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Requirement of Macromolecular Complex Formation for Selective Lymphatic Transfer of Bleomycin from Large Intestine by Bifunctional Delivery System

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The requirement of complex formation for the selective transfer of bleomycin from the large intestine of rat by a bifunctional delivery system was investigated. The delivery system developed by us is a combination of macromolecular dextran sulfate, mean molecular weight ($\overline{MW}$), 500000, as a lymphotropic carrier forming an ionic complex with bleomycin, and lipid-surfactant mixed micelles as an absorption promoter. Co-existence of the bifunctional delivery system (bleomycin·dextran sulfate complex + mixed micelles) with saline resulted in the dissociation of the complex. Administration of this system with saline into the lumen of the large intestine showed no selective rise of lymph level of bleomycin compared with the blood level, and pretreatment with dextran sulfate into the lumen did not increase the lymphatic transfer of bleomycin. Lower molecular weight dextran sulfate ($\overline{MW}$, 5000) formed a complex with bleomycin, like macromolecular dextran sulfate ($\overline{MW}$, 500000). However, administration of this complex with mixed micelles failed to selectively enhance the lymphatic transfer of bleomycin. Co-administration of bleomycin with other macromolecules (dextran or diethylaminoethyl dextran, $\overline{MW}$, 500000) that do not form a complex with bleomycin also failed to selectively increase the lymphatic transfer of bleomycin. These findings suggest that the complex formation of bleomycin with a macromolecule over a certain molecular weight is required for the selective transfer of bleomycin into the lymphatics from the large intestine by a bifunctional delivery system.

Keywords——bleomycin; bifunctional delivery system; lymphatic transfer; complex formation; lipid-surfactant mixed micelle; dextran sulfate; macromolecule; bleomycin·dextran sulfate complex; large intestinal absorption; blood–lymph barrier

We have developed a bifunctional delivery system for the selective transfer of bleomycin (BLM) into the lymphatics *via* the enteral route.[1] BLM ($\overline{MW}$, approximately 1500), a basic glycopeptide anticancer agent, is itself poorly absorbable from the intestine, and the BLM levels detected in the blood and the lymph were almost identical.[1] To obtain selective uptake of BLM by the lymphatic system *via* the enteral route, we used the following two adjuvants; 1) monoolein-sodium taurocholate mixed micelles as an absorption promoter[2-6] and 2) macromolecular anionic dextran sulfate (DS_{500}, $\overline{MW}$, 500000) as a lymphotropic carrier with the ability to form an ionic complex with BLM.[7] Administration of this bifunctional delivery system (BLM·DS_{500} complex + mixed micelles) into the lumen of the intestine produced a selective concentration rise of BLM in the lymph, and this effect was more pronounced after administration into the large intestine than into the small intestine.[1] We have also reported that the lymphatic transfer of BLM is closely related to the stability of the absorbed BLM·DS_{500} complex in the intestinal tissue.[8,9]

In this work, in order to clarify further the requirement for the selective lymphatic transfer of BLM by a bifunctional delivery system, we studied the effects on the transfer of BLM into the lymph of dissociation of the complex, pretreatment of the lymphotropic carrier (DS_{500}), molecular weight of DS and co-administration with other macromolecules not forming a complex with BLM.

Experimental

Materials——BLM was supplied by Nippon Kayaku Co. DS_{500} ($\overline{MW}$, 500000) was obtained from Nakarai Chemicals Ltd. Dextran (D_{500}, $\overline{MW}$, 500000), diethylaminoethyl dextran (DEAE D_{500}, $\overline{MW}$, 500000) and DS of lower molecular weight (DS_5, $\overline{MW}$, 5000) were purchased from Sigma Chemical Co. The monoolein used was of high purity grade (Nikko Chemicals Co.). Sodium taurocholate was synthesized according to the method of Norman.[10] The purity of sodium taurocholate was checked by thin-layer chromatography (TLC) and infrared (IR) spectroscopy.[11] All other chemicals were of reagent grade.

