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Research Papers

INCREASED LYMPHATIC DELIVERY OF BLEOMYCIN BY MICROSPHERE IN OIL EMULSION AND ITS EFFECT ON LYMPH NODE METASTASIS

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SUMMARY

The efficiency of a gelatin-microsphere in oil (S/O) emulsion as a drug delivery system of bleomycin in surgical adjuvant chemotherapy was evaluated in normal and VX2 carcinoma-bearing rabbits. Following injection of bleomycin into the appendix wall in the form of a S/O emulsion, a remarkable accumulation of the drug was observed at the injection site and the regional lymph node for 3 days, while administration of an aqueous solution of the drug exhibited a slight accumulation and a rapid disappearance from these sites. A water in oil (W/O) emulsion exhibited lower potency than the S/O emulsion and the oily suspension was unable to promote lymphatic transport of bleomycin. The implanted VX2 carcinoma at the appendix of the control rabbits showed a metastasis at the regional lymph node and all of the control group died although the primary tumor had been surgically excised. Adjuvant chemotherapy using bleomycin–S/O emulsion system resulted in obvious histological damage on metastatic VX2 cells and prolongation of survival time of about two times over the control. These results suggest the superiority of the S/O emulsion in surgical adjuvant chemotherapy by satisfying many of the criteria of an ideal drug delivery system.

INTRODUCTION

The ideal dosage form in cancer chemotherapy is the one that provides a tumor site with sufficient amount of anticancer agents over a long period of time without considerable interaction with normal tissues. In the past, considerable efforts have been directed toward a development of a timed-release device which could be implanted in the closest possible proximity to a malignant tissue (Kojima et al., 1978; Takahashi et al., 1976; Yolles et al., 1976) or a carrier system which could deliver anticancer agents selectively into malignant cells thriving far from the injection site (Gregoriadis et al., 1974; Kramer, 1974).

We have been studying the utility of various types of vegetable oil emulsions as delivery systems which might enhance the accumulation of anticancer agents in lymphatics for designing a potential treatment for metastasis along the lymphatic pathways: In previous studies, an enhanced delivery of anticancer agents such as bleomycin (Nakamoto et al., 1975) and 5-fluorouracil (Hashida et al., 1977a) to the lymphatics after parenteral administration of their emulsion formulations was demonstrated in the rat. In these studies, the largest enhancement of lymphatic transport was exhibited by a microsphere in oil (S/O) emulsion, in which a water in oil (W/O) emulsion was improved through a replacement of its inner water droplets by gelled gelatin microspheres of approximately 1.5 μm in diameter (Hashida et al., 1977b).

The present investigation was undertaken to demonstrate a further utility of the S/O emulsion in surgical adjuvant chemotherapy, for these studies VX2 carcinoma-bearing rabbits were employed, these are characterized by rapid and predictable metastasis to lymph node (Zeidman, 1965). The appendix was chosen as the model experimental organ. The transfer patterns of bleomycin in several tissue compartments following topical injection of S/O emulsion were determined in normal rabbit or hemiappendectomized rabbit bearing VX2 carcinoma at the excised portion. The preventive effect against lymph node metastasis was compared by histological examination or animal survival data.

MATERIAL AND METHODS

Materials

Bleomycin was supplied from Nihon Kayaku Co., Japan. A mixture of medium chain triglyceride (MCT) was supplied from Nissin Seiyu Co., Japan. Non-ionic surfactants, polyoxyethylene derivative of hydrogenated castol oil (HCO-60) and sorbitan sesquioleate (SO-15) were supplied from Nikko Chemicals Co., Japan. All other chemicals were reagent grade products obtained commercially.

Preparation of parenteral formulations

Both S/O and W/O emulsions were prepared with 40 volumes oily phase and 7 volumes aqueous phase. MCT or sesame oil incorporating SO-15 and HCO-60, 6.7% and 1.7% (v/v) respectively, was used as the oily phase. The aqueous phase was 20% (w/v) gelatin solution (S/O emulsion) or distilled water (W/O emulsion), dissolving bleomycin at the concentration of 60 mg (potency)/ml. After heating at about 50°C, both phases were mixed with each other and emulsified by ultrasonification. Sonification was carried out in the water bath maintained at 70°C followed by rapid cooling to about 0°C. The drug content of final product was 8.9 mg/ml.

