International Journal of Pharmaceutics, 8 (1981) 291–302
Elsevier/North-Holland Biomedical Press

BIFUNCTIONAL DELIVERY SYSTEM FOR SELECTIVE TRANSFER OF BLEOMYCIN INTO LYMPHATICS VIA ENTERAL ROUTE

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(Recieved December 3rd, 1980)
(Revised version received and accepted April 7th, 1981)

SUMMARY

Development of lymphotropic delivery system for poorly absorbed drug administered via enteral route was attempted. Lymphotropic property of dextran sulfate (DS) combined with the absorption-inducement property of lipid–surfactant mixed micelles was studied for the delivery of bleomycin (BLM). Monoolein-sodium taurocholate mixed micelles induced a remarkable absorption of BLM from the small and the large intestine of rat, but its concentration in the blood and the lymph was almost identical. However, administration of BLM-DS complex together with the mixed micelles induced a very high concentration rise only in the lymph. This lymphotropic selectivity, observed by complexation of BLM with DS, was much more effective in the large intestinal rather than in the small intestinal administration. Lower lymphotropic selectivity of BLM from the small intestine was due to the dissociation of the complex since instability of BLM-DS complex in the small intestinal lumen was demonstrated by gel filtration studies. Development of this bifunctional delivery system, as an advantageous device for a selective delivery of poorly absorbed anticancer drug into the lymph by way of rectal administration, holds considerable promise.

INTRODUCTION

In chemotherapy-selectivi affinity of drugs with target site is most desirable. Unfortunately, most of drugs tend to interact with non-target sites as well; this is well illustrated particularly in cancer chemotherapy; the slow progress might be attributed to the poor selectivity of anticancer drugs to enter the target area. Lately a review article (Gregoriadis, 1977) has exemplified various drug carriers with effective selectivity to target area in the body such as macromolecules, cells and biodegradeble systems. Our recent studies show that microspheres in oil-type emulsion as drug carriers increases the accumulation of anticancer drugs in the lymphatics. It is a new potential design for

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metastasis treatment through the lymphatic pathway (Hashida et al., 1979). All those above-mentioned carriers are of parenteral use, but not of enteral use because of their difficulties to penetrate the intestinal barrier.

There are at least 3 requirements to overcome for driving a drug from the enteral route toward the lymphatic system, which serves an important route where tumor cells extend. First, drug carriers themselves should penetrate the intestinal barrier such as epithelial cells; it is known that highly water-soluble or large molecular substances cannot be absorbed through them. Second, the size and structure of the lymphotropic carriers must be chosen considering the anatomical barrier of lymphatic capillary. Third, the highly specific binding of anticancer drug to the carrier is required so as to achieve the lymphatic delivery.

Based on these considerations, development of a new lymphotropic delivery system for enteral administration is presented. In this work, the anticancer drug, bleomycin (BLM), a basic glycoprotein, is chosen for absorption studies carried out in the large and small intestine of rats. A lipid–surfactant mixed micelles composed of monoolein-sodium taurocholate is a very effective intestinal absorption-inducer for poorly absorbed and high molecular weight substances (Muranishi et al., 1979a; Muranishi et al., 1979b; Taniguchi et al., 1980). A combined use with dextran sulfate (DS) (mol. wt. 500 000), a lymphotropic functional carrier having the ability to complex with BLM (Muranishi et al., 1979c), is proposed as a selective delivery system for BLM into the lymphatics. The present results indicate the presence of a very selective mechanism inducing an increase of drug (BLM) concentration of about 5 times in the lymph but not in the blood, which shows the remarkable properties of this newly developed bifunctional delivery system.

MATERIALS AND METHODS

Materials. BLM was supplied by Nihon Kayaku. DS (average mol. wt. of about 500 000) was commercially obtained (Nakarai Chemicals). The monoolein used was of high purity grade (Nikko Chemicals). Sodium taurocholate was synthesized according to the method of 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.