Preparation of Test Solution——A solution of mixed micelles was prepared by dissolving monoolein (40 mM) and sodium taurocholate (40 mM) in distilled water followed by sonication at 37 °C with an Ohtake sonicator, model 5202 (100 W, 4 min). The BLM·DS_{500} and the BLM·DS_5 complexes were prepared by mixing BLM and DS_{500} or the DS_5 in 40 mM mixed micellar solution, respectively (2.5 mg/ml for BLM and 8.3 mg/ml for DS_{500} or DS_5). Formation of the BLM·DS_{500} and the BLM·DS_5 complexes was checked by gel filtration. Samples were chromatographed on a 1.8 × 20 cm column of Sephadex G-50 or G-25 using distilled water as the eluent. Fractions (3 ml each) were automatically collected, and BLM was determined by ultraviolet spectroscopy (as described later in this section). Other test solutions for administration were prepared as follows. BLM·DS_{500} complex in saline: BLM (2.5 mg/ml) and DS_{500} (8.3 mg/ml) were dissolved in saline containing 40 mM mixed micelles. BLM + D_{500} and BLM + DEAE D_{500}: BLM (2.5 ml/ml) and D_{500} (8.3 mg/ml) or DEAE D_{500} (8.3 mg/ml) were dissolved in 40 mM mixed micellar solution, respectively.

Absorption Experiment——Male Wistar albino rats weighing 200—250 g were anesthetized intraperitoneally with sodium pentobarbital (32 mg/kg of body weight). The large intestine was exposed through midline incision, and a closed loop of the entire large intestine (colon and rectum) was prepared by ligation at the proximal and the distal ends. Doses of 5 mg BLM and 16.7 mg DS_{500}, DS_5, D_{500} or DEAE D_{500} per rat, were used; 2 ml of the test solution was introduced into the loop of the large intestine. A polyethylene catheter (i.d. 0.5 mm, o.d. 0.8 mm, Dural Plastics Co.) was inserted into the carotid artery and blood samples were collected periodically. Plasma was separated by an Eppendorf centrifuge, model 5412 (at 15000 *g* for 2 min). A modification of the method of Bollman *et al.*[12] was used for the collection of the lymph from the thoracic duct. The thoracic duct was cannulated with a heparin-filled flexible vinyl catheter (i.d. 0.5 mm, o.d. 0.9 mm, Dural Plastics Co.) and was fixed with a drop of tissue cement (Aron Alpha, Sankyo Co.). This cannula allowed continuous drainage of the lymph throughout the experiments. The plasma and the lymph samples were immediately immersed in an ice bath after collection. Disappearance of BLM from the lumen of the large intestine was examined at 3 h after the administration. At the end of the experiment, the remaining test solution in the loop was collected for BLM assay by flushing with air and saline solution.

Pretreatment Experiment——Two ml of DS_{500} (8.3 mg/ml) with 40 mM mixed micelles was first introduced into the loop of the large intestine. After pretreatment for 1 h, the solution remaining in the loop was entirely removed by forcing air and saline through the loop with a syringe, and 2 ml of free BLM (2.5 mg/ml) with 40 mM mixed micelles was infused into the loop.

Gel Filtration of Remaining Test Solution in Lumen, Tissue Homogenate of Large Intestine and Lymph——At 2 h after the administration of the test solution into the lumen of the large intestine, the remaining solution in the lumen was thoroughly expelled and the entire large intestine was removed and homogenized with isotonic mannitol solution. This homogenate was centrifuged for 10 min at 15000 *g*. These samples (test solution from the lumen, the supernatant of the tissue homogenate of the large intestine and the lymph) were fractionated by gel filtration on a 1.8 × 20 cm column of Sephadex G-50 or G-25 with distilled water as the eluent. Fractions (3 ml each) were automatically collected, and the concentration of BLM was determined.

Analytical Method for BLM——An antimicrobiological assay was used for the determination of BLM in the remaining solution of the lumen, the tissue homogenate, the plasma and the lymph. The disc plate method using *Bacillus subtilis* PCI-219 as a test microorganism was employed for BLM assay. The *in vitro* formation of complexes between BLM and macromolecules was tested by optical density measurement at 290 nm for BLM using an ultraviolet spectrophotometer (Hitachi model 200-20).