The oily suspension of bleomycin was supplied from Nihon Kayaku Co., Japan which was prepared by suspending bleomycin in sesame oil using aluminum monostearate as a dispersing agent.

Animals

Male domestic rabbits weighing between 2.0 and 3.2 kg were used in all animal experiments. The animals were anesthetized with intravenous injection of sodium pentobarbital and placed on their backs during the course of the operation.

Procedures for injection and sampling

The appendix (appendix vermiformis) of the rabbit was exposed through a midline incision of abdominal wall and various formulations were injected into the sub-serosal layer of the appendix. The dose of bleomycin was 3 mg/kg for all experiments. At varying time periods after injection, rabbits were sacrificed, and the appendix, regional lymph node, lung, and liver were excised. Blood samples were obtained simultaneously.

Analytical method

The concentration of bleomycin was determined by microbiological assay using *Bacillus subtilis* PCI-219 as a test organism (Ohnuma et al., 1974). The excised tissue was homogenized by a glass homogenizer after weighing and diluted with isotonic phosphate buffer (pH 7.4). After centrifugation at 2800 rpm for 5 min, the supernatant was used for the microbiological assay. For measurement of plasma levels, blood was collected at appropriate time intervals in heparinized syringes and centrifuged at 3000 rpm for 2 min. The plasma obtained was either assayed immediately or after storage in freezer. All assays were undertaken by a disc-plate method and the results were calculated using a standard curve.

Procedures for preparing lymph node metastasis

VX2 carcinoma was maintained by monthly transplantation of a tumor cell suspension into the thigh muscle. In our experience the established tumor has never regressed spontaneously. The tumor fragments were excised except for areas of suspected necrosis and chopped in sterile Hanks' balanced salt solution. After centrifugation at 800 rpm for 5 min, the supernatant was discarded and the residual sediment was resuspended in 4 times the volume of fresh medium. In order to remove clumps, the suspension was sieved through two layers of surgical gauze. The cell suspension obtained contained approximately 5×10^6 cells per ml and 0.3 ml of this suspension was implanted into the midpoint of the appendix. Ten days after transplantation, VX2 carcinoma metastasized almost completely to the regional lymph node located at the root of the appendix.

Evaluation of the effect of the adjuvant chemotherapy

Ten days after implantation of VX2 cells the primary tumor (approximately 1 cm × 1 cm in size) was excised by hemi-appendectomy operation, and bleomycin was administered simultaneously as an adjuvant chemotherapy. The efficiency of bleomycin treatment was examined by histological observation of the metastasized lymph node and survival data.

RESULTS

Transport of bleomycin after administration with S/O emulsion into the appendix

As discussed in previous papers (Hashida et al., 1977b, c), it is considered that the drug injected as an emulsion formulation into the interstitial space of the appendix could be transported from the injection site directly into the lymphatic system, either with oil droplet carriers or as free drug after separation at the injection site. Also the absorption

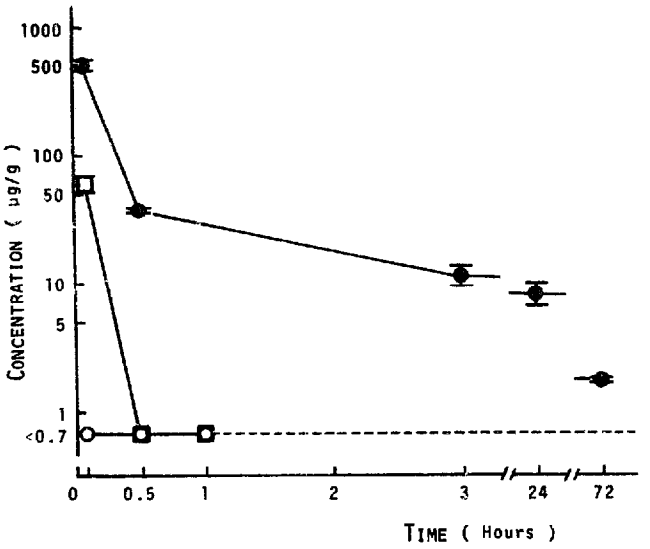

Fig. 1. Concentration of bleomycin in the appendix after injection with various conditions. ○, intravenous injection of aqueous solution; □, topical injection of aqueous solution; ●, topical injection of S/O emulsion. Results are expressed as the mean ± S.E. of three animals.

