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Mechanism for selective transfer of bleomycin into lymphatics by a bifunctional delivery system via the lumen of the large intestine

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Summary

The mechanism for the selective lymphatic transfer of bleomycin from the lumen of the large intestine of rat by the administration of a bifunctional delivery system was investigated. This system is a combination of a macromolecular bleomycin–dextran sulfate complex as a lymphotropic carrier, and a lipid–surfactant mixed micelles as an absorption promoter. The administration of the bifunctional delivery system into the lumen of the large intestine showed that the macromolecular complex was stable in the lumen, but half of the complex dissociated in the tissue and 95% of the absorbed bleomycin was detected as free drug in the lymph and the blood. The absorption percentages of bleomycin and dextran sulfate from the lumen were approximately identical, but the lymphatic transfer of dextran sulfate was greater than for bleomycin. Intravenous pretreatment with dextran sulfate indicated no effect on the lymphatic transfer of bleomycin from the lumen of the large intestine. The necessity for a specific binding was tested with 5-fluorouracil; the dextran sulfate showed no effect on the transfer of this drug into the lymphatics when administered concomitantly into the lumen. The formation of a stable macromolecular complex which exists intact in the intestinal lumen was found to be necessary in order to obtain enhanced selective transfer of bleomycin into the lymph in the presence of mixed micelles. A basis for understanding the selective lymphotropic mechanism is presented, and the implication of a bifunctional delivery system in the enhancement of absorption is discussed.

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Introduction

A bifunctional delivery system for the selective transfer of poorly absorbed drug into the lymphatic system via enteral route has been the interest of our research in recent years (Yoshikawa et al., 1981). Poor intestinal absorption of bleomycin (BLM), a basic glycopeptide anticancer drug, mol. wt. 1500, has been found. Upon its intestinal administration, the concentration of BLM in the blood and the lymph have been identical, but its administration along with a new bifunctional delivery system, utilizing monoolein–sodium taurocholate mixed micelles as an absorption promoter together with a macromolecular anionic dextran sulfate (DS) (mean mol. wt. 500,000) as a lymphotropic carrier, has revealed a selective uptake of BLM by the lymphatic system (Yoshikawa et al., 1981); namely, upon its administration into the lumen of the large intestine, a more pronounced effect has been detected.

This very interesting result prompted us to look for the enhancement mechanism involved in this newly developed bifunctional delivery system. The selective lymphotrophy observed as BLM is administered along with the bifunctional delivery system is attributed to the ability of DS to form an ionic complex with BLM. The stability of this BLM–DS complex in the presence of mixed micelles might play a very important role in the enhancement mechanism for transfer of BLM into the lymph. Thus in order to provide the necessary basis to understand the lymphatic transfer of BLM, the synthesis and use of fluorescein isothiocyanate-labelled DS, the intravenous pretreatment with DS, and the co-administration of 5-fluorouracil (5-FU) (mol. wt. 130) were examined, and a plausible mechanism for the selective transfer of BLM into the lymph by the bifunctional delivery system was formulated.

Materials and methods

Materials

BLM and 5-FU were supplied by Nippon Kayaku and Kyowa Hakko Kogyo, respectively. DS (Nakarai Chemicals), fluorescein isothiocyanate (Sigma Chemicals) and monoolein of high purity grade (Nikko Chemicals) were commercially available. Sodium taurocholate was synthesized according to the method of Norman (Norman, 1955). The purity of sodium taurocholate was checked by thin-layer chromatography (Hoffman, 1962) and infrared spectroscopy. All other chemicals were of reagent grade.

Synthesis of a DS tracer: fluorescein isothiocyanate-labelled DS (FDS)

FDS was synthesized according to the method of Belder and Granath (Belder and Granath, 1973). DS (1 g) was dissolved in dimethyl sulfoxide (10 ml) containing a few drops of pyridine. Fluorescein isothiocyanate (0.1 g) was added, followed by dibutyltin dilaurate (20 mg), and the mixture was heated for 2 h at 95°C. After several precipitations in ethanol to remove the free dye, the precipitate was dissolved in distilled water, and after its purification through a Sephadex G-50 column (3.5 × 40 cm), the macromolecular fraction was dried in vacuo at 80°C.

Preparation of test solutions

Test solutions of BLM–DS or BLM–FDS complex were prepared by mixing BLM and either DS or FDS in distilled water (BLM—2.5 mg/ml; DS or FDS—8.25 mg/ml). Test solutions were chromatographed on a 1.8×20 cm column of Sephadex G-50 using distilled water as eluent. Fractions (3 ml each) were automatically collected, and BLM and FDS were determined by ultraviolet and fluorescent spectroscopy, respectively (see details later in this section). Solution of mixed micelles was prepared by dissolving monoolein (40 mM) and sodium taurocholate (40 mM) in distilled water containing the drug, and sonication at 37°C with an Ohtake sonicator model 5202 (100 W, 4 min).