Preparation of test solutions. BLM-DS complex was prepared by mixing BLM and DS in distilled water at a concentration of 2.5 mg/ml and 8.4 mg/ml, respectively. Formation of BLM-DS complex was checked by gel filtration. Samples were chromatographed on a 1.8 × 20 cm column of Sephadex G-75 using distilled water as eluent. Three ml of fractions was automatically collected and BLM was determined by spectroscopy. Mixed micellar solutions were prepared by dissolving monoolein and sodium taurocholate in distilled water containing BLM-DS complex or free BLM. A clear solution was obtained by sonicating the mixture at 37°C for 4 min with Ohtake sonicator model 5202.

Absorption experiments. 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 the midline incision, and a closed loop of the entire large intestine (colon and rectum) or the small intestine was separately prepared by ligation at the proximal and distal ends. Aliquots of the test solution was introduced into the intestinal

loops. Doses of 5 mg and 16.7 mg DS per rat were used in a volume of 2.0 ml for the large intestine and 5.0 ml for the small intestine studies. 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 collected periodically. Plasma was separated by an Eppendorf centrifuge, model 3200. A modification of Bollman's method (Bollman et al., 1948) was used for the collection of the thoracic duct lymph. The thoracic duct lymph 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 A, Sankyo). This cannula allowed a continuous drainage of lymph throughout the experiments. Plasma and thoracic duct lymph samples were immediately immersed in an ice bath after collection.

Stability of BLM-DS complex in the intestinal lumen. Three hours after intralumial administration of test solution, the remaining solution in each intestinal lumen was thoroughly collected by forcing it out with syringe air. BLM-DS complex and free BLM in those solutions were fractionated by gel filtration on a 1.8 X 20 cm column of Sephadex G-75 using distilled water as eluent. Three ml of fractions was automatically collected and BLM was determined by antimicrobial assay.

Analytical method of BLM. Antimicrobial assay was used for the determination of BLM in plasma, lymph and intestinal lumen. Disc plate method using *Bacillus subtilis* PCI-219 as a test micro-organism was employed. The minimal sensitivity of the method was 0.8 μg/ml of BLM. Mixed micelles did not affect the antimicrobial activity of BLM. The in vitro formation of BLM-DS complex was tested by optical density of BLM at 290 nm using Hitachi spectrophotometer, model 200-20. Both these methods could accurately measure BLM activity in BLM-DS complex.

RESULTS

Bleomycin–dextran sulfate (BLM-DS) complex formation in mixed micellar solution

Our previous report (Muranishi et al., 1979c) has shown that BLM, a cationic molecule, and DS, a strong anionic macromolecule, formed a nearly complete complex in distilled water. However, it has also been suggested that such an ion-pair complex could dissociate in the presence of ionic substances like buffer components and tissue components. Consequently the stability of the complex in the presence of mixed micelles was questioned.

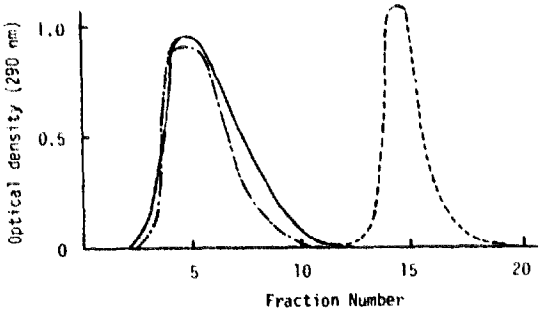

Fig. 1. BLM-DS complex formation in mixed micellar solution. ------, free BLM; - - - - - -, BLM-DS complex; ———, BLM-DS complex with 40 mM mixed micelles. Chromatography was performed on Sephadex G-75.