Results

Effect of Complex Dissociation

Upon administration of the bifunctional delivery system with saline into the lumen of the large intestine, a selective rise of BLM concentration in the lymph compared with that in the blood was not observed (Fig. 1). The BLM level in the lymph was not significantly higher than that in the plasma at any sampling time (Fig. 1(a)). The lymph/plasma concentration (L/P) ratios of BLM calculated from the data of Fig. 1(a) are shown in Fig. 1(b), and the range of L/P ratio was 0.4 to 1.6. The state of BLM in the lumen and the tissue of the large intestine

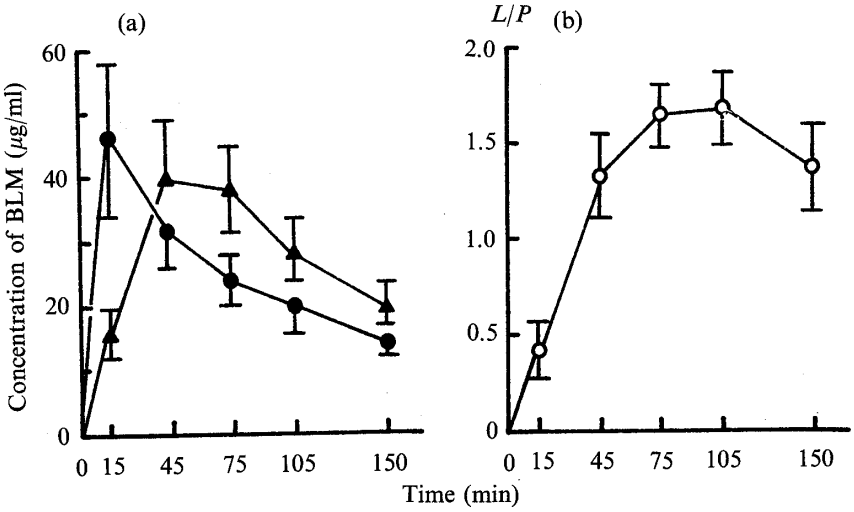

Fig. 1. Effect of Dissociation of BLM·DS_{500} Complex on Lymphatic Transfer of BLM by a Bifunctional Delivery System

(a) Concentration of BLM in the plasma and the lymph of the thoracic duct after administration of the BLM·DS_{500} complex with 40 mM mixed micelles in saline solution into the large intestinal lumen. ●, plasma; ▲, lymph.

(b) Ratio of concentrations in the lymph relative to the plasma (L/P) of BLM calculated from the data of Fig. 1(a). Each value represents the mean ± S.E. of 4—7 experiments.

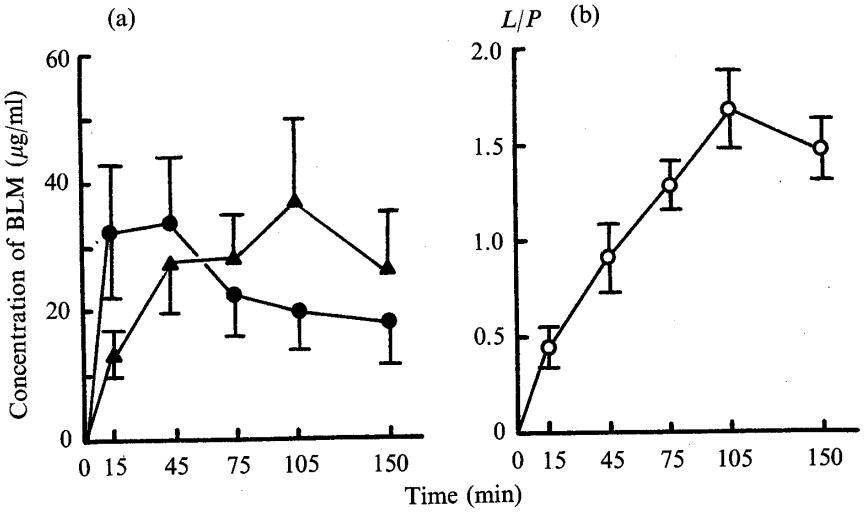

Fig. 2. Effect of DS_{500} Pretreatment on Lymphatic Transfer of BLM

(a) Concentration of BLM in the plasma and the lymph of the thoracic duct after administration of free BLM with 40 mM mixed micelles into the large intestinal lumen pretreated with DS_{500} + 40 mM mixed micelles for 1 h. ●, plasma; ▲, lymph.