route via blood capillaries should be included. To evaluate the efficiency of the S/O emulsion more clearly, the consecutive movement of bleomycin after injection into the appendix was investigated in normal rabbits. In this experiment, MCT was employed as the base of the S/O emulsion.

The time course of concentration of bleomycin in the appendix after injection of S/O emulsion is shown in Fig. 1, together with the results of topical or intravenous injection of aqueous solution for comparison. The S/O emulsion showed the highest bleomycin concentration of more than 500 μg/g wet tissue at 5 min after injection. Three days after injection, bleomycin was still detectable in the appendix. When aqueous solution was injected, a considerably higher concentration was exhibited at 5 min after injection, but it decreased rapidly and no antimicrobial activity could be detected even at after 30 min. Intravenous injection did not exhibit any significant concentration over the period of the experiment, suggesting that distribution of bleomycin from the circulating blood to the appendix was negligible. These data clearly indicate that bleomycin is maintained at the injection site for a longer period when injected with the S/O emulsion.

Fig. 2 shows the concentration–time course of bleomycin in the regional lymph node. Five minutes after intravenous injection of aqueous solution bleomycin quickly arrived at the lymph node, but the concentration decreased rapidly and no antimicrobial activity could be detected after 1 h. On the other hand, topical administration showed a higher concentration and, consequently, direct transport of bleomycin from the appendix through the peripheral lymph vessels was indicated. The S/O emulsion showed the highest concentration immediately after injection and then showed gradual decrease; 3 days after injection the concentration was greater than 10 μg/g. The enhanced and sustained delivery of bleomycin into the regional lymph node by the S/O emulsion was demonstrated.

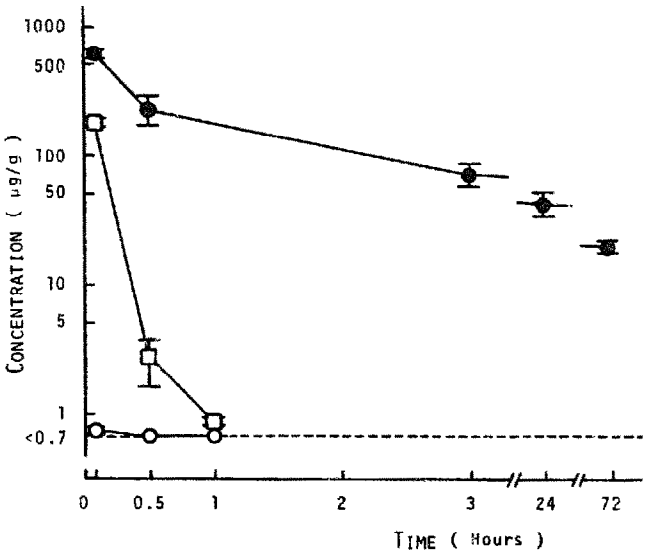

Fig. 2. Concentration of bleomycin in the regional lymph node after injection with various conditions. ○, intravenous injection of aqueous solution; □, topical injection of aqueous solution; ●, topical injection of S/O emulsion. Results are expressed as the mean ± S.E. of three animals.