Absorption experiment

Male Wistar albino rats weighing 200–250 g were anesthesized intraperitoneally with sodium pentobarbital (32 mg/kg of body weight). The intestine was exposed through a midline incision, and the closed loop of the entire large intestine (colon and rectum) was prepared by its ligation at the proximal and distal ends. Doses of 5 mg BLM and 16.7 mg FDS per rat, and 2.6 mg 5-FU and 13 mg DS per rat were used, respectively. Two 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, Australia) was placed into the carotid artery and blood samples were periodically collected. Plasma was separated by an Eppendorf centrifuge, model 3200 (15,000 *g* for 2 min). A modification of the method of Bollman et al. (1948) 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, Australia) and fixed with a drop of tissue cement (Aron Alpha, Sankyo). This cannula allowed a continuous drainage of the lymph throughout the experiment. The plasma and the lymph samples were immediately immersed in an ice-bath after collection. For the pretreatment studies, the intravenous injection through the femoral vein of DS (5 mg/0.5 ml distilled water) was performed moments before the instillation of test solution into the lumen of the large intestine. Then the disappearance of BLM and DS (as FDS) from the lumen of the large intestine was studied at 15 min, 1 h and 3 h after administration. At the end of each experiment, any test solution remaining in the loop was removed by forcing air out with a syringe.

Stability of the BLM–FDS complex in the lumen of the large intestine, tissue, plasma and lymph

At 2 h after the administration of test solutions into the lumen, the remaining solution in the lumen was thoroughly expelled and the entire large intestine was removed and homogenized. Samples of plasma, lymph and test solution from the lumen, collected along with the supernatant of the homogenate of the entire large intestine (obtained in isotonic mannitol solution) (15,000 g, 10 min), were fractionated by gel filtration on a 1.8×20 cm column of Sephadex G-50 using distilled water as eluent. Fractions (3 ml each) were automatically collected, and the concentrations of BLM and FDS were determined.

Analytical method of BLM, 5-FU and FDS

The determination of BLM and 5-FU in the remaining solution from the lumen, the supernatant of the tissue homogenate, the plasma and the lymph were carried out by microbiological assays. Disc plate methods using *Bacillus subtilis* (PCI-219) and *Staphylococcus aureus* (209 p) were employed for the BLM (Fujita, 1969) and for the 5-FU assay (Kohono et al., 1977), respectively. The sensitivities of these methods were 0.8 μg/ml for BLM and 0.05 μg/ml for 5-FU. The presence of mixed micelles did not alter the antimicrobiological activity.

The in vitro formation of the BLM–FDS or the 5-FU–FDS complex was tested by optical density using an ultraviolet spectrophotometer (Hitachi model 200-20) at 290 nm for BLM and 265 nm for 5-FU. Fluorescence intensity of FDS was measured with a fluorescence spectrophotometer (Hitachi model 650-10s) using an excitation and an emission wavelength of 490 nm and 520 nm, respectively.

Results

BLM–FDS complex formation and stability in lumen of large intestine

Our previous reports (Muranishi et al., 1979; Yoshikawa et al., 1981) have indicated the presence of an extensive interaction of BLM, a cationic molecule, and DS a strongly anionic macromolecule. In order to provide the basis for understanding this phenomenon, a DS molecule was labelled with fluorescein isothiocyanate, and this labelled compound (FDS) was used as a tracer of DS. As shown in Fig. 1a, the elution diagram of the BLM–FDS complex in distilled water using Sephadex G-50 gel filtration indicated its integrity as a fraction of the BLM–FDS eluted with the fraction of macromolecules. Free BLM was not detected. This great stability was also observed in the mixture of the BLM–FDS complex and 40 mM mixed micelles instilled into the lumen of the large intestine even at 2 h after its administration as shown in Fig. 1b.

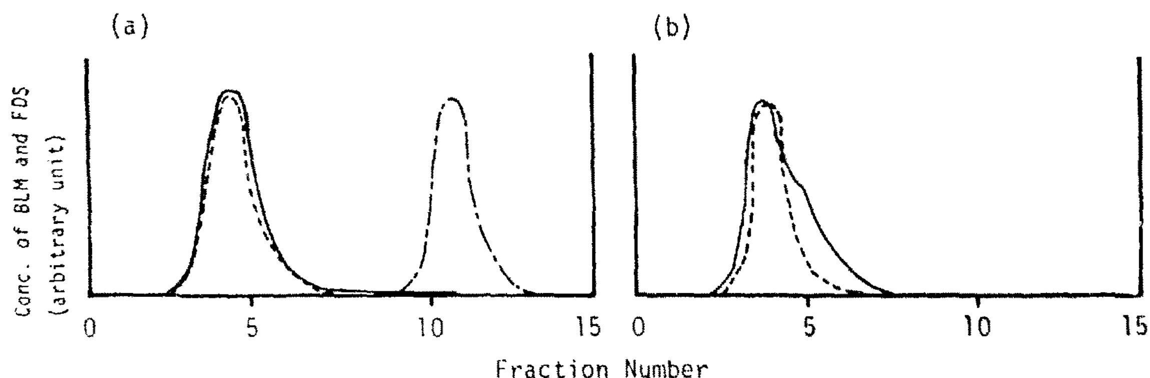

Fig. 1. Formation and stability of the BLM–FDS complex. Gel filtration chromatograms (Sephadex G-50) of (a) free BLM in distilled water (·–·–·) and the BLM–FDS complex in distilled water (analyzed for BLM, ———, and for FDS, - - - - - -); and (b) the contents of the lumen of the large intestine at 2 h after the administration of the BLM–FDS complex with 40 mM mixed micelles into the lumen (analyzed for BLM, ———, and for FDS, - - - - - -).