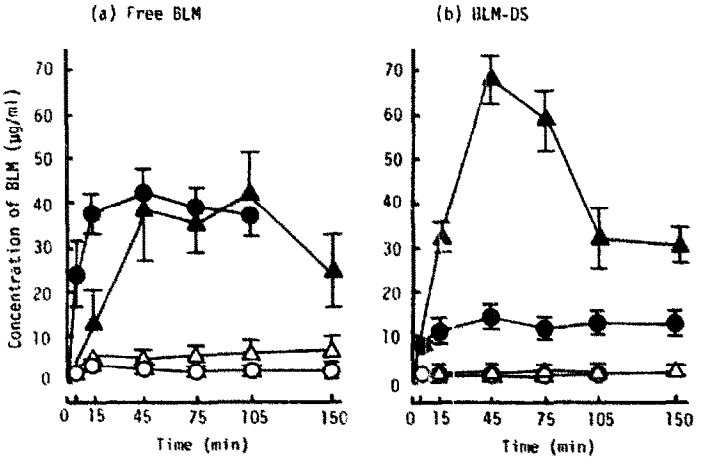

Fig. 2. Concentration of BLM in plasma and thoracic duct lymph after administration into the large intestine. a: administration of free BLM. b: administration of BLM-DS complex. ○, plasma; △, thoracic duct lymph; open, administration without mixed micelles; closed, administration with 40 mM mixed micelles. Each value is represented as mean ±S.E. for 4–8 experiments.

Fig. 1 shows the elution diagram of BLM-DS complex with mixed micellar solution. The figure showed that most of BLM was detected in the elution fraction of BLM-DS complex, a fraction different from free BLM. This fact suggested that BLM-DS complex is not dissociated by the addition of monoolein-sodium taurocholate mixed micelles.

Administration into the large intestine

It is well known that BLM is an impermeable drug to the gastrointestinal barrier. The promoting effect of mixed micelles on heparin absorption in the large intestine was demonstrated to be larger than in the small intestine (Muranishi et al., 1977; Taniguchi et al., 1980).

As seen in Fig. 2a, the administration of free BLM showed very low concentrations in both plasma and thoracic duct lymph at any time (open symbols). But in the presence of 40 mM mixed micelles, BLM concentration in both fluids increased about 10-fold with no apparent concentration differences in both fluids (closed symbols). These results suggested that the lipid–bile salts mixed micelles provided an increase in the large intestinal absorption of BLM, inducing a similar entrance toward the blood and lymph capillaries as observed also in the previous study (Muranishi et al., 1979b). Administration of the complex BLM-DS alone was not effective, but in the presence of 40 mM mixed micelles the concentration of BLM in the lymph was much higher than in the plasma; about 2–5-fold higher as seen in Fig. 2b. When these patterns of mixed micellar promotion were compared with that in Fig. 2a, blood concentration reduction was evident favouring a remarkable increase of lymph concentration. These striking results indicated that a selective delivery device for the lymphatic system was found. BLM conjugated with DS in the presence of mixed micelles provided a very important selective delivery system.

Transfer rates and cumulative amounts of BLM in the thoracic duct lymph was registered during the 3 h after administration as shown in Fig. 3. The administration of BLM in the presence of 40 mM mixed micelles showed an increase of up to approximately 5–6