In Fig. 3, the plasma concentration following injection of the S/O emulsion and an aqueous solution are compared with that obtained after intravenous injection. The intravenous injection exhibited a maximum concentration 5 min after injection, after which the concentration decreased rapidly. Topical injection of the aqueous solution showed a higher peak level at 5 min and also a rather rapid decrease. Compared with these

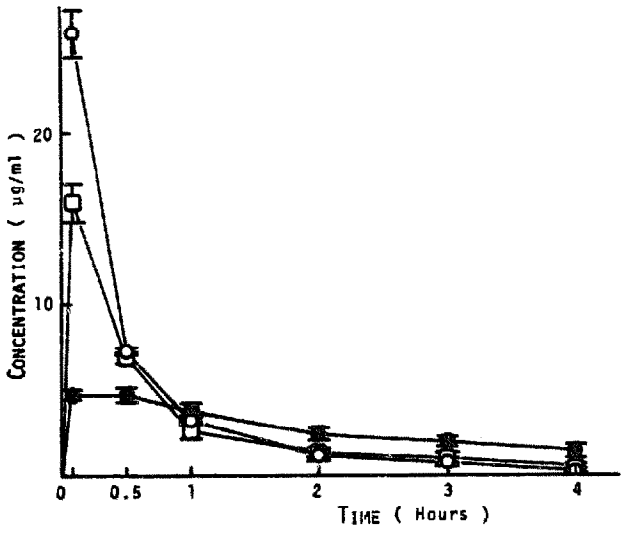

Fig. 3. Plasma concentration of bleomycin after injection with various conditions. ○, intravenous injection of aqueous solution; □, topical injection of aqueous solution; ●, topical injection of S/O emulsion. Results are expressed as the mean ± S.E. of three animals.

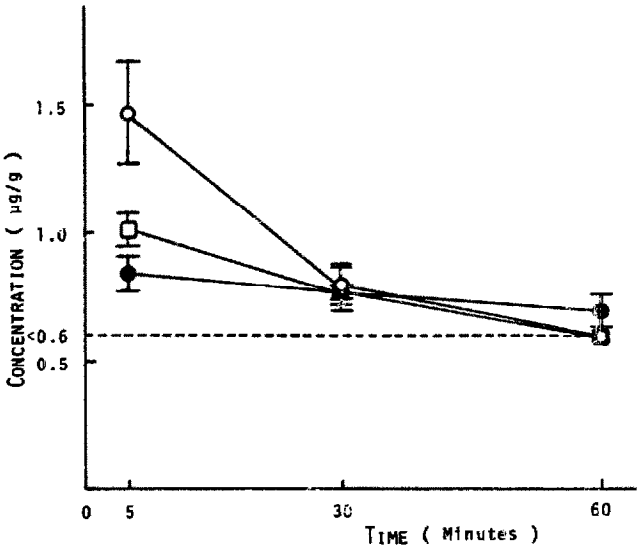

Fig. 4. Concentration of bleomycin in the lung after injection with various conditions. ○, intravenous injection of aqueous solution; □, topical injection of aqueous solution; ●, topical injection of S/O emulsion. Results are expressed as the mean ± S.E. of three animals.

results, the S/O emulsion showed a significant delay of appearance of bleomycin in the blood and a subsequent gradual plasma level decrease. The rate of disappearance of the drug from tissue shown in Fig. 1 is fairly well reflected in these results on blood concentration.

In the same experiment shown in Figs. 1 and 2, the bleomycin concentration was also determined for the lung and liver. Fig. 4 shows the concentration of bleomycin in the lung, which is known to be adversely effected by bleomycin treatment (Umezawa et al., 1967). As shown in this figure, bleomycin could be detected at earlier periods in the experiment although the concentration did not exceed the level of 1.5 μg/g. No significant difference could be noticed 30 min after injection for the three cases. On the other hand, bleomycin could never be detected in the liver during the experiments, suggesting a rapid inactivation in this organ (Ohnuma et al., 1974).

