Chem. Pharm. Bull. 23(12)3125—3131(1975)

UDC 615.277.3.033 : 615.076.7

Studies on Pharmaceutical Modification of Anticancer Agents. II.[1] Enhanced Delivery of Bleomycin into Lymph by Emulsions and Drying Emulsions

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(Received March 31, 1975)

The enhanced delivery into lymph of bleomycin was studied by the intraperitoneal and intramuscular injection of oil emulsified preparations. A comparison of concentration of bleomycin in thoracic duct lymph showed that W/O emulsion was the most effective and followed by O/W emulsion. An application of spray drying powder was also attempted to the enhanced delivery, and the lymph concentration was increased as compared with the injection of the aqueous solution although lower than O/W liquid emulsion. A measuring of binding to oil particles indicated that approximately 40% of bleomycin was bound to the particles of O/W liquid emulsion and of reconstituted emulsions prepared from drying products. Furthermore the clearance rates of bleomycin from the muscle were delayed about one half by W/O, O/W and reconstituted emulsion. The possible mechanism of enhanced delivery into lymph was discussed in this paper.

The previous study has shown that the enhancement of lymphatic transport of mitomycin C was accomplished by the injection of oil in water (O/W) and water in oil (W/O) type emulsion to tissue spaces.[1] Bleomycin is water soluble and basic anticancer agent having relatively low molecular weight, and it is necessary to create a selectively high concentration of bleomycin in the lymphatic system for the prevention of metastasis and for the treatment of malignant lymphoma. Bleomycin is, however, an unique type of glycoprotein structurally different from mitomycin C, has a larger molecular size than the latter, and has an unique behaviour of distribution in the body.[3] Consequently it is difficult to predict the lymphatic transport of bleomycin directly from the basis of mitomycin C.

Although the utilization of fat emulsions would be promising for the facilitation of drug transportation into lymph, the instability of emulsions is one of the problems from the viewpoint of pharmaceutical technology. Drying emulsions so that they can be reconstituted when required may solve some of the problems in ageing and storage. For this reason, we attempted to utilize drying emulsions in order to promote the lymphatic transportation.

In this study, the lymphatic transport of bleomycin in different formulations; O/W, W/O emulsion and drying emulsion, was investigated by intraperitoneal and intramuscular administration to rats. An effort has been made to elucidate the mechanism of lymphatic transport from their preparations.

Experimental

Materials——Bleomycin was supplied from Nihon Kayaku Co. Ltd. Sesame oil and gelatin were obtained from Nakarai Chemical Co. Ltd. HCO-60 and SO-15 were obtained from Nikko Chemical Co. Ltd. Drying milk produced by Morinaga Milk Co. was purchased on the market.

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2) Location: *a) Kawai-cho, Matsubara, Osaka; b) Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto.*
3) H. Umezawa, Y. Suhara, T. Takita, and K. Maeda, *J. Antibiotics, Ser., A* **19**, 210 (1966); H. Umezawa, M. Ishizuka, S. Hori, H. Chimura, and T. Takeuchi, *J. Antibiotics, Ser., A* **21**, 638 (1968); H. Fujita and K. Kimura, Proceedings of the 6th International Congress of Chemotherapy, Tokyo, August, 1969.

Preparation of Emulsions and Drying Emulsions——The preparations of O/W and W/O emulsion were identical to those described previously.[1] Oil contents were 20% (v/v) for O/W and 78% (v/v) for W/O respectively.

The spray drying was carried out in order to prepare drying emulsion. Formula of the emulsion was sesame oil, 20% (v/v); gelatin, 4% (w/v); distilled water to make 100 ml. The mixture was sonicated for 2—5 min by Ohtake sonicator (20 kc). Using a centrifugal atomizer (Iwai Kikai Co. Ltd, Model IS III) spray dryer, the dryer was equilibrated with the inlet temperature at 150° and the outlet temperature at 90°. The atomizing wheel revolved at a rate of 20000 rpm. The drying emulsion was collected in a receiver at the bottom of the main chamber. When the drying emulsion was shaked with distilled water, the diameter of the particles of the reconstituted emulsion was 1—10 μ according to microscopic observation.