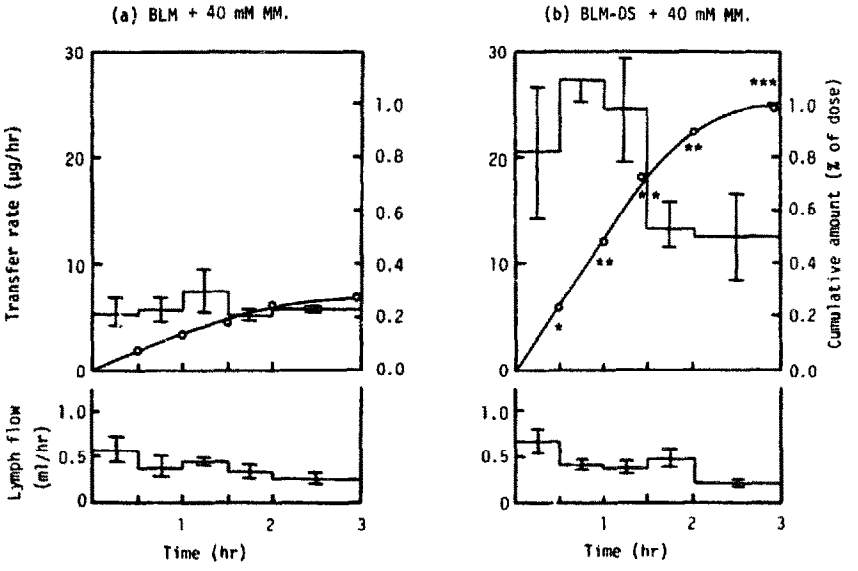

Fig. 3. Transfer rate and cumulative amount of BLM in thoracic duct lymph after administration with 40 mM mixed micelles into the large intestine. a: administration of free BLM with 40 mM mixed micelles; b: administration of BLM-DS complex with 40 mM mixed micelles. Bars over or under horizontal line are transfer rate (μg/h) or flow-rate of lymph (ml/h), respectively. Curves (o——o) are cumulative amount of BLM (percentage of dose). Each value is represented as mean ±S.E. for 4–8 experiments. There is no significant difference of lymph flow-rate during same time interval between (a) and (b) (as in Figs. 4, 6 and 7). Statistical comparison of cumulative amount of BLM transferred in lymph at each time was done by Student's *t*-test.: $*P < 0.05$, $**P < 0.005$, $***P < 0.001$.

μg/h transfer rate and only a cumulative amount of 0.3% in 3 h (Fig. 3a). But the administration of BLM together with 40 mM mixed micelles and DS showed an initial transfer rate of more than 20 μg/h with a cumulative amount of about 1.0% (Fig. 3b). The presence of as low a concentration of mixed micelles as 10 mM administered in the large intestine also promoted the lymphatic absorption of free BLM and BLM-DS as shown in Fig. 4. Although the cumulative amount of BLM without DS was 0.29% (Fig. 4a), similar to the former case, co-administration of DS induced only a slight change to 0.47% (Fig. 4b), indicating a lower increase in lymphatic delivery as compared to the higher concentration of mixed micelles.

Administration into the small intestine

Promotion of BLM-DS delivery into lymphatics from the small intestine was also tested. Administration into the small intestine of free BLM in the absence of mixed micelles indicated that plasma and lymph concentrations were small but larger than the amounts of BLM detected by the administration in the large intestine (Fig. 5a). As shown in Fig. 5b, with the administration of BLM-DS complex, both plasma and lymph concentrations were lower. This result showed that the absorption of the higher molecular weight compound (BLM-DS) was essentially smaller than that of free BLM in the small intestine unless they were administered with mixed micelles. However, the presence of 40 mM mixed micelles administered into the small intestine also increased plasma and

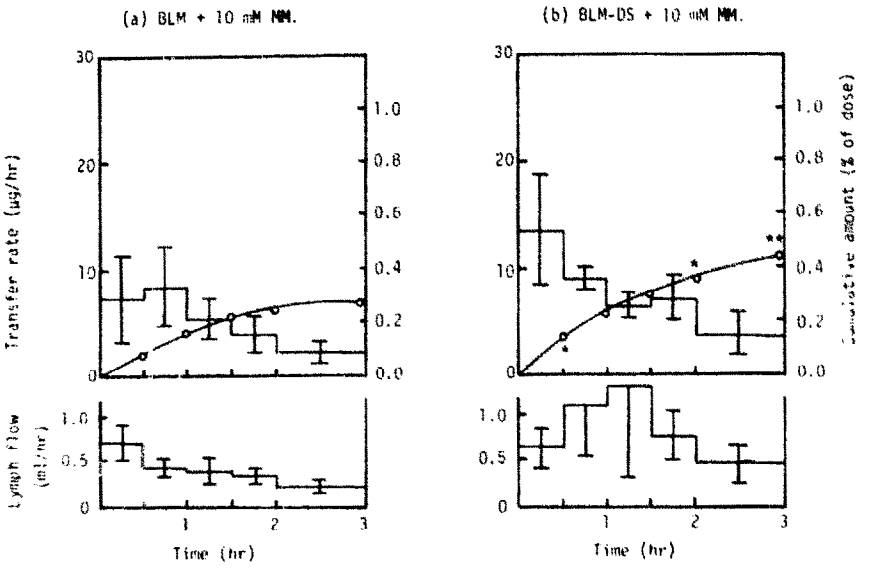

Fig. 4. Transfer rate and cumulative amount of BLM in thoracic duct lymph after administration with 10 mM mixed micelles into the large intestine. a: administration of free BLM with 10 mM mixed micelles. b: administration of BLM-DS complex with 10 mM mixed micelles. Each value is represented as mean ±S.E. for 4–6 experiments. Statistica' comparison of cumulative amount of BLM in lymph: $*P < 0.05$; $**P < 0.01$.

lymph concentrations (Fig. 5a and b). Administration of free BLM with 40 mM mixed micelles showed no significant concentration difference in both fluids. These results showed that the concentration of BLM in the lymph was about two-fold higher than that in the plasma at 45–105 min after administration of BLM-DS complex with 40 mM mixed micelles.