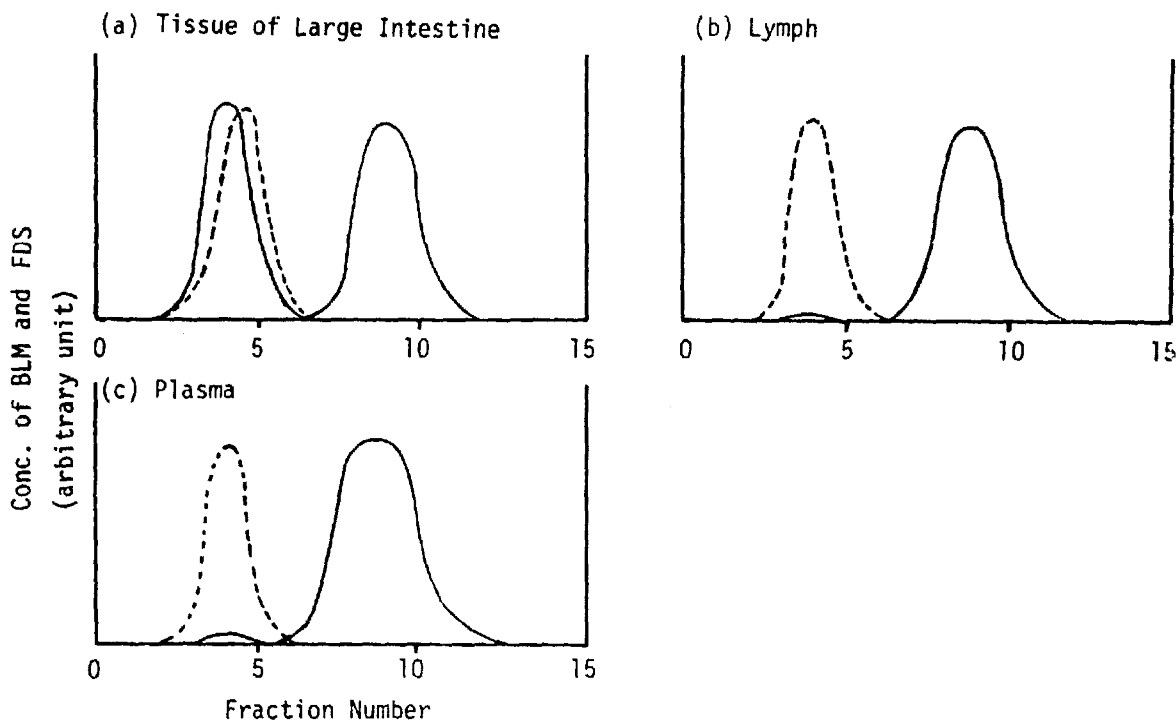

Fig. 2. Stability of the BLM–FDS complex in the different tissues. Gel filtration chromatograms (Sephadex G-50) of (a) the supernatant of the tissue homogenate of the large intestine; (b) the lymph of the thoracic duct; and (c) the plasma, at 2 h after the administration of the BLM–FDS complex with 40 mM mixed micelles into the lumen of the large intestine (analyzed for BLM, ———, and for FDS, - - - - - -).

Stability of absorbed BLM–FDS complex in tissue of large intestine, lymph and plasma

The stability of the absorbed BLM–FDS complex in the body was analyzed in the tissue of the large intestine, the lymph and the plasma, at 2 h after the administration of the mixture of the BLM–FDS complex together with mixed micelles into the lumen of the large intestine. In the tissue of the large intestine, although a great portion of FDS remained stable as shown by the fluorescence detected in the elution fraction of macromolecules, 55% of the BLM–FDS complex dissociated to free BLM (Fig. 2a). In the analysis of the lymph and the plasma, 94–95% of the complex dissociated to free BLM (Fig. 2b and c), and the FDS was detected also in the fraction containing the macromolecules.

Absorption of BLM–FDS complex from lumen of large intestine into blood and lymph

Upon the administration of the BLM–FDS complex with 40 mM mixed micelles into the lumen of the large intestine, a selective transfer of BLM and FDS into the lympnatic system was observed. The concentration of BLM and FDS found in the plasma and the lymph are shown in Fig. 3a. BLM activity in the lymph was 2–5-fold higher than that in the plasma, while FDS concentration in the lymph was 5–14-fold higher. The lymph/plasma concentration ratio (L/P) of BLM and FDS calculated from the data of Fig. 3a are shown in Fig. 3b. These results show that the administration of the BLM–FDS complex into the lumen of the large intestine