Procedure of Lymphatic Transport Experiments——Male Wistar albino rat, 200—250 g, were used in all animal experiments. The lymphatic transport experiments were almost identical to those described previously.[1] One ml aliquot of the parenteral emulsion per 250 g of rat body weight was administered after completion of the canulation into the thoracic duct. For intraperitoneal, the preparation was injected into the center of the perioneal cavity, and for intramuscular, each of 0.5 ml of the preparations was injected into the centers of both thigh muscle of rat. Blood samples were taken from the tail vein.

Muscle Clearance Experiments——At various times after the injection of emulsions into the center of the rat thigh muscle, the muscle was removed. The muscle was excised and then homogenized in 10 ml of distilled water, and the drug remaining at the injection site and associated area was determined.

Preparation of Injection Emulsions——For the injection emulsions, bleomycin was dissolved in water phase to make 3 mg per 1 ml of emulsion immediately before administration. In the case of drying emulsion and milk, distilled water was added to 300 or 400 mg of the powder to make 1 ml of injection.

Analytical Method of Bleomycin——The concentrations of bleomycin in lymph and plasma were determined by the cylinder plate method using *Bacillus Subtillis PCI* 219 as the test organism. Plasma protein and emulsion did not interfere the assay of bleomycin. The concentration of bleomycin in the muscle was read from the calibration curve in muscle homogenates.

Measurement of Binding of Bleomycin to Oil Droplets——The binding was measured by using ultrafiltration method through Millipore filter (pore size 0.22 μ) under reduced pressure. The concentration of bleomycin in the filtrate were determined spectrophotometrically at 294 mμ. As 9.2% of bleomycin was adsorbed on the filter during filtration, all the concentrations in the filtrates were corrected. The binding percent was calculated from the following equation, $I-F/I\times 100$, where I is total concentration in aqueous phase; the ratio of amount of added bleomycin to unit volume of aqueous phase, and F is the corrected filtrate concentration.

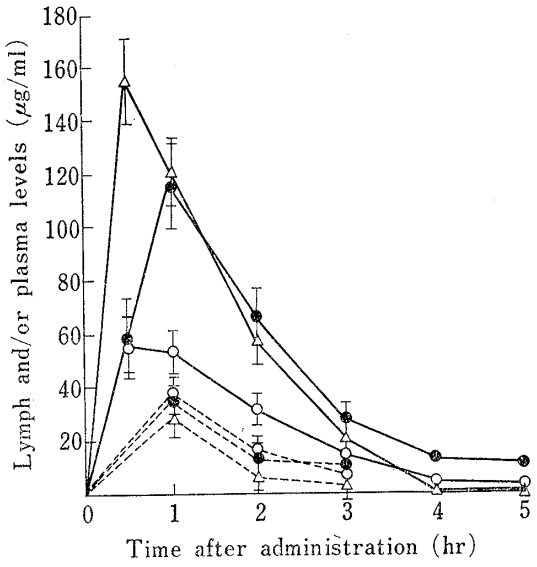

Fig. 1. Effect of O/W Emulsion and W/O Emulsion on the Lymph and Plasma Levels of Bleomycin by Intraperitoneal Administration

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

Results

Lymphatic and Blood Transport from Emulsions

(a) Intraperitoneal Administration

Samples of thoracic duct lymph and tail venous blood were taken at 30 or 60 min intervals and were assayed for their contents of biologically active drug after intraperitoneal injection of 3 mg per 250 g rat of bleomycin. As shown in Fig. 1, when the aqueous solution was injected, the drug concentration in thoracic duct lymph was not high, which was close to the concentration in plasma. When O/W emulsion containing 4% of gelatin was injected, the lymph concentration was markedly increased after one hour following the administration although the plasma concentration was nearly equal with the aqueous solution. Furthermore at the administration of W/O emulsion the maximum lymph concentration was risen above the O/W emulsion's, and the plasma concentration exhibits a reduced tendency as compared with the aqueous solution.