(b) L/P ratio of BLM concentration calculated from the data of Fig. 2(a). Each value represents the mean ± S.E. of 4—5 experiments.

was examined by gel filtration on Sephadex G-50 at 2 h after administration of the bifunctional delivery system with saline. In the lumen and the tissue, no BLM was detected in the macromolecular fraction (not shown).

Pretreatment with DS_{500}

The effect of DS_{500} pretreatment in the lumen of the large intestine on the lymphatic transfer of BLM was studied. Figure 2 shows the effect of a 1 h pretreatment with DS_{500} + 40 mM mixed micelles in the lumen on the lymph and the plasma levels of BLM after the administration of free BLM with 40 mM mixed micelles. BLM level in the lymph was not

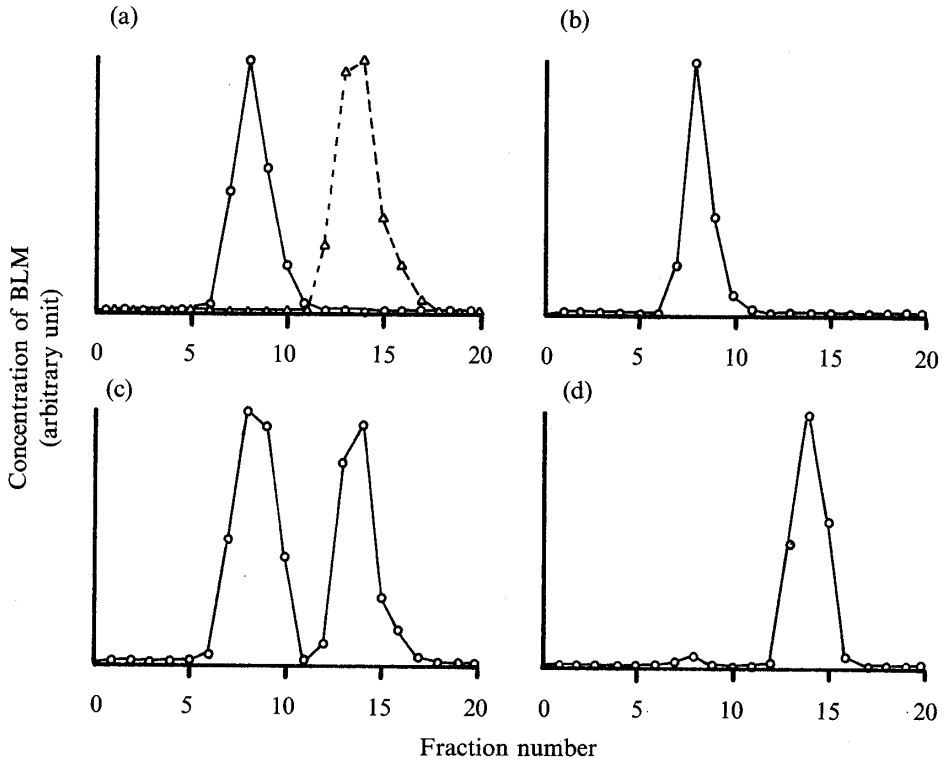

Fig. 3. (a) Formation of BLM·DS_5 Complex *in Vitro*

Gel filtration chromatogram (Sephadex G-25) of free BLM with 40 mM mixed micelles (△---△), BLM + DS_5 with 40 mM mixed micelles (analyzed for BLM, ○—○).

(b)—(d) Stability of BLM·DS_5 Complex in Different Tissues

Gel filtration chromatogram (Sephadex G-25) of (b) remaining solution in the lumen of the large intestine, (c) the supernatant of the tissue homogenate of the large intestine and (d) the lymph of the thoracic duct, at 2 h after administration of the BLM·DS_5 complex with 40 mM mixed micelles into the large intestinal lumen (analyzed for BLM, ○—○).