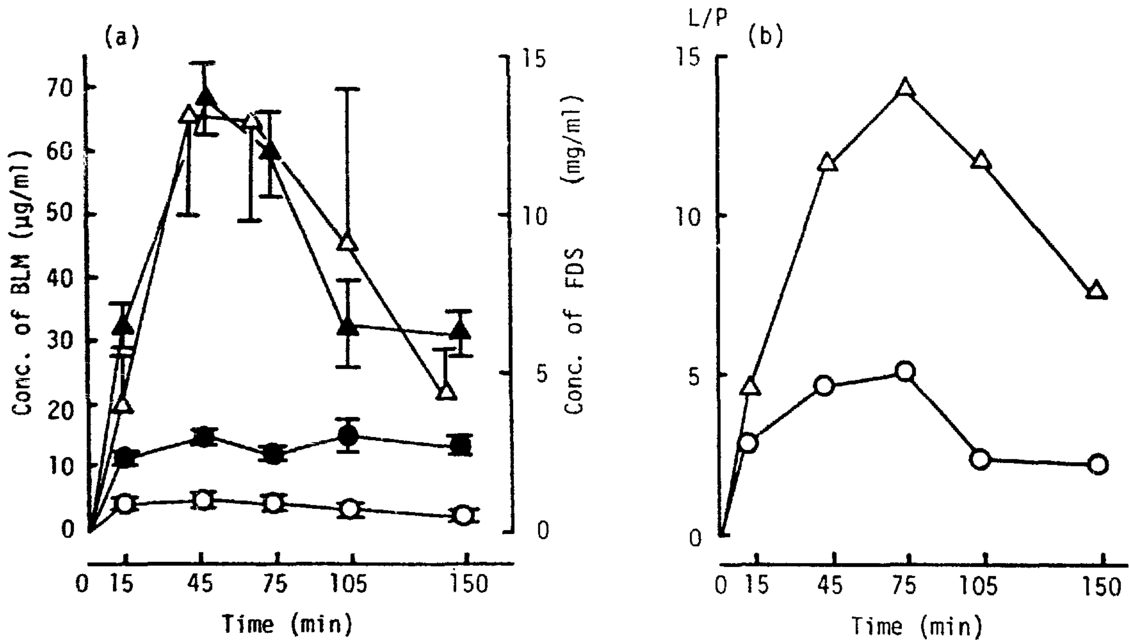

Fig. 3. Absorption of the BLM–FDS complex from the lumen of the large intestine into the blood and the lymph. (a) Concentration of BLM and FDS in the plasma and the lymph of the thoracic duct after the administration of the BLM–FDS complex with 40 mM mixed micelles. Key: open symbols (○,△), FDS; closed symbols (●,▲), BLM; circle = plasma; triangle = lymph. Each value represents the mean ± S.E. for 4–8 experiments. (b) Ratio of concentrations in the lymph relative to the plasma (L/P) of BLM and FDS calculated from the data in Fig. 3a. ○, BLM; △, FDS.

favored the preferential lymph transfer of FDS relative to BLM. In order to decipher that absorption mechanism, the absorption of free BLM and of the BLM–FDS complex from the lumen (both in the presence of 40 mM mixed micelles) was compared. The time course study, as shown in Fig. 4, indicated a significant difference in the absorption rate of BLM, when administered as free BLM or as the

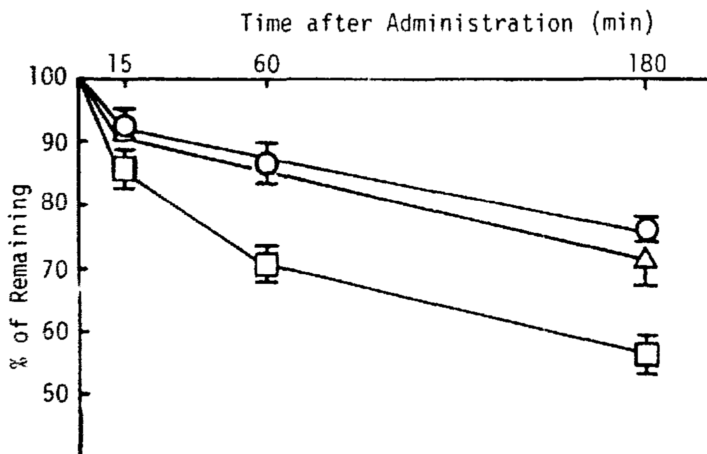

Fig. 4. Time course for the absorption of BLM and FDS from the lumen of the large intestine after the administration of the BLM–FDS complex or free BLM (both with 40 mM mixed micelles). ○, BLM (administered as BLM–FDS); △, FDS (administered as BLM–FDS); □, free BLM. Each value represents the mean ± S.E. for 4–6 experiments.