The transfer rate and cumulative amount of BLM in the thoracic duct lymph are shown in Figs. 6 and 7 upon administration into the small intestine which was registered for 3 h. Free BLM and BLM-DS complex accumulated in the lymph to an extent of

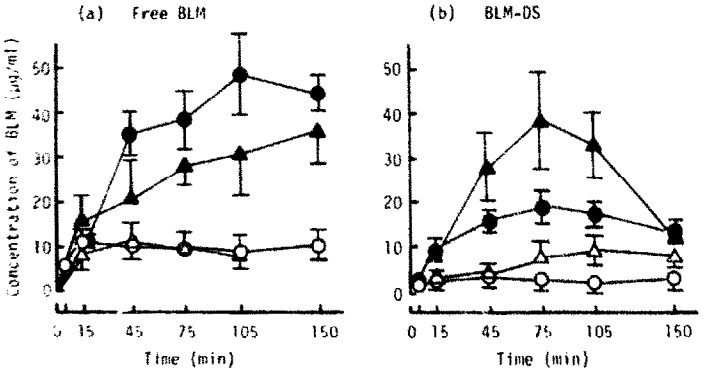

Fig. 5. Concentration of BLM in plasma and thoracic duct lymph after administration into the small intestine. a: administration of free BLM. b: administration of BLM-DS complex. ○, plasma; △, lymph; open, without mixed micelles; closed, with 40 mM mixed micelles. Each value is represented as mean ±S.E. for 4–6 experiments.

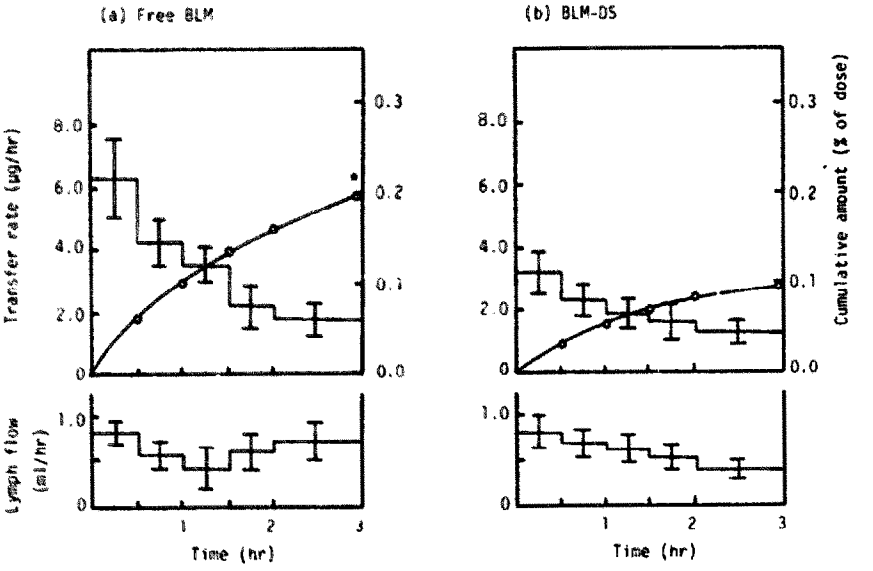

Fig. 6. Transfer rate and cumulative amount of BLM in thoracic duct lymph after administration into the small intestine. a: administration of free BLM without mixed micelles. b: administration of BLM-DS complex without mixed micelles. Each value is represented as mean with S.E. for 4–6 experiments. Statistical comparison of cumulative amount of BLM in lymph: $*P < 0.05$.

0.20% and 0.10%, respectively (Fig. 6). In the presence of 40 mM mixed micelles, the cumulative amount of free BLM and BLM-DS complex increased to 0.23% and 0.33% respectively (Fig. 7). Comparative cumulative amounts of BLM in the lymph after the administration into the large intestine and the small intestine are summarized in Table 1.