Comparison of delivery properties of various formulations

In previous investigations (Hashida et al., 1977a, b, c) sesame oil was used as the emulsion base in spite of its disadvantage for injection because of its intrinsic high viscosity. For overcoming such practical inconvenience, MCT was chosen as the base of the S/O emulsion as it has a lower viscosity and no toxicity problems for medical use (Greenberger and Skillman, 1969; Sailer and Berg, 1977). Table 1 summarizes the concentrations of bleomycin in the appendix and lymph node after topical injection of various formulations. The oily suspension showed the highest concentration in the appendix 30 min after injection, and the levels decreased in the rank order of S/O emulsion (sesame oil), S/O emulsion (MCT), and W/O emulsion. After 24 h, the concentrations in the

TABLE 1

BLEOMYCIN CONCENTRATIONS IN THE APPENDIX AND LYMPH NODE AFTER TOPICAL INJECTION WITH VARIOUS FORMULATIONS

Formulation (oil component)	Concentration [a] (μg/g wet tissue)			
	Appendix		Lymph node	
	30 min	24 h	30 min	24 h
S/O emulsion (MCT)	38.1 ± 4.3	8.3 ± 1.5	233.7 ± 60.0	45.0 ± 9.2
S/O emulsion (sesame oil)	28.1 ± 4.6	6.1 ± 0.6	169.5 ± 18.1	18.2 ± 5.2
W/O emulsion (MCT)	22.0 ± 0.8	4.4 ± 0.8	121.9 ± 10.5	16.3 ± 1.6
Oily suspension (sesame oil)	47.2 ± 19.8	4.5 ± 0.4	8.8 ± 0.7	2.8 ± 0.2

[a] Results are the mean ± S.E. of three experiments.

appendix were between 4.4 and 8.3 μg/g in all cases. On the other hand, the S/O emulsion (MCT) exhibited the highest concentration in the lymph node both at 30 min and 24 h after injection, while the oily suspension was the lowest. From these results it can be considered that the orginal delivery characteristics of the S/O emulsion is improved by utilizing MCT instead of sesame oil as the base of the emulsion.

Delivery of bleomycin by S/O emulsion in VX2 metastasis-bearing rabbit

For comparing the results obtained in the normal animals with those in the metastasized animals, concentrations of bleomycin in the appendix, lymph node and plasma 30 min after injection are summarized in Table 2. Obviously, these concentrations were almost identical between the two animal conditions, so it can be presumed that S/O emulsion may also exhibit its advantage for VX2-bearing rabbits. The lymph node concentrations were more than 40 times as great as those of plasma in both animal groups, and an enhanced supply of bleomycin to the regional lymph node was thus proved in the pathological condition.

TABLE 2

BLEOMYCIN CONCENTRATION AT 30 MIN AFTER INJECTION WITH S/O EMULSION IN NORMAL AND VX2 CARCINOMA-BEARING RABBIT

Animal	Concentration [a] (μg/ml or g)		
	Plasma	Appendix	Lymph node
Normal rabbit	4.7 ± 0.3	38.1 ± 4.3	233.7 ± 60.0
VX2-bearing rabbit	4.4 ± 0.4	32.4 ± 4.5	209.9 ± 41.2

[a] Results are the mean ± S.E. of three experiments.