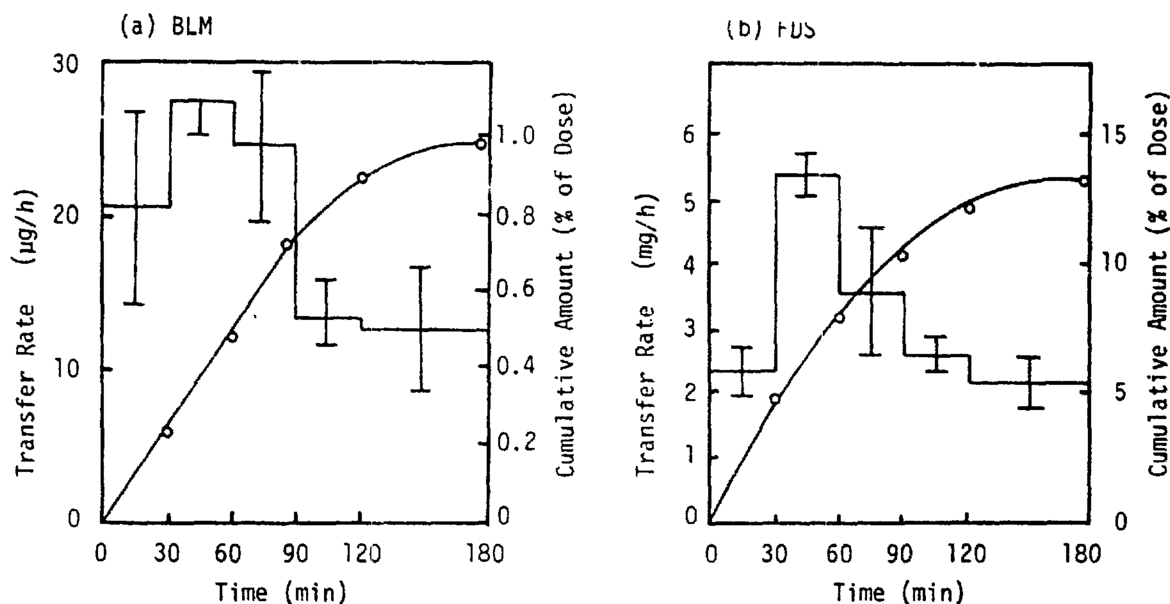

Fig. 5. Transfer rates and cumulative amount of BLM and FDS into the thoracic duct lymph after the administration of the BLM–FDS complex with 40 mM mixed micelles into the lumen of the large intestine. (a) BLM, (b) FDS. Bars are transfer rates and curves (O——O) are cumulative amounts of BLM and FDS (percentage of dose). Each value represents the mean ± S.E. for 4–8 experiments.

BLM–FDS complex (at 1 h, $P < 0.01$; at 3 h, $P < 0.001$). At 3 h after the administration of the BLM–FDS complex with 40 mM mixed micelles, still 75.6 ± 0.7% as BLM and 71.9 ± 3.0% as FDS could be detected in the lumen, while upon the administration of free BLM, only 56.9 ± 1.8% of BLM remained. The transfer rates from the large intestine, i.e. the accumulation of BLM and FDS in the thoracic duct lymph, were monitored for 3 h following the administration of the BLM–FDS complex along with 40 mM mixed micelles as shown in Fig. 5. Comparative cumulative amounts of BLM and FDS in the lymph at 3 h post-administration of the complex and of free BLM is shown in Table I. While the administration of free BLM showed only 0.29% absorption of the dose (0.67% of the quantity absorbed from the lumen), use of the BLM–FDS complex resulted in 0.98% absorption of the dose as BLM (3.92% of that absorbed from the lumen), and 13.21% absorption of the dose of FDS (47.18% of that absorbed from the lumen), respectively, into the lymph.

TABLE I

CUMULATIVE AMOUNT OF DRUG (BLM) IN LYMPH AT 3 h AFTER ADMINISTRATION OF EITHER FREE BLM OR BLM-FDS COMPLEX WITH 40 mM MIXED MICELLES INTO THE LUMEN OF THE LARGE INTESTINE

	BLM–FDS complex		Free BLM
	BLM	FDS	
% of dose	0.98	13.21	0.29
% of absorbed quantity	3.92	47.18	0.67