The cumulative amounts of biologically active bleomycin delivered from the peritoneal cavity into thoracic duct lymph are summarized in Fig. 2. Lymph flow did not change in any time and any of the preparations by supplying of saline into the duodenum. Every cumulative curve reached maximum in 3 or 4 hours after administration. The cumulative amount from W/O emulsion was the highest among the three preparations about 2.6 times of the aqueous solution, and that from O/W emulsion followed the former about 2.0 times of the aqueous solution. The cumulative maximum of the aqueous solution, O/W emulsion and W/O emulsion were 3.1, 6.2,

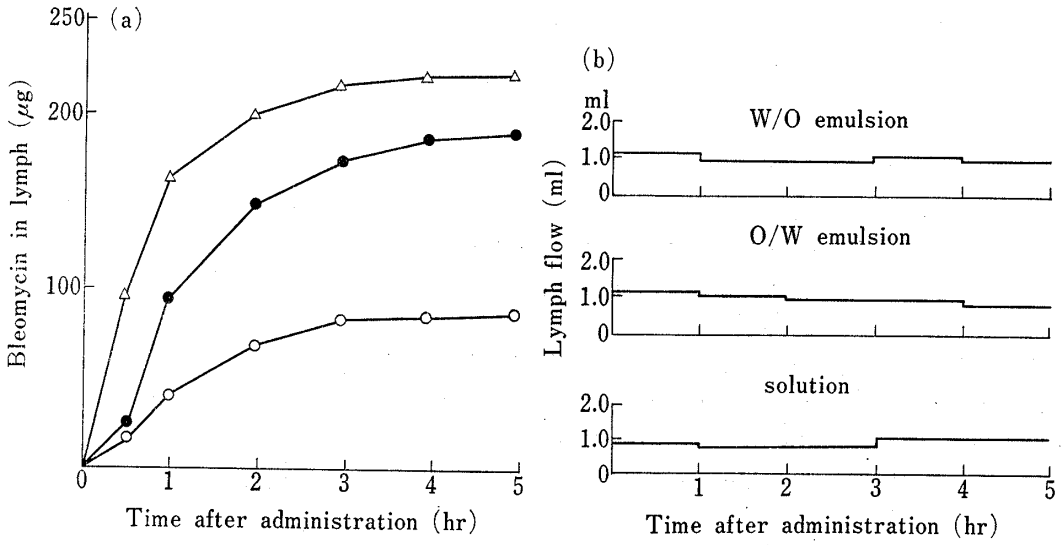

Fig. 2. Cumulative Amount of Bleomycin transferred into Thoracic Lymph after Intraperitoneal Administration (a) Cumulative Curve (b) Lymph Flow

○: solution, ●: O/W emulsion, △: W/O emulsion
Results are expressed as the mean of at least 5 animals.

and7. 2% of administration dose respectively. These values are relatively high as compared with mitomycin C observations.

(b) Intramuscular Administration At the intramuscular injection of the aqueous solution the lymph concentration was always higher about two or three times than the plasma concentration as shown in Fig. 3. An administration of O/W emulsion containing gelatin and W/O emulsion resulted in a pronounced increase of the lymph concentration though the plasma concentration slightly increased. The patterns of lymph curves were very similar to those obtained by intraperitoneal administration.

The cumulative amounts transferred into thoracic lymph from the muscle are shown in Fig. 4. Every cumulative curve reached maximum in 3 or 4 hours after administration, and the increasing order of cumulative amount is as follows; W/O emulsion>O/W emulsion> aqueous solution, which is the same as that found by intraperitoneal administration.

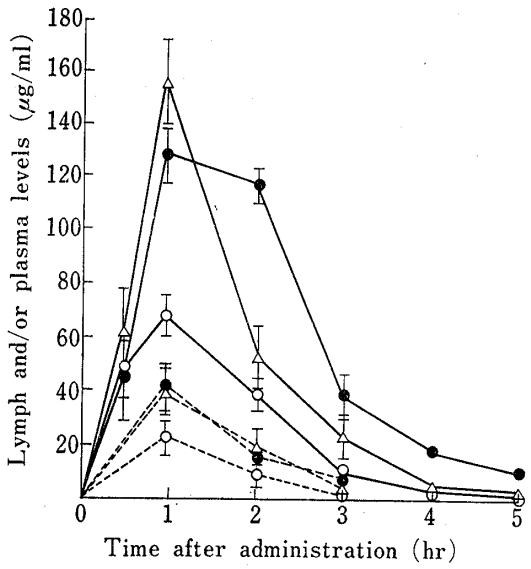

Fig. 3. Effect of O/W Emulsion and W/O Emulsion on the Lymph and Plasma Levels of Bleomycin by Intramuscular Administration