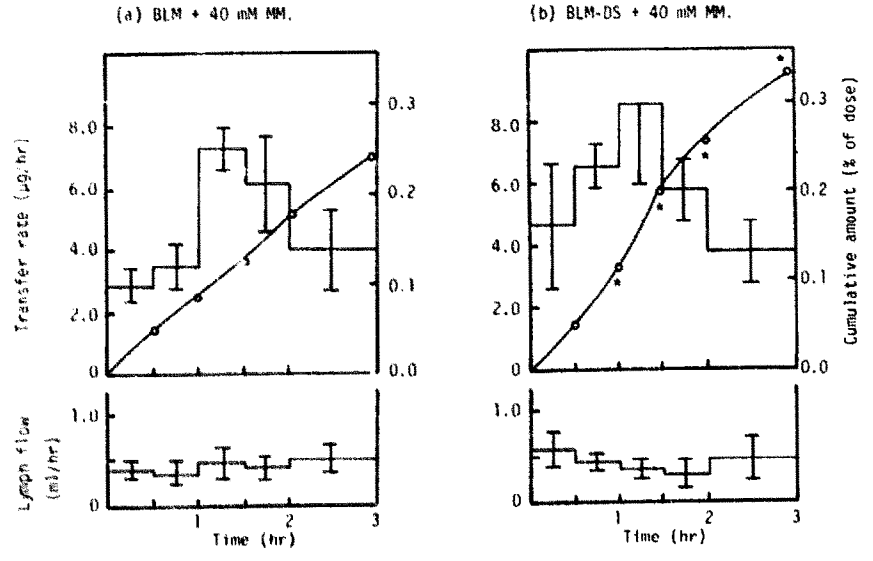

Fig. 7. Transfer rate and cumulative amount of BLM in thoracic duct lymph after administration with 40 mM mixed micelles into the small intestine. a: administration of free BLM with 40 mM mixed micelles. b: administration of BLM-DS complex with 40 mM mixed micelles. Each value is represented as mean ±S.E. for 4–6 experiments. Statistical comparison of cumulative amount of BLM in lymph: $*P < 0.05$.

TABLE 1

CUMULATIVE AMOUNT OF BLM TRANSFERRED IN LYMPH AFTER 3 h ADMINISTRATION

Administration site	Large intestine				Small intestine			
Formulation	BLM-DS 40 mM MM. *	BLM 40 mM MM.	BLM-DS 10 mM MM.	BLM 10 mM MM.	BLM-DS 40 mM MM.	BLM 40 mM MM.	BLM-DS	BLM
% of dose **	0.98 ± 0.18 [a,e]	0.29 ± 0.05 [a]	0.47 ± 0.04 [b]	0.29 ± 0.07 [b]	0.33 ± 0.05 [c,e]	0.23 ± 0.02 [c]	0.10 ± 0.02 [d]	0.20 ± 0.03 [d]

* Monoolein-sodium taurocholate mixed micelles.

** Means ± S.E.

Statistical comparison was done by Student's t-test: [a] $P < 0.001$; [b] $P < 0.01$; [c] $P < 0.05$; [d] $P < 0.05$; [e] $P < 0.001$.

Stability of BLM-DS complex in the intestinal lumen

It is necessary to know the stability of the BLM-DS complex in the intestinal lumen. The intraluminal solutions of BLM-DS complex with mixed micellar solution were recol-

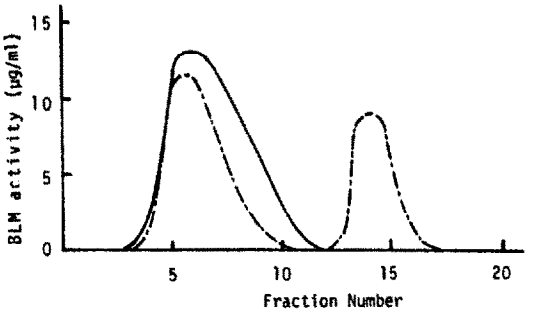

Fig. 8. Stability of BLM-DS complex in intestinal lumen at 3 h after administration. BLM was administered with 40 mM mixed micelles. Samples in intestinal lumen were chromatographed on Sephadex G-75 column. ————, large intestine; – · – · –; small intestine.

lected after 3 h of administration, and the recovered solutions chromatographed on Sephadex G-75. The elution profiles of recovered solution from the large and the small intestinal lumen are shown in Fig. 8. BLM-DS complex in the large intestinal lumen showed a single peak though slightly tailing to the position of the smaller molecule. However, BLM-DS complex in the small intestinal lumen revealed two peaks whose position agreed with the fractions detected for the complex and free BLM, respectively. The percentage of intact BLM-DS complex remaining in the small intestinal lumen, calculated by integration of the peak area, was approximately 56%.