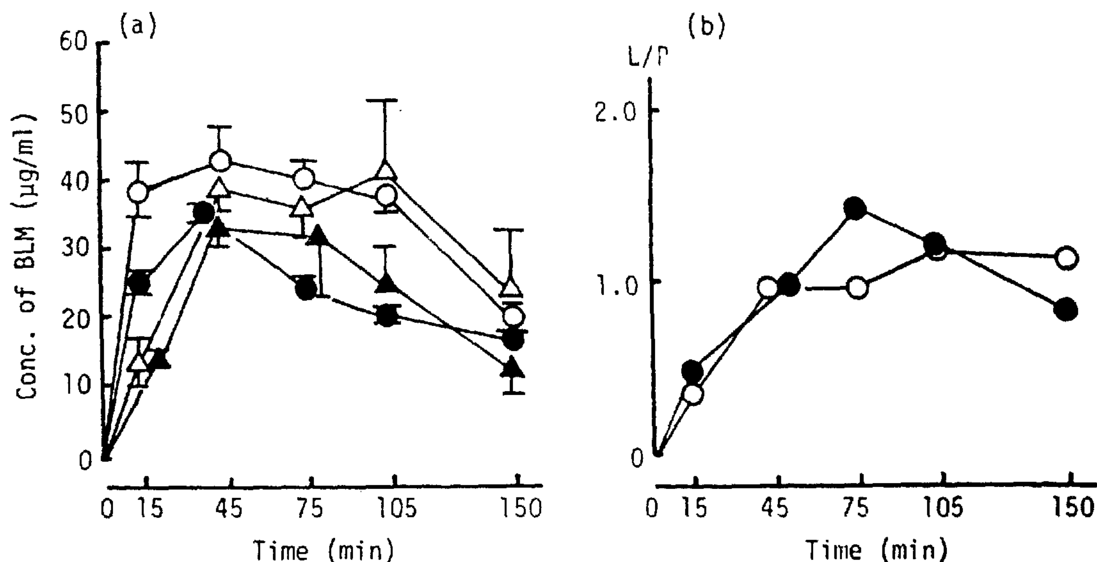

Fig. 6. Effect of intravenous pretreatment with DS on the lymphatic transfer of BLM administered with 40 mM mixed micelles into the lumen of the large intestine. (a) BLM concentration: ○, plasma, no pretreatment; ●, plasma, pretreatment; △, lymph, no pretreatment, ▲, lymph, pretreatment. Each value represents the mean ± S.E. for 4–6 experiments. (b) Ratio of BLM concentration in the lymph relative to the plasma (L/P) calculated from the data in Fig. 6a; ○, no pretreatment; ●, pretreatment.

Effect of intravenous pretreatment of DS on lymphatic transfer of BLM administered in lumen of large intestine

Fig. 6a shows the plasma and the lymph concentrations of BLM after the administration of free BLM with 40 mM mixed micelles into the lumen of the large intestine. An administration of BLM with no intravenous pretreatment of DS showed no significant difference in BLM concentrations in the plasma and the lymph. The pretreatment also showed no significant difference of BLM concentrations in both fluids. The L/P ratio of BLM concentration was ≈ 1 (Fig. 6b).

Lymphatic transfer of 5-FU administered with DS in lumen of large intestine

The effect of co-administration of DS on the lymphatic transfer of 5-FU from the lumen of the large intestine was studied. In aqueous solution, as seen in Fig. 7a, 5-FU did not form a complex with DS. The administration with and without DS into the lumen of the large intestine demonstrated similar 5-FU levels in the lymph and the plasma (Fig. 7b), and the L/P ratio was near the unity (Fig. 7c).

Discussion

Blood is considered to be the main transport route for drugs absorbed from the intestine. The fast-flowing portal venous drainage of the intestine is estimated to be 500 times greater than the flow of the intestinal lymph (Bollman et al., 1948). However, the lymph is another important route for the transfer of anticancer agents used to prevent or treat the lymphatic metastasis of cancer (Haagensen et al., 1972).

For a drug, which is poorly absorbed through the enteral route and not selectively transferred into the lymphatic system, 3 points are considered in order to deliver such drug selectively into the lymphatic system and show its efficacy: (1) penetration through the intestinal barrier; (2) specific binding of the drug to selective lymphotropic carrier; and (3) dissociation to free drug in the lymphatic system. The selective transfer of BLM, a poorly absorbed anticancer agent, into the lymph via the enteral route has been achieved by the development of bifunctional delivery system (Yoshikawa et al., 1981). This system appeared as fulfilling those above-mentioned points, namely with the selection of monoolein–sodium taurocholate mixed micelles as an absorption promoter and of DS as a selective lymphotropic macromolecular compound favoring the formation of an ionic complex with BLM which dissociates in the lymph. In order to trace the enhancing mechanism of DS, a macromolecular carrier, fluorescein isothiocyanate-labelled DS (FDS), was synthesized. This FDS formed with BLM a stable ionic complex (BLM–FDS) in aqueous solution (Fig. 1). Upon its administration into the lumen of the large intestine, the analysis also showed that this BLM–FDS complex remained stable in the lumen for about 2 h; however, the analysis of tissues of the large intestine, the lymph and the plasma indicated that 55%, 94% and 95%, respectively, of the complex was dissociated to free BLM (Fig. 2). This is reasonable since BLM–FDS is an ionic complex, and as such dissociates in the circulatory system. Accordingly, the enhancement provided by the bifunctional delivery system on the levels of BLM, should induce an improvement of anticancer activity.

Studies on the absorption time course of the BLM–FDS complex instilled with mixed micelles into the lumen of the large intestine showed a curve of similar characteristics for either BLM and FDS, but different from that observed when free BLM was administered (Fig. 4). This result indicates the great stability of the BLM–FDS complex in the lumen of the large intestine (Fig. 1). The BLM–FDS complex was stable in the lumen; therefore, the extent of loss of BLM and FDS after the administration of the complex was almost identical.

The selective lymphotrophy of the BLM–DS complex observed might be due to a molecular sieving mechanism of the blood–lymph barrier in the tissue of the intestine, since the pore radius of the blood capillaries in rat intestine has been reported to be under 40–50 Å (Simionescu et al., 1974) as opposed to the lymphatic capillary having a pore radius of 100–150 Å (Leak, 1970). The average molecular radius of DS (mean mol. wt. 5×10^5) are estimated to be around 130 Å based on the molecular size of dextran (Garlick and Renkin, 1970). Therefore, once the intact BLM–DS complex is taken up by the tissue of the intestine, they would be preferentially transferred into the lymph capillary rather than the blood capillary. Thus, after the administration of the BLM–FDS complex with mixed micelles, the lymph/plasma (L/P) ratio for BLM and for FDS concentration reached values from 2–5 and 5–14, respectively (Fig. 3b). The cumulative amounts of BLM and FDS transferred into the lymph (% of the absorbed quantity) was estimated as 4% and 47%, respectively (Table 1). This large difference of the transference of the absorbed BLM and FDS toward the lymphatic system was detected, in spite of their almost similar absorption from the lumen (Fig. 4). Those phenomena may be

attributed to the dissociation of the absorbed complex in the tissue (Fig. 2a).