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

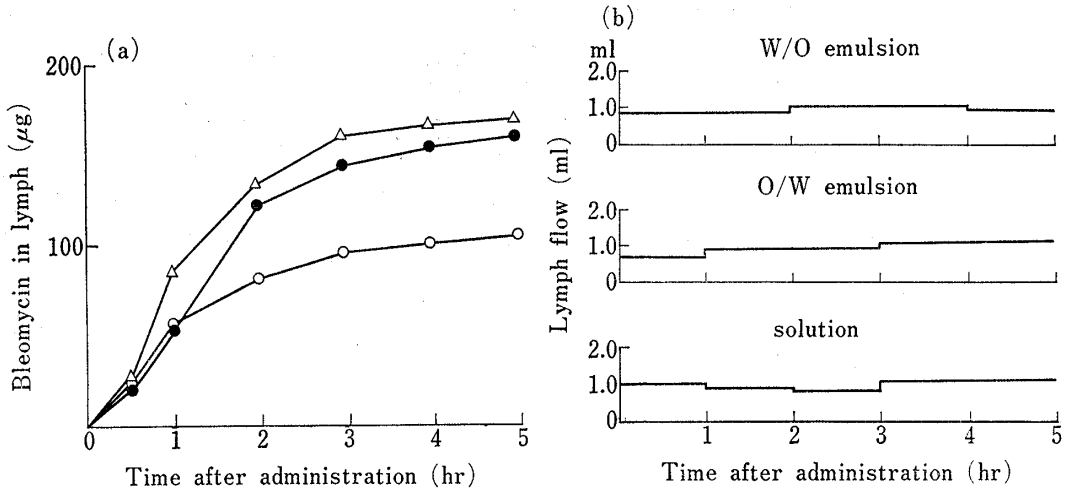

Fig. 4. Cumulative Amount of Bleomycin transferred into Thoracic Lymph after Intramuscular Administration (a) Cumulative Curve (b) Lymph Flow

○: solution, ●: O/W emulsion, △: W/O emulsion
Results are expressed as the mean of at least 5 animals.

Lymphatic and Blood Transport from Reconstituted Emulsions

An application of drying emulsion to an enhanced delivery into lymph is also interesting, since it has an advantage in its stability during storage. In order to examine the possibility of the enhancement, the lymphatic transport experiment was studied. Immediately before injection, O/W emulsion was reconstituted by agitating the drying powder with bleomycin and distilled water at 37° for 5 min.

First the commercial drying milk was chosen, and to a 300 or 400 mg of the milk powder an aliquot of water was added to make one ml. When these emulsions were injected intraperitoneally and intramuscularly, no much difference in lymphatic transportation of bleomycin between both figures was observed as shown in Fig. 5. Contrary to expectation, even in the case of 400 mg of the powder the lymph concentrations were only slightly increased.

Next we tried to utilize the dry product obtained from O/W emulsion by spray drying for an enhanced delivery. When the reconstituted emulsions were injected intraperitoneally, there was also no much difference between 300 mg, 400 mg of the drying emulsion and the