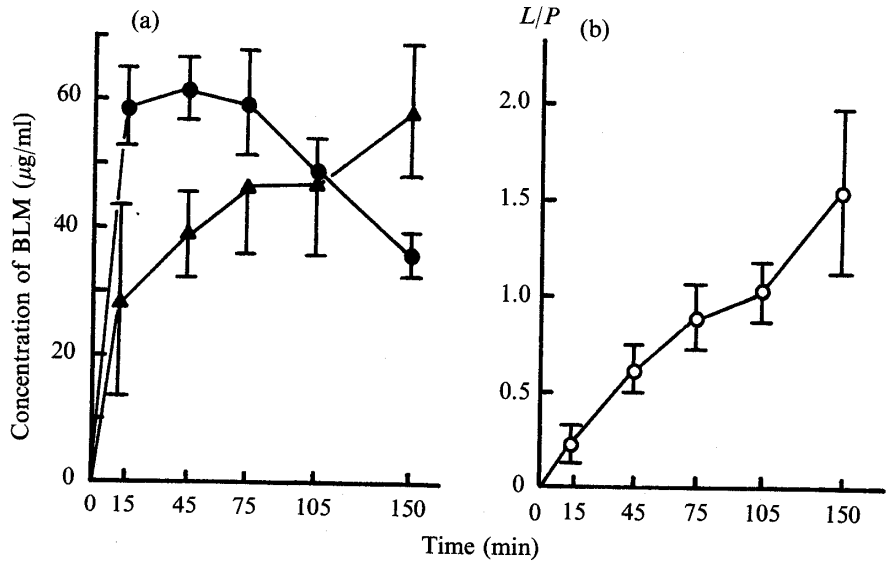

Fig. 4. Absorption of BLM·DS_5 Complex into Blood and Lymph

(a) Concentration of BLM in the plasma and the lymph of the thoracic duct after administration of the BLM·DS_5 complex with 40 mM mixed micelles into the large intestinal lumen. ●, plasma; ▲, lymph.

(b) *L*/*P* ratio of BLM concentration calculated from the data of Fig. 4(a). Each value represents the mean ± S.E. of 4—6 experiments.

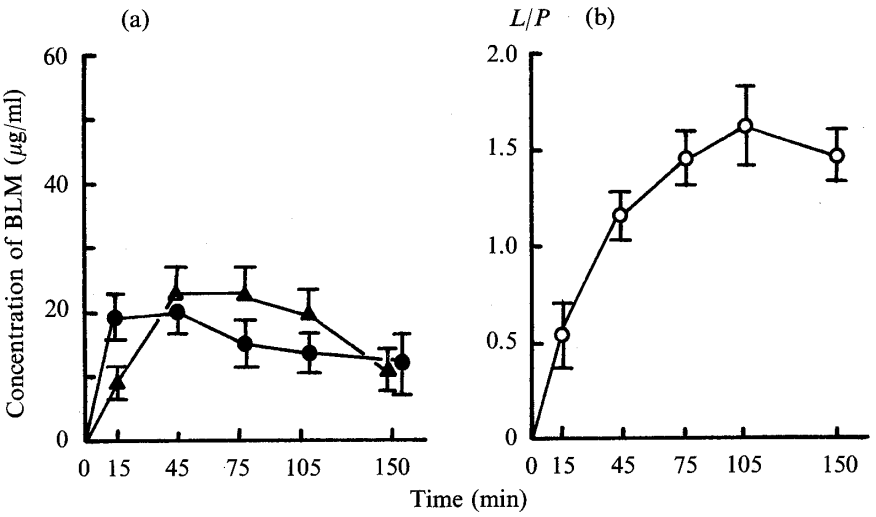

Fig. 5. Effect of D_{500} on Lymphatic Transfer of BLM

(a) Concentration of BLM in the plasma and the lymph of the thoracic duct after co-administration of D_{500} and BLM with 40 mM mixed micelles into the large intestinal lumen. ●, plasma; ▲, lymph.
(b) L/P ratio of BLM concentration calculated from the data of Fig. 5(a). Each value represents the mean ± S.E. of 4—7 experiments.