Equivalent distribution of free BLM into the lymph and the blood was considered, but previous work has shown that macromolecules such as DS or the intact BLM–DS complex is preferentially transferred into the lymph rather than into the blood (Yoshikawa et al., 1981). So, although from data presented in Fig. 2a, a ratio of total DS to total BLM in the lymph (% of the absorbed quantity) should be estimated as 2:1, yet from the data presented in Table 1 a ratio of 12:1 is indicated. Various factors might be implicated in this discrepancy. In this study, the net dissociation of the absorbed BLM–DS complex at the exact site of the blood–lymph barrier of the intestine, is a difficult matter to be determined. Although, it was found that 55% of the complex absorbed from the lumen was dissociated in the tissue of the large intestine, this value is an average percentage of the amount dissociated in the whole tissue, and the percentage of the intact complex at the serosal side should be smaller than 45%, due to the concentration gradient from the luminal side to the serosal side. Therefore, the net percentage of the complex dissociated at the site of the blood–lymph barrier might be larger than 55%.

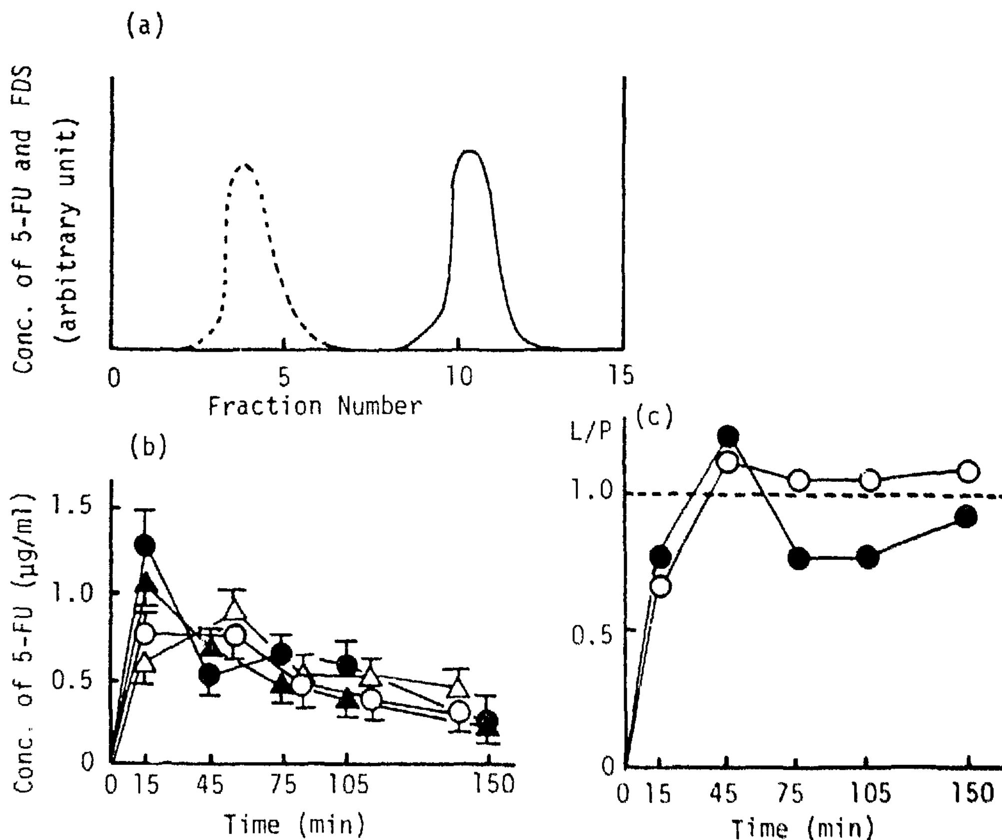

Fig. 7. Lymphatic transfer of 5-FU administered with DS and 40 mM mixed micelles into the lumen of the large intestine. (a) Gel filtration chromatogram (Sephadex G-50) of 5-FU + DS in distilled water (analyzed for 5-FU, ———, and for FDS, - - - - - -). (b) 5-FU concentration in the plasma and the lymph. Key: open symbols (○,△), without DS; closed symbols (●,▲), with DS; circle = plasma; triangle = lymph. Each value represents the mean ± S.E. for 4–5 experiments. (c) Ratio of 5-FU concentration in the lymph relative to the plasma (L/P) calculated from the data in Fig. 7b. ○, without DS; ●, with DS.