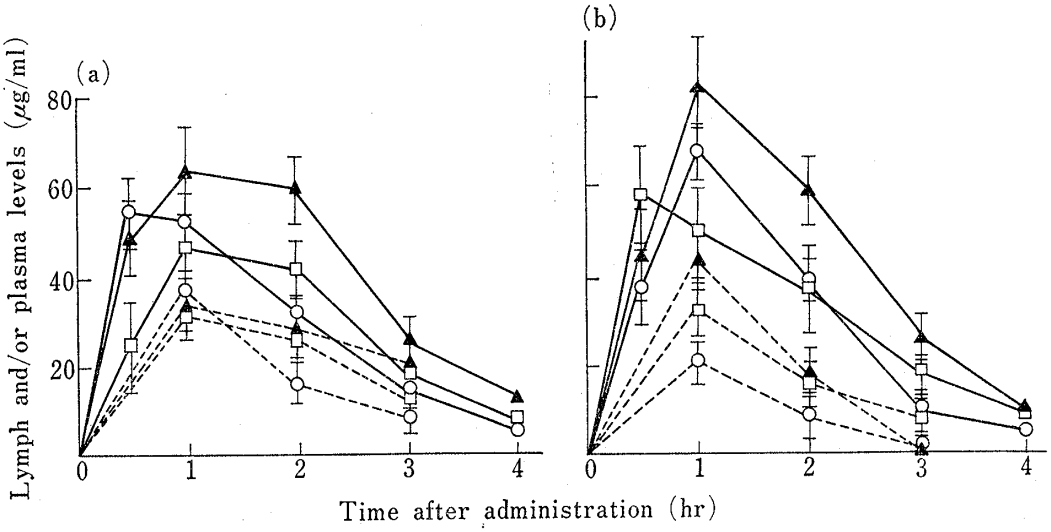

Fig. 5. Effect of Drying Milk Emulsion on the Lymph and Plasma Levels of Bleomycin (a) Intraperitoneal (b) Intramuscular

○: solution, □: milk 300 mg, ▲: milk 400 mg, ——: lymph, ----: plasma
Results are expressed as the mean of at least 5 animals. The vertical bar indicates ± S.D.

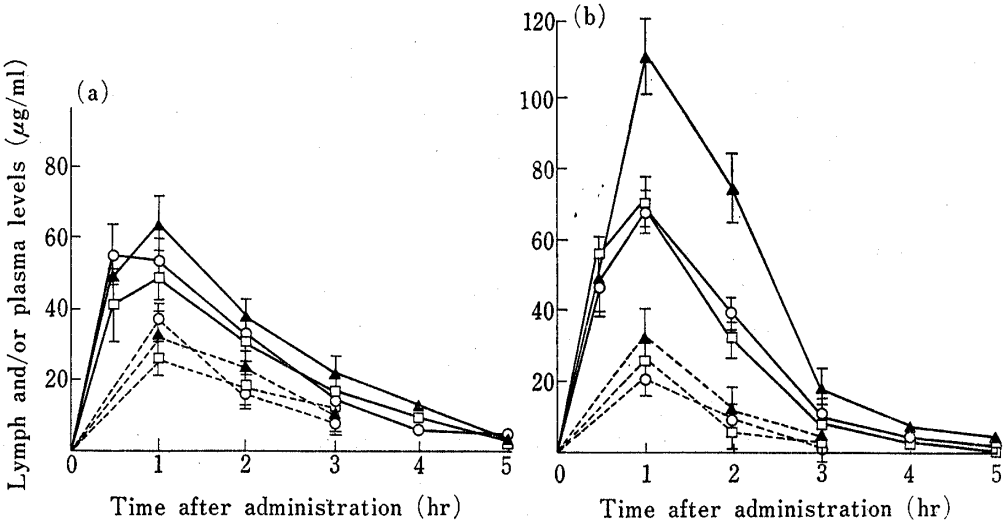

Fig. 6. Effect of Spray Drying Emulsion on the Lymph and Plasma Levels of Bleomycin (a) Intraperitoneal (b) intramuscular

○: solution, □: powder 300 mg, ▲: powder 400 mg, ——: lymph, ----: plasma
Results are expressed as the mean of at least 5 animals. The vertical bar indicates±S.D.

aqueous solution as similar to the drying milk, as shown in Fig. 6 (a). However at an intramuscular injection of 400 mg of the drying emulsion, the lymph concentration was increased about twice of the aqueous solution as shown in Fig. 6 (b).

Binding of Bleomycin to Oil Particles

As it was found in the previous paper[1] that mitomycin C was partially bound to oil droplets in O/W emulsion containing gelatin, bleomycin is also presumed to be bound to oil droplets and milk particles. The equilibrium dialysis using cellulose membrane is not adequate for determining the binding percentage, because a considerable amount of bleomycin was observed to be bound to the membrane. Therefore the ultrafiltration method using Millipore filter, which is easy to filter bleomycin but does not filter the particles, was utilized in this study.