DISCUSSION

Blood is known to be the main transport route for absorbed drugs from the intestine, because the fast-flowing portal venous drainage of the intestine is estimated to be as much as 500 times greater than the flow of intestinal lymph (Bollman et al., 1948). The lymphatic transport of drugs, however, is noteworthy, because the lymphatic transport is essential for some special compounds; namely, important as a route to avoid the first pass effect of the liver, and the necessity to transfer drugs into the lymphatic system of patients suffering from the cancer metastasis, infection, or inflammation. It follows that in tumor metastasis, the lymphatic pathway is an important route, and a selective lymphatic transport of anticancer agents might be of great significance for cancer chemotherapy.

BLM is well known as an anticancer basic glycoprotein. We have reported that BLM can be complexed with higher molecular weight compounds such as DS (Muranishi et al., 1979c). Some macromolecules can be absorbed from the mature mammalian small intestine (Warshaw et al., 1971) and transferred selectively into the lymph (Katayama and Fujita, 1972; Yuzuriha et al., 1975). But their absorbed quantity is very small; the amount absorbed from the large intestine being lower than from the small intestine (Warshaw et al., 1977). On the other hand, lipid–surfactant mixed micelles markedly promotes the

absorption of poorly absorbed drugs, like streptomycin and gentamicin (Muranishi et al., 1979a) or a macromolecular drug like heparin (Tokunaga et al., 1978; Taniguchi et al., 1980).

Free BLM and BLM-DS complex were poorly absorbed from the large intestine as well as the small intestine (Figs. 2 and 5). Monoolein-sodium taurocholate mixed micelles induced a remarkable absorption of BLM as reported in the absorption of heparin. No differences between their concentration in the blood and in the lymph after the administration of free BLM were observed; however, the administration of BLM-DS complex with mixed micelles showed a remarkably higher concentration in the lymph than in the plasma (Figs. 2 and 5). Lymph/plasma concentration ratio of BLM after the administration of BLM or BLM-DS complex with mixed micelles in the large intestine and the small intestine were calculated (Fig. 9a and b). These figures showed that the administration of free BLM reached about an equal ratio value in the large and the small intestine. But the administration of BLM-DS complex gave a higher ratio in the large intestine. The increased cumulative amount of BLM in the lymph by the complexation with DS and by the addition of mixed micelles was more effective in the large intestine than the small intestine (Table 1).

The lymphotropic selectivity of BLM-DS complex was attributed to a molecular sieving mechanism of the blood–lymph barrier. The average molecular radius of DS (mol. wt. 500000) might be about 130 Å, estimated from that of dextran which has a similar mol. wt. (Garlick and Renkin, 1970). The pore radius of blood capillary in rat intestine has been reported to be under 40–50 Å (Simionescu et al., 1974), and the lymphatic capillary has a larger pore (radius 100–150 Å) and large intercellular clefts (width several μm) (Leak, 1970). Therefore, if BLM-DS complex passes through the epithelium of the intestine and does not dissociate in the intestinal tissue fluid, BLM in the presence of DS preferentially transferred into the lymph capillary but not into the blood capillary due to the larger size of the complex.

