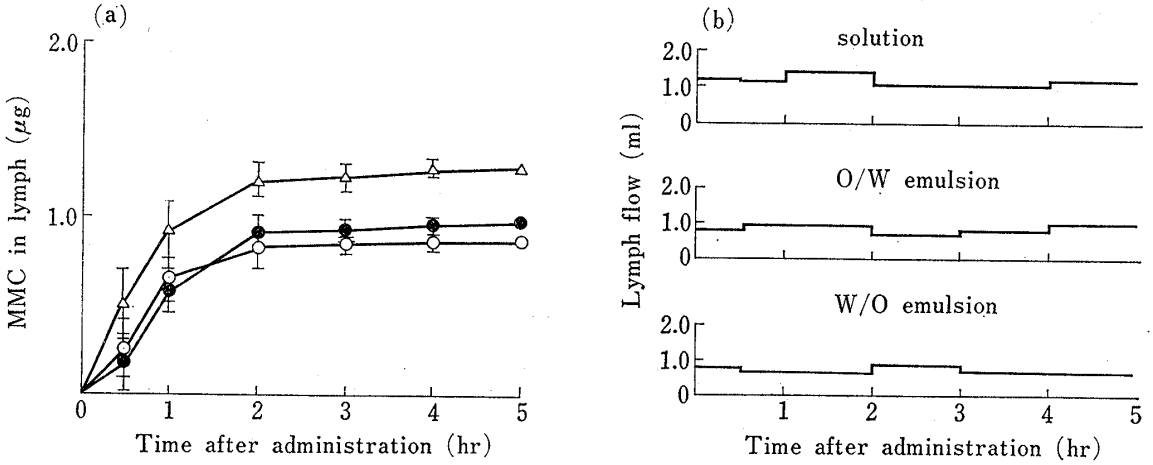

Fig. 5. Cumulative Amount of MMC transferred into Thoracic Lymph after Intramuscular Administration

(a) Cumulative Curve (b) Lymph Flow
○: solution; ●: O/W emulsion (gelatin), △: W/O emulsion
Results are expressed as the mean of at least 5 animals. The vertical bar indicates ±S.D.

10) B.B. Ballard, *J. Pharm. Sci.*, **57**, 357 (1968).

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in Fig. 5. Every cumulative curve reached maximum in 3 or 4 hours after administration, and the cumulative amount from W/O emulsion was the highest as similar to intraperitoneal administration.

Binding of MMC to Oil Droplets

The authors observed in the previous study[11] that methyl orange partly distributed on the oil droplets of ethyl laurate emulsified by polysorbate 80. So it is thought that MMC may be adsorbed on the surface of oil droplets in some conditions. To examine this possibility, the dialysis equilibrium was used for determining the percentage of binding to oil droplets in O/W emulsion. Fig. 6 shows the relationship between the binding percentage and the concentration of emulsifier used. In either case of 0.1 or 1.0% polysorbate 80, MMC did not bind to oil droplets contrary to expectation. On the other hand, in the case of oil droplets emulsified by gelatin or egg albumin an increase of emulsifier concentration resulted in a gradual increase of binding. Gelatin forced more strongly to bind the drug to sesame oil droplets than egg albumin. At 4% of gelatin, the actual concentration used in animal injection, the drug bound was 34%, while no bound drug observed in the case of polysorbate 80.

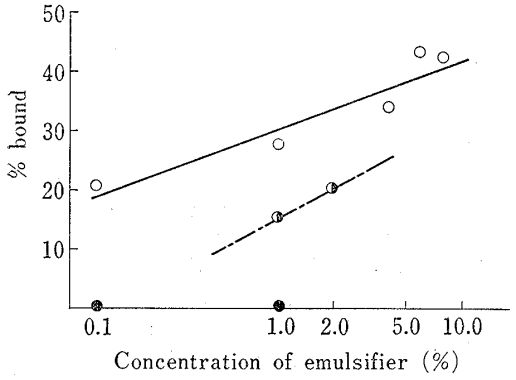

Fig. 6. Binding of MMC to Oil Droplets in O/W Emulsion

Every O/W emulsion contains 4% of sesame oil and 40 μg/ml MMC.
○ : gelatin, ◑ :egg albumin, ● :polysorbate 80