TABLE I. Binding of Bleomycin to Oil Particles

O/W emulsion	Bound %
Oil 20%+gelatin 4%	40±3.5
Oil 20%+gelatin 6%	42±4.0
Oil 20%+gelatin 8%	44±2.2
Powder[a] 300 mg	32±2.3
Powder[a] 400 mg	36±3.0
Milk[b] 300 mg	35±2.4
Milk[b] 400 mg	33±1.8

Results are expressed as the mean±S.D. of 4 samples.
a) spray drying powder, *b*) drying milk

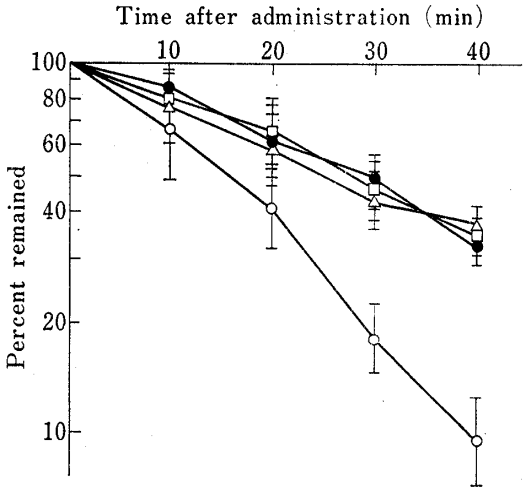

Fig. 7. Effect of O/W Emulsion, W/O Emulsion and Powder on the Disappearance of Bleomycin from the Rat Thigh Muscle

○: solution, ●: O/W emulsion, △: W/O emulsion, □: powder 400 mg
Results are expressed as the mean of at least 5 animals. The vertical bar indicates±S.D.

The binding percentages of bleomycin to oil particles in O/W emulsions and reconstituted emulsions were summarized in Table I. The result indicates that bleomycin was bound 32 to 44% to the particles of various kinds of emulsions used in this experiment. An increase of gelatin content resulted in a gradual increase of binding as similar to mitomycin C's case described previously.[1] An increase of powder slightly increased the binding for drying emulsion, but not for drying milk.

Clearance of Bleomycin from Muscle

To determine the possible effects of emulsion on lymphatic and blood transportation, it is necessary to investigate the time course of the drug clearance from the injection site. The rat thigh muscle was chosen for this clearance study, because it is easier to assay the drug remaining in the muscle than in the peritoneal cavity.

As shown in Fig. 7, the clearance from the aqueous solution showed approximately monoexponential as similar to the other compounds.[4] When O/W emulsion, W/O emulsion and reconstituted emulsion of 400 mg drying product were injected, all the clearance rates were equally suppressed about one half. Therefore this result indicates that the release of bleomycin from the injection site into systemic circulation was delayed by the delivery system such as O/W and W/O emulsion.

Discussion

Bleomycin is a group of antitumor antibiotics discovered by Umezawa and his collaborators in 1962. Thirteen kinds of bleomycins have been isolated from the cultured broth of *Streptomyces verticillus*, and were named A_1 through A_6, A_2', and B_1 through B_6. The sample used in this study is bleomycin clinically used now which contains A_2 as the main component. Although molecular weights of bleomycins are approximately 1500, and these are water soluble glycopeptide analogue, the structural formula has not fully determined.

When bleomycin was administered into the interstitial space as the aqueous solution, the concentration ratios of lymph to plasma were maintained in the level from 1 to 3, almost identical to the case of mitomycin C injection in spite of its larger molecular size than mitomycin C. This result suggests that the transfer of such a relatively small and water-soluble compound as mitomycin C or bleomycin from the injection site possesses a nearly equal opportunity for *via* blood and lymph route with respect to its concentration. Consequently it is necessary to use an adequate delivery system in order to create a selectively high concentration of the anticancer agent in the lymphatic system. When O/W and W/O emulsion were utilized as a delivery system, W/O emulsion was most effective for an increase of lymph level, followed by O/W emulsion in both cases of intraperitoneal and intramuscular administration. The cumulative amounts of bleomycin into lymphatic transport was much higher than mitomycin C. This may be mainly due to the difference in inactivation in the area of injection site.