Another consideration is that the inactivation of BLM in the lymph tissues may be higher than in the tissue of the large intestine (Yoshioka et al., 1978). Inactivated BLM cannot be measured by the antimicrobiological assay method. Such a situation could result in the discrepancy observed.

With regard to DS, this study indicated that half of the FDS absorbed from the lumen was transferred into the blood (Table 1), in spite of its molecular size being larger than the pore radius of the intestinal blood capillary as mentioned above. This might be due to the heterogeneous distribution of its molecular size, containing macromolecular as well as smaller molecular size DS with no selective lymphotropic characteristics. Another concern about the role of DS was its possible pharmacological effect on the blood–lymph barrier for the transport of BLM. However, its irrelevance was demonstrated by the intravenous pretreatment with DS since no effect on the selective transfer of free BLM into the lymphatics was detected (Fig. 6). On the other hand, the requirement for a specific binding of the drug with this lymphotropic agent was demonstrated by the work carried out with 5-FU. This drug did not form a complex with 5-FU and concomitant administration of 5-FU with DS had no effect on the selective lymphatic transfer of 5-FU from the lumen of the large intestine (Fig. 7).

These findings clearly indicated that DS in the body fluid does not affect the lymphatic transfer of BLM, but the formation of a complex between DS and BLM is essential in order to achieve a selective transfer of BLM into the lymph. In conclusion, from the data gathered, the selective lymphotropic mechanism of bifunctional delivery system (mixed micelles + BLM–DS complex) upon administration into the lumen of the large intestine was derived as shown in Fig. 8. The BLM–DS

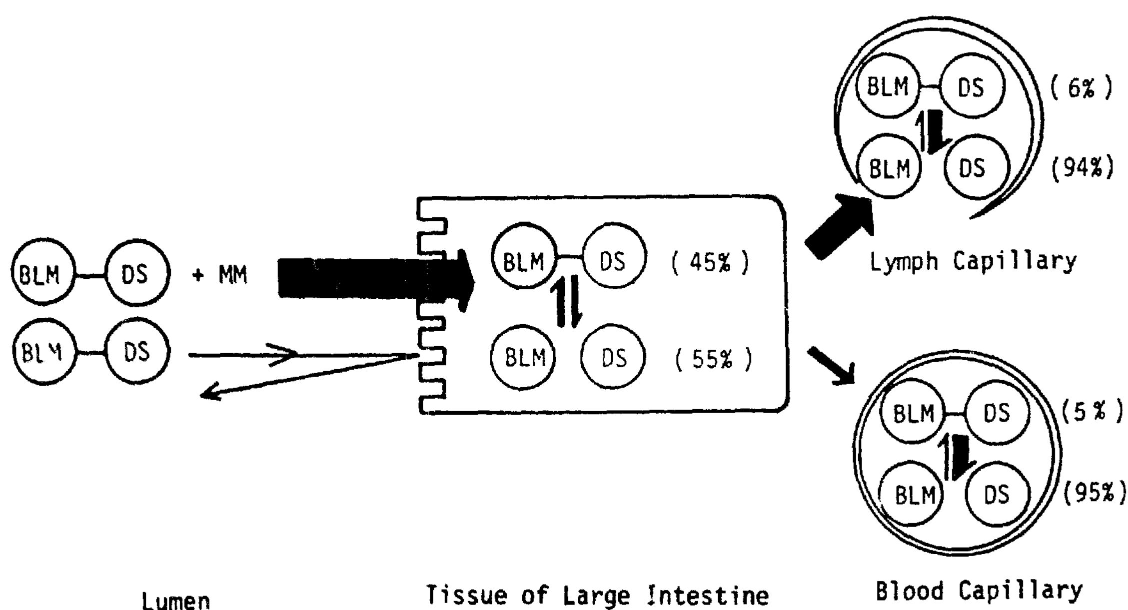

Fig. 8. Proposed mechanism for the selective lymphatic transfer of poorly absorbed BLM from the lumen of the large intestine by the administration of bifunctional delivery system. MM = monoolein–sodium taurocholate mixed micelles.

complex is stable in the lumen. Then the complex penetrates the epithelial barrier in the intact form probably with the aid of mixed micelles. Of the BLM–DS complex absorbed from the lumen, 45% remains as an intact form, while the remaining 55% is dissociated to free BLM and DS in the tissue of the large intestine. The free BLM generated from the dissociation of the BLM–DS complex, transfers into the lymph and the blood at equal concentrations. However, the intact BLM–DS complex preferentially transfers into the lymph. Of the BLM–DS complex in the circulatory system, 94–95% dissociates to free BLM. Therefore, the selective transfer of BLM into the lymphatic system by the bifunctional delivery system via the lumen of the large intestine might be attributed to the following: (1) promotion of the absorption of the intact BLM–DS complex by mixed micelles in the tissue; and (2) the presence of a carrier-like mechanism favored by the intact macromolecular BLM–DS complex at the blood–lymph molecular sieving barrier of the tissue of the large intestine. The presented scheme provided a basis for understanding the selective lymphatic transfer of BLM using a bifunctional delivery system. Further studies about the pretreatment effect of DS into the lumen on the absorption and lymphatic transfer of BLM, or the membrane structure are important subjects, and now under study.

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