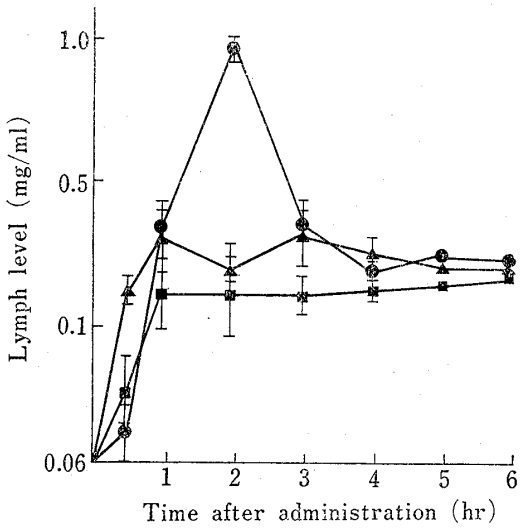

Fig. 7. The Levels of Oil in Thoracic Duct Lymph at Various Times after Intraperitoneal Administration (6μCi Triolein-^{131}I/250 g Rat)

■ : oily solution, ▲ : O/W emulsion (gelatin), ● : W/O emulsion
Results are expressed as the mean of at least 3 animals. The vertical bar indicates ± S.D.

Arrival of Oil in Thoracic Duct Lymph and Lymph Nodes

Transfers of oil into thoracic duct lymph and lymph nodes were evaluated by measuring the radioactivity of triolein-^{131}I after intraperitoneal administration of sesame oil preparations incorporating triolein-^{131}I. The oil levels in the lymphatic system were calculated by regarding triolein-^{131}I as sesame oil. Although the lymph levels of W/O reached maximum at 2 hours after injection, there were not much differences according to the type of preparation as shown in Fig. 7. The lymph levels were kept relatively constant, being still high even at 6 hours after injection.

11) H. Ogata, K. Kakemi, A. Furuya, M. Fujii, S. Muranishi, and H. Sezaki, *Chem. Pharm. Bull.* (Tokyo), 23, 716 (1975).

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Next the levels in lymph nodes, which are located in the pathway to the thoracic duct, were surveyed for what extent was deposited. The levels in lymph nodes and thoracic duct lymph at 6 hours after intraperitoneal administration were summarized in Table I. The concentrations in lymph nodes were always extremely higher than that in thoracic duct lymph in every preparations; lymph nodes: thoracic duct lymph concentration ratios were 157 for oily solution, 18 for O/W emulsion and 185 for W/O emulsion. The results which the very high concentration was deposited in lymph nodes even at 6 hours after administration suggest that oil droplets transferred from injection site into lymph capillary may be stored at lymph nodes, where oil are sustained to release gradually by lymph flow.

TABLE I. The Levels of Oil in Thoracic Duct Lymph and Lymph Nodes at 6 Hours after Intraperitoneal Administration (6μCi Triolein-^{131}I/250 g Rat)

	Oily solution	O/W emulsion (gelatin)	W/O emulsion
Concentration of oil in thoracic duct lymph (mg/ml)	0.22±0.04	0.22±0.03	0.26±0.04
Concentration of oil in lymph nodes (mg/ml)	35.16±4.2	4.63±1.0	40.61±5.0
Lymph nodes/Thoracic duct lymph	157.4	17.8	185.4

Results are expressed as the mean ± S.D. of at least 3 animals.

Discussion

In this study an attempt has been made to deliver efficiently the anticancer agents into the lymphatic systems. It has been known that lipids, fatty acid and high molecular substances are absorbed mainly *via* the lymphatic capillary contrary to the blood capillary absorption of most small molecular substances. Since MMC and most of the other anticancer agents are water soluble compounds with relatively low molecular weight, the transfer *via* the blood capillary is main route when the aqueous solution of the anticancer agent is injected into tissue spaces. In order to facillitate the transport of drug into the lymphatic capillary, we first utilized fat emulsion as a drug delivery system.