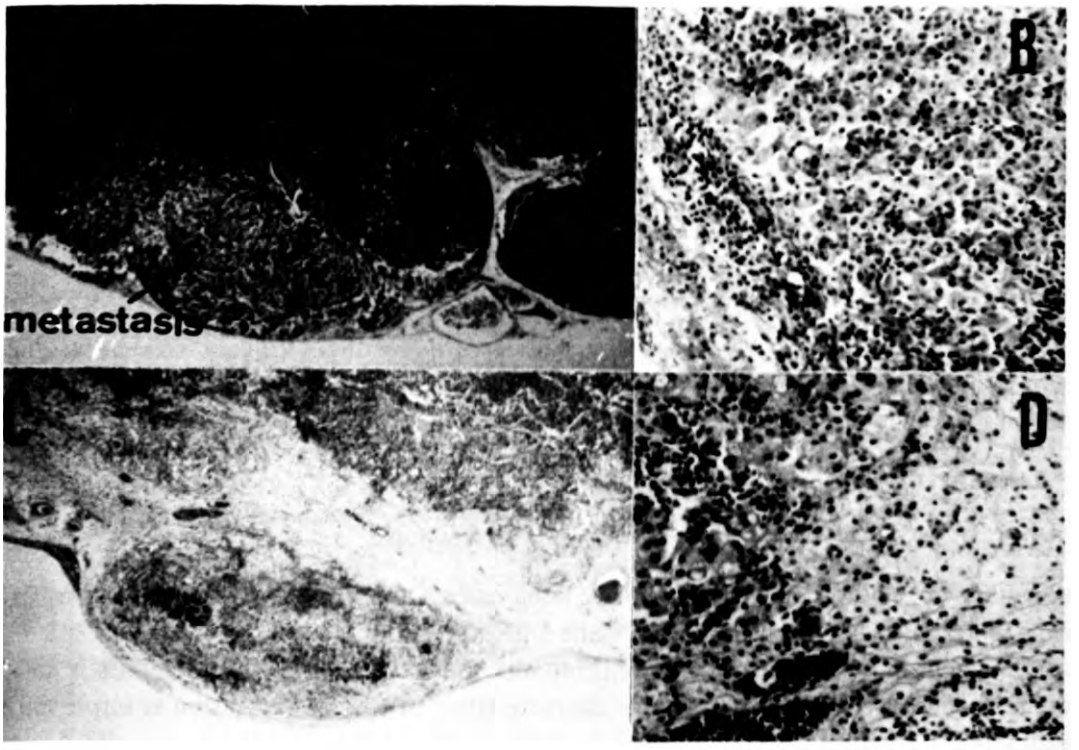

Fig. 5. Microphotographs of the metastasized regional lymph node at 1 week after excision of the primary tumor without any chemotherapy (A and B) or at 1 month after surgical treatment and an adjuvant chemotherapy using bleomycin–S/O emulsion system (C and D). The excised lymph nodes were fixed in 10% formalin and stained with hematoxylin and eosin. A and C; ×40. B and D; ×200.

Effect of bleomycin–S/O emulsion on lymph node metastasis

In order to demonstrate the advantage of the S/O emulsion simultaneous chemotherapy of bleomycin was carried out together with surgical treatment, and its activity was evaluated by histological examination. The control group, which had the excision of the primary tumor but no adjuvant chemotherapy, was observed without exception to develop micrometastasis in the lymph node after 1 week. At this stage most metastases were observed in the marginal sinus (Fig. 5A and B). A month after the operation, an extended metastatic growth was observed in the medullary zone such as the medullary sinus. At this time an enlargement of lymph node was recognized by gross examination. On the contrary, bleomycin–S/O emulsion chemotherapy resulted in obvious damage to the metastatic VX2 cells, and 2 weeks after treatment cell borders became partially obscured and the cytoplasms of most of the cells were characterized by vacuolation. After one month the VX2 cells were almost completely degenerated and micronecrosis of cytolysis was observed accompanied with collagenous fibrosis and an inflammatory response in the cells (Fig. 5C and D). Simultaneous topical administration of aqueous solution of bleomycin showed a damaging effect against metastatic cells of various degrees at the earlier stage, while reproduced metastasis was demonstrated in some cases after one month.

The survival time of animals receiving the bleomycin–S/O emulsion was remarkably increased, with 5 of the 7 rabbits still surviving 100 days after the operation. There were no survivals among the rabbits not receiving chemotherapy, their mean survival time was approximately 50 days. These results are interpreted to suggest the efficiency of bleomycin administered in the form of S/O emulsion.

DISCUSSION

A sufficient supply of cancer-destroying agents to the lymph node seems to offer a promising means of preventing lymphatic spread of cancer. The advantage of direct introduction of chemotherapeutic agents into the lymphatic vessels has been demonstrated in several reports (Ariel et al., 1964; Kitchen and Garrett, 1971), but only little profit could be expected by employing this treatment modality in surgical adjuvant chemotherapy because of its technical difficulties. On the other hand, Ballard (1968) mentioned that molecules having relatively low molecular weights are not absorbed primarily by the lymphatic vessels but through capillaries from the interstitial spaces. Consequently, it is necessary to develop a specific carrier system which can deliver anticancer agents to the lymphatics for prevention of lymphatic metastasis.