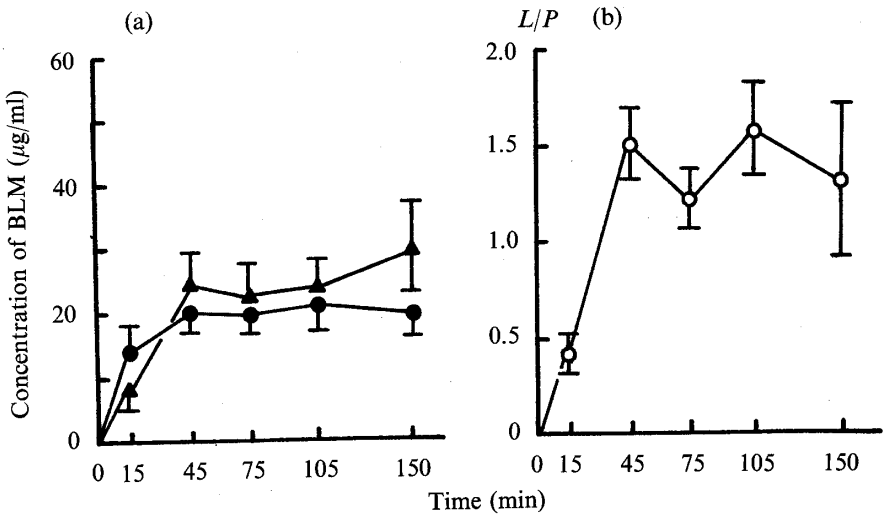

Fig. 6. Effect of DEAE D_{500} on Lymphatic Transfer of BLM

(a) Concentration of BLM in the plasma and the lymph of the thoracic duct after co-administration of DEAE D_{500} and BLM with 40 mM mixed micelles into the large intestinal lumen. ●, plasma; ▲, lymph.
(b) L/P ratio of BLM concentration calculated from the data of Fig. 6(a). Each value represents the mean ± S.E. of 4—6 experiments.

significantly higher than that in the plasma at any sampling time (Fig. 2(a)), and the L/P ratio was 0.5 to 1.7, as shown in Fig. 2(b).

Formation of BLM · DS_5 Complex and Transfer into Lymph

Figure 3(a) shows the elution diagram of the solution of 40 mM mixed micelles containing BLM + DS_5 on Sephadex G-25 gel; the results indicate the integrity of the BLM · DS_5 complex, since free BLM was not detected. The stability of the BLM · DS_5 complex was studied in the lumen and the tissue of the large intestine and the lymph at 2 h after the administration of the mixture of BLM · DS_5 complex with 40 mM mixed micelles into the lumen. In the lumen, complete stability of the BLM · DS_5 complex was observed (Fig. 3(b)).

However, 46% of the BLM·DS_5 complex dissociated to free BLM in the tissue (Fig. 3(c)). The analysis of the lymph showed that 95% of BLM was detected as free BLM (Fig. 3(d)). Upon the administration of the BLM·DS_5 complex with 40 mM mixed micelles into the lumen of the large intestine, the lymph level did not rise significantly above the plasma level during 150 min (Fig. 4(a)), and the L/P ratio of BLM level was 0.2 to 1.5 as shown in Fig. 4(b).

Effect of Co-existence of D_{500} or DEAE D_{500}

D_{500} and DEAE D_{500} are nonionic and cationic macromolecules, respectively, and as expected, complex formation of BLM with these compounds was not observed in a gel filtration study using Sephadex G-50 (not shown). The effect of co-administration of D_{500} or DEAE D_{500} with 40 mM mixed micelles on the lymphatic transfer of BLM from the large intestine is shown in Figs. 5 and 6. BLM level in the lymph was not significantly higher than that in the plasma during 150 min (Figs. 5(a), 6(a)), and the ranges of L/P ratio of BLM concentrations were 0.6 to 1.6 (with D_{500}) and 0.4 to 1.6 (with DEAE D_{500}), respectively (Fig. 5(b), Fig. 6(b)).

Discussion

It has been reported that the lymphatic transfer of lipid-soluble compounds absorbed from the small intestine is closely related to the affinity for chylomicrones.[13] However, water-soluble compounds are considered to transfer into the lymphatics by a molecular sieving mechanism at the blood–lymph barrier in the intestinal tissue according to their molecular size. Macromolecules and particles do not easily enter the blood vessels and instead pass into the lymphatic channels through clefts in the lymphatic endothelium.[14] We have therefore developed a bifunctional delivery system to selectively increase the lymphatic transfer of poorly absorbable BLM *via* the enteral route.