In emulsion system, the hydrophilic anticancer agent is predominantly located not in oily phase but in aqueous phase. Accordingly bleomycin 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. From the results obtained in the lymphatic delivery of bleomycin in this study and of mitomycin C in the previous study,[1] it is concluded that an encapsulating of the hydrophilic drug in oily phase is the most advantageous for lymphatic transport from the interstitial space. Even in O/W emulsion, bleomycin was observed to be bound to oil droplets approximately 40% of its presence in aqueous phase (Table I). This may be the reason why the lymphatic transport of bleomycin was promoted by O/W emulsion.

4) K. Kakemi, H. Sezaki, K. Okumura, and S. Ashida, *Chem. Pharm. Bull.* (Tokyo), **17**, 1332 (1969); H. Kobayashi, T. Nishimura, K. Okumura, S. Muranishi, and H. Sezaki, *J. Pharm. Sci.*, **63**, 580 (1974).

In general, the stability of liquid emulsion is the problem during ageing and storage. Aggregation, coalescence and creaming may occur by the natural process of ageing, *i.e.*, as a function of time.[5] Richter and Steiger-Trippi examined the drying of emulsion by spray-drying techniques for the purpose of stabilization.[6] We examined to use this technique for O/W emulsion containing gelatin to obtain the drying product in this study. Thus the enhanced delivery of bleomycin by reconstituted O/W emulsion was investigated with respect to this drying product and drying milk. The results of lymph concentrations at 1 and 2 hours after administration were summarized in Table II. When the emulsion prepared by 400 mg of the spray drying powder was injected intramuscularly, the lymphatic transport was increased significantly though did not reach the level of O/W liquid emulsion. Drying milk only slightly changed the transport into lymph of bleomycin.

Table II. The Levels of Bleomycin in Thoracic Duct Lymph at 1 and 2 Hours after Intramuscular and Intraperitoneal Administration

	Intramuscular		Intraperitoneal	
	1 hr	2 hr	1 hr	2 hr
Solution	68± 6.3	39±4.2	53± 5.5	32±3.9
O/W emulsion	128± 8.0	117±5.0	116±12.0	68±8.0
W/O emulsion	147±11.5	54±8.5	122± 9.2	58±6.5
Powder 400 mg	111±10.3	74±8.6	63± 7.3	37±5.4
Milk 400 mg	83±10.0	60±7.5	64± 7.2	59±8.5

(μg/ml)
Results are expressed as the mean ±S.D. of at least 5 animals.

The binding of bleomycin to dispersed particles was more than 30% even 300 mg of drying emulsion and drying milk (Table I). These powders, however, do not possess the powerful ability of enhanced deliver into lymph. On the other hand, the clearances of bleomycin from the injection site were retarded quite similarly by all the emulsion (Fig. 7). Thus the affinity of drug to oil particles may be one of the most important factors for sustained release from the injection site. As the delivery of the drug from the injection site was protected by emulsion from the leakage into blood circulation, the affinity of drug to oil must act advantageously on kinetical lymphatic transportation. The difference in lymph concentration between O/W liquid emulsion, drying product's emulsion and drying milk's emulsion might depend on the degree of dispersion, the degree of aggregation and the size of oil particle. In addition, a transfer rate of oil particles may be related to an increase of lymph concentration of bleomycin besides the binding to oil particles. Detailed experiments for lymphatic behavior of oil are supposed to be necessary to elucidate the mechanism of the promotion of lymphatic transport.

Birmingham's group[7] have investigated multiple emulsion incorporated anticancer agent to produce prolonged action. The study of emulsion, however, has not become operative by these investigators in order to prevent metastasis along the lymph pathways. The present study strongly suggests that the formulation of emulsion incorporated bleomycin can be useful for prevention of metastasis, malignant lymphoma and Hodgkin's disease.

Acknowledgement The authors would like to thank Mr. Masanobu Kawamata, Nihon Shinyaku Co. Ltd., for his technical support of spray drying.

5) E.R. Garrett, *J. Pharm. Sci.*, **54**, 1557 (1965).
6) A. Richter and K. Steiger-Trippi, *Pharm. Acta Helv.*, **36**, 322 (1961).
7) C.J. Benoy, L.A. Elson, and R. Schneider, *Brit. J. Pharmacol.* **45**, 135 (1972); C.J. Benoy, R. Schneider, L.A. Elson, and M. Jones, *Europ. J. Cancer*, **10**, 27 (1974).