The results shown in Fig. 2 indicate an increased transfer of bleomycin from the injection site to the regional lymph node by the S/O emulsion. Topical injection of aqueous solution exhibited rapid but insufficient transfer of bleomycin, and no accumulation was observed after intravenous administration. These results are in good agreement with previous results observed in rats (Hashida et al., 1977a). When the S/O emulsion was injected into the sub-serosal space of the appendix, oil particles were delivered very quickly into the peripheral lymph vessels and drained to the regional lymph node as shown in Fig. 6. Fig. 7 is a photomicrograph of such peripheral lymph fluid obtained by puncture and it can be readily observed that the S/O emulsion was converted into the so-called multiple-type emulsion in which a large number of microspheres are dispersed within the oil droplets. Bleomycin is considered to be contained in or adsorbed to the gelatin microspheres.

In previous studies on the role of oil used as the S/O emulsion base, the movement of oil between tissue compartments was traced using ^{14}C-labeled tripalmitin following topical injection; a remarkable accumulation of oil was demonstrated in the regional lymph node, which had a good relation with the extent of enhanced drug localization (Hashida et al., 1977b, c). On the basis of these observations, it can be concluded that these oil droplets play the role of carrier incorporating bleomycin in their inner microspheres, resulting in specific enhanced delivery of the drug into the lymphatics.

As is obvious from Table 1, the most improved lymphatic transport of bleomycin was obtained in the form of S/O emulsion, followed by the W/O emulsion. The oily suspension form exhibited a pronounced retention of bleomycin in the injection site, i.e. appendix, for a considerably longer period than the S/O emulsion, but only a low level of accumulation was found in the lymph node. This result suggests that the oily suspension can only be used as a depot-type formulation to give a prolonged release of drug into the circulation, but cannot be utilized as a specific delivery system for increasing lymphatic localization. These differences of efficiency between emulsions and suspension are con-

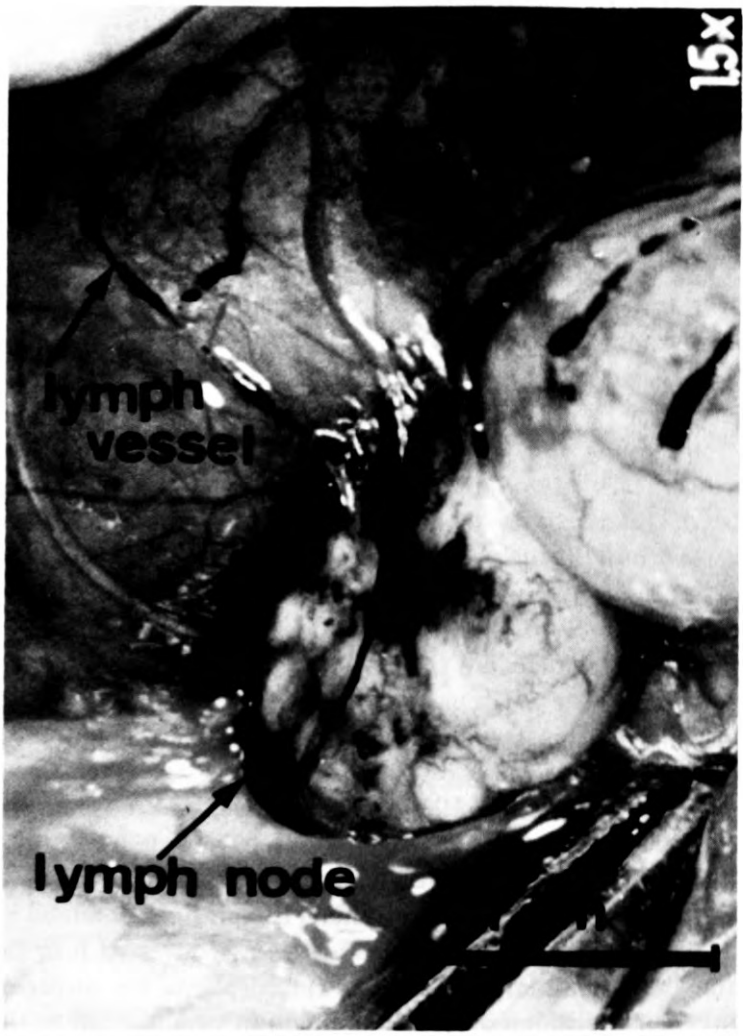

Fig. 6. Photograph of the metastasized lymph node immediately after injection of S/O emulsion into the appendix. S/O emulsion (stained with Sudan blue) drains into the lymph node through the peripheral lymph vessels.

sidered to reflect their physicochemical characteristics, and further examination concerning this problem is in progress.