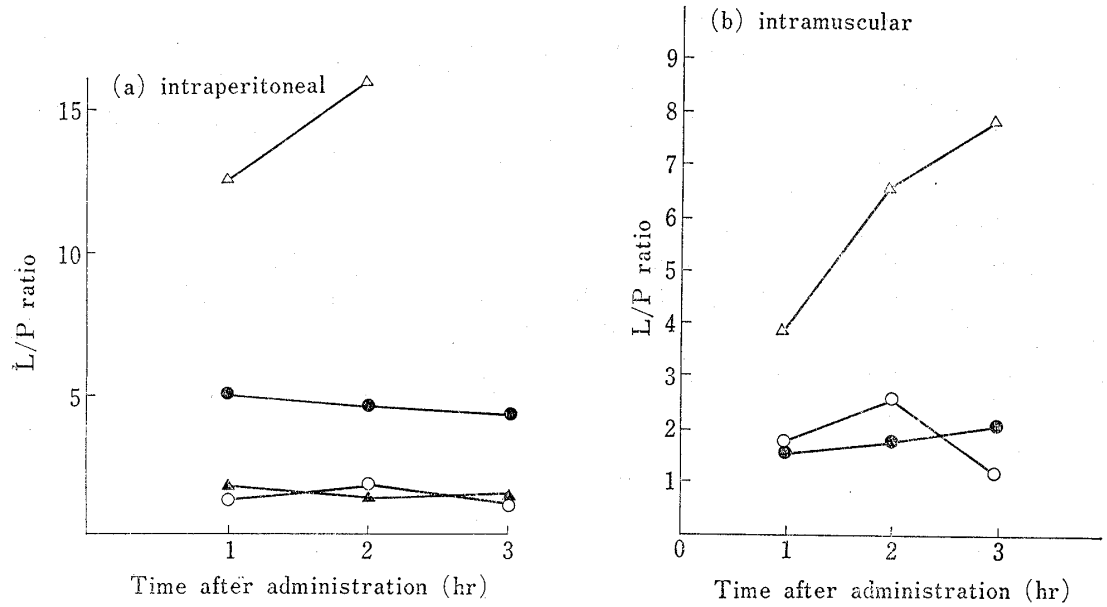

Fig. 8. The Ratios of Lymph to Plasma Level of MMC at Various Times after Intraperitoneal and Intramuscular Administration

○: solution, ●: O/W emulsion (gelatin), ▲: O/W emulsion (Polysorbate 80), △: W/O emulsion

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Lymph: plasma concentration ratio (L/P ratio) has been used for evaluating blood-lymph transport by Garlick.[12] Accordingly the time courses of L/P ratios are shown in Fig. 8 for understanding the transport from interstitial space to lymph and to blood. At the injection of the aqueous solution the ratios were maintained in the level from 1 to 2.5 in the both figures, indicating that the concentration of MMC is difficult to be risen over the ratio 2 by the injection to tissue spaces without adequate delivery system. W/O emulsion, however, considerably increased the ratios, more than 12 at intraperitoneal case and more than 4 at intramuscular case. O/W emulsion also promotes the lymphatic transport of MMC although worse than W/O emulsion. In emulsion system, the hydrophilic anticancer agent is predominantly located not in oily phase but in aqueous phase. Consequently, the drug is distributed in outer phase in the case of O/W emulsion, and it is encapsulated in inner phase in the case of W/O emulsion. It is thought that an encapsulating of drug in oil is highly advantageous for lymphatic transport from interstitial space. In O/W emulsion incorporating gelatin, however, MMC was observed to be partly bound to oil droplets besides its presence in aqueous phase (Fig. 6). It has been found that the water soluble substance bound to oil droplets can be readily absorbed from the large intestine of rat.[11] The difference of lymphatic transport between the two O/W emulsions (gelatin and polysorbate 80) may be due to the degree of binding to oil droplets. These results suggest that the drug having high affinity to oil droplets also can be more favorable for the lymphatic transport.

On the other hand, the behaviours of oil from injection site to the lymphatic system was investigated quantitatively with various types of oil preparations. The results indicate that oil can reach lymph nodes and thoracic duct lymph from the peritoneal cavity (Fig. 7, Table I). The concentration in lymph nodes, however, was much higher than that in thoracic duct lymph. Namely lymph nodes: thoracic duct lymph concentration were more than 15, in the increasing order of W/O emulsion, oil solution, and O/W emulsion respectively. Oil in thoracic duct lymph was keeping a relatively constant levels for a long time, suggesting that oils transferred into lymph capillaries stay in lymphatic nodes and are released continuously downstream. On the contrary to such a oil removing in the lymphatic system, the anticancer agent in thoracic duct lymph reached peak at 30 or 60 min after injection, and subsequently decreased promptly. Accordingly, it is concluded that the anticancer agent does not accompany with oil in the lymphatic system all the time and one of the part where oil and the drug would be separated is probably lymph nodes.

In general the water soluble drug injected into interstitial space or absorbed from the intestine would have equal access to both blood and lymph capillaries[13] such as the case of the administration of MMC aqueous solution. Since blood flow in rat is about 500 times greater than lymph flow,[14] the drug would be mainly removed *via* blood if there is no apparent selective uptake into lymph capillaries. In cancer chemotherapy, the anticancer agents which reach in high concentration within the lymphatic system might prove to be more useful in the treatment of prevention of metastasis located to this system. Actually MMC was able to be supplied in high concentration into thoracic duct lymph by the injection of W/O and O/W types emulsion into the peritoneal cavity or the muscle. Further experiments to elucidate the mechanism of promotion of lymphatic transport by parenteral emulsions are being carried out in this laboratory.

12) D.G. Garlick and E.M. Renkin, *Am. J. Physiol.*, **219**, 1595 (1970).
13) T.J. DeMarco and R.R. Levine, *J. Pharmacol. Exptl. Therap.*, **169**, 142 (1969).
14) E.J. Reininger and L.A. Sapirstein, *Science*, **126**, 1178 (1957).

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