The stability of BLM-DS complex in the intestinal lumen was also considered as one of

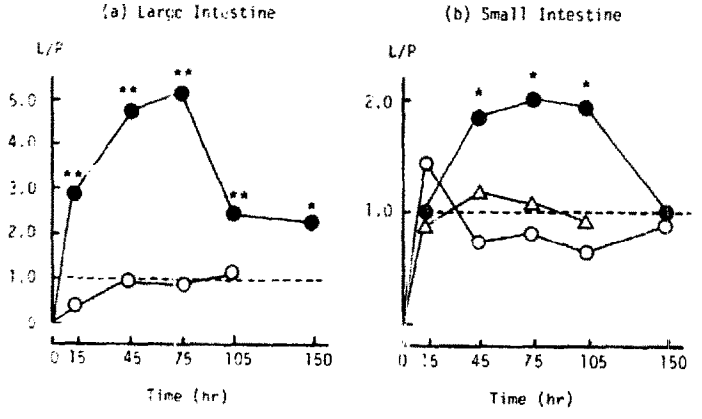

Fig. 9. Ratio of BLM concentration in thoracic duct lymph to plasma (L/P). a: administration into the large intestine. b: administration into the small intestine. △, free BLM without mixed micelles; ○, free BLM with 40 mM mixed micelles; ●, BLM-DS complex with 40 mM mixed micelles. Statistical evaluation of BLM concentration in lymph compared with plasma was done by Student's *t*-test: $^{*}P < 0.05$; $^{**}P < 0.01$.

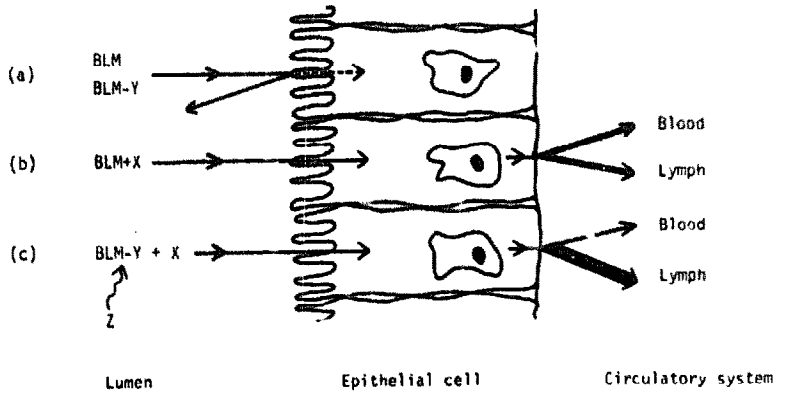

Fig. 10. Proposed scheme for BLM absorption and lymphatic transport. X, absorption inducer such as monoolein-sodium taurocholate mixed micelles; Y, high molecular weight complexing agents such as DS; Z, intestinal labilizer.

the factors affecting the lymphotropic selectivity. As indicated in Fig. 8, the complex was rather stable in the large intestinal lumen, while in the small intestinal lumen more than half of complex was dissociated. Therefore, the smaller lymphotropic selectivity of BLM in the small intestinal administration of the complex may be due to the dissociation of the complex in the lumen before any absorption occurs. Dissociation of the BLM-DS complex might be due to the ionic components in the secreting fluid of the small intestine and its dilution effect.

Finally, a schematic representation of the phenomenon observed in the present study is shown in Fig. 10. Fig. 10a shows that either BLM or BLM-DS (BLM-Y) complex can hardly transfer into the epithelial cells of the intestine in the normal condition; Fig. 10b shows that when an absorption potentiator (X) such as lipid – surfactant mixed micelles is present, BLM can transfer through the epithelial cells and even be delivered at an equal concentration into the blood and lymph; and Fig. 10c shows that when BLM is complexed with a high molecular weight compound (Y) such as DS, and co-administered with X, the complex can transfer through the epithelial cells and be delivered with high selectivity into the lymphatic system. Some intestinal labilizers (Z) may dissociate the ion complex in the intestinal lumen as observed in the small intestine. We reported previously that this BLM-DS complex almost dissociated in pH 7.4 isotonic buffer solution (Muranishi et al., 1979c). Therefore, absorbed BLM-DS complex probably dissociates in the blood and the lymph. This formulation Fig. 10c could be designated as a bifunctional delivery system, since it has a double function, namely absorption potentiation demonstrated by X and lymphotropic selectivity exhibited by Y, and fulfills the 3 requirements proposed. Development of the bifunctional delivery system holds considerable promise as a selective device for drug delivery into the lymph via the rectal route. Wide applications are open since numerous versatile macromolecular carriers, such as the Y component, are available to improve the concentration in the target system.

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