The administration of this system into the large intestine produced a greater effect of increasing the lymphatic transfer of BLM than that into the small intestine.[1,8,9] We have also reported that the administration of the bifunctional delivery system in the large intestine resulted in greater stability of the BLM·DS_{500} complex in the lumen and the tissue of the large intestine compared with that in the small intestine.[8,9] Therefore, the stability of the complex in the lumen and the tissue of the intestine should be closely related to the selective lymphatic transfer of BLM by the bifunctional delivery system.

As the BLM·DS_{500} complex is an ionic complex, the co-existence of the other ions would cause dissociation of the macromolecular complex. In a saline solution of the bifunctional delivery system, the complex dissociated to free BLM, and specific delivery of BLM into the lymphatics compared with that into the blood was not observed upon the administration of this system with saline (Fig. 1). This finding suggests that co-existence of the DS_{500} originally forming a complex with BLM under conditions prohibiting complex formation had no effect of selectively enhancing the BLM delivery into the lymph. Pretreatment with DS_{500} for 1 h in the lumen of the large intestine did not increase the lymphatic transfer of BLM (Fig. 2). This finding rules out the possibility that pretreatment with DS_{500} affects the permselectivity function of the blood-lymph barrier for BLM in the large intestine. This result is supported by the result in our previous report[8] that intravenously pre-administered DS_{500} had no effect on the selective increase of the lymphatic transfer of BLM. Other macromolecules (D_{500} or DEAE D_{500}) having equal molecular weight to DS_{500} did not show complex formation with BLM, and the administration of these mixture produced no selective lymphatic transfer of BLM (Figs. 5 and 6). We have also reported that 5-fluorouracil (MW 130.1) does not form a complex with DS_{500}, and co-administration with DS_{500} failed to enhance the lymphatic transfer of 5-fluorouracil.[8] These results suggested that the formation of the complex between

TABLE I. Cumulative Amount of BLM in Lymph at 3 h after Administration with Mixed Micelles into the Lumen of the Large Intestine

Formulation	BLM·DS_{500} complex in saline	DS_{500} pretreatment	BLM·DS_5 complex	BLM+D_{500}	BLM+DEAE·D_{500}
% of dose	0.48±0.09	0.54±0.12	0.62±0.16	0.47±0.15	0.35±0.20
% of absorbed quantity	1.12±0.45	0.96±0.15	1.00±0.25	0.65±0.19	1.14±0.69

Each value represents the mean±S.E. for 4—7 experiments.

BLM and the DS_{500} is essential for selectively enhancing the lymphatic transfer of BLM by the bifunctional delivery system. Lower molecular weight DS_5 ($\overline{MW}$, 5000) could form a complex with BLM, like DS_{500} ($\overline{MW}$, 500000), as shown in Fig. 3(a). However, the administration of the BLM·DS_5 complex with mixed micelles did not selectively increase the lymphatic transfer of BLM (Fig. 4), although the percentage of the macromolecular fraction of the BLM·DS_5 complex in the large intestinal tissue was 54% (Fig. 3(c)), and this value is larger than that of the BLM·DS_{500} complex (44%) after administration of the bifunctional delivery system.[8] This result suggests that DS of large molecular weight is necessary to increase the transfer of BLM into the lymphatics.

The lymph concentration of BLM was not significantly higher than the plasma level in any experiments of this study. The range of the L/P ratio of BLM level was 0.2—1.7 (Fig. 1, 2, 4—6), and these values are smaller than that observed after administration of the bifunctional delivery system[1] ($L/P=2.3$—5.1). The cumulative amount of BLM transferred to the lymph from the large intestine for 3 h in all experiments of this study was in the range of 0.65—1.14% (of the absorbed quantity) (Table I). These values are significantly ($p<0.01$) smaller than that after administration of the bifunctional delivery system (3.92%)[8] and are not significantly larger than that after free BLM administration with mixed micelles (0.67%).[8] These results indicate that no treatment in this study was able to selectively increase BLM level in the lymph compared with that in the blood as effectively as the bifunctional delivery system.[1,8]

In conclusion, from the results of this study and our previous reports,[1,8,9] the requirements for selective lymphatic transfer of BLM by a bifunctional delivery system upon administration into the lumen of the large intestine appear to be as follows; 1) complex formation of BLM with a macromolecule having lymphotropic properties due to large molecular size and 2) maintenance of the macromolecular complex of BLM in the lumen and the tissue of the large intestine.

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