In the present investigation, the utility of the S/O emulsion as a drug delivery system for surgical adjuvant chemotherapy was evaluated in VX2 carcinoma-bearing rabbits. The fact that lymphatic metastasis occurred in almost all the animals having surgical excision of the primary tumor proved the adequacy of the present experimental model for surgical adjuvant chemotherapy. Postoperative intravenous administration of bleomycin exhibited no significant histological change of metastatic VX2 cells at the lymph node, suggesting that systemic adjuvant therapy has little effect on metastasis under the present condition (dose; 3 mg/kg). On the contrary, topical treatment with the bleomycin–S/O emulsion near the excised primary tumor produced an appreciable effect on metastatic

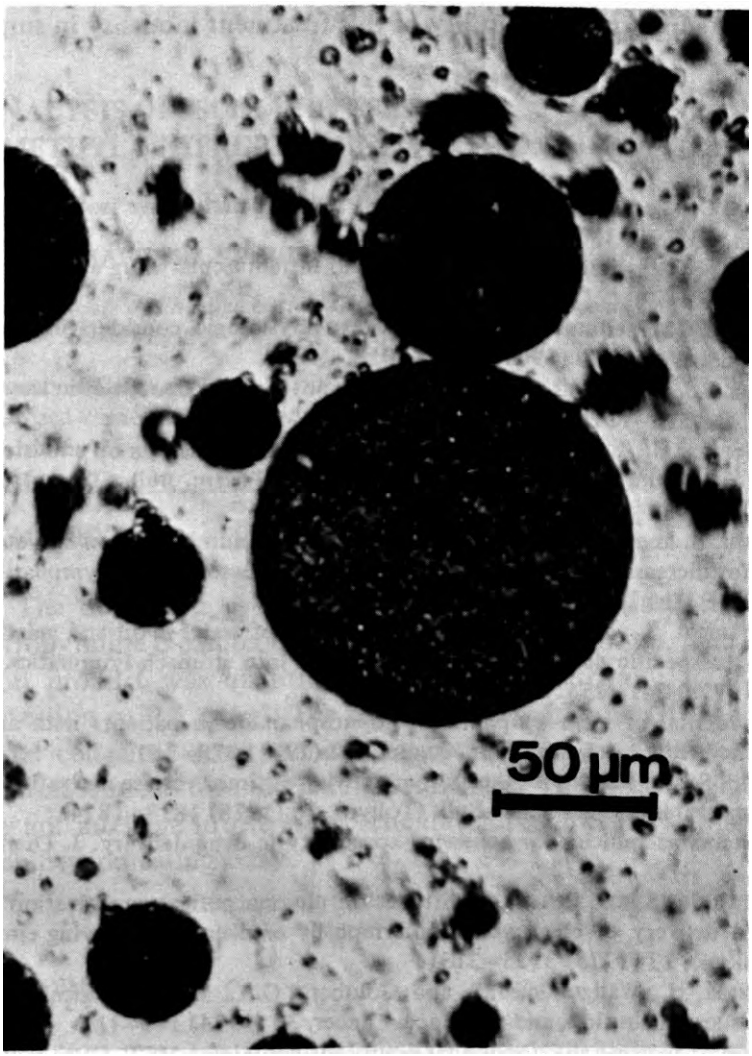

Fig. 7. Microphotograph of peripheral lymph fluid drained from the appendix which had an injection of S/O emulsion at the sub-serosal layer. Lipophilic dye (Sudan blue) was dissolved in the oily phase of emulsion so that the oil droplets could be seen easily.

VX2 cells and the animal survival times. The different extent of localization of bleomycin in the regional lymph node between these treatment forms seems to explain these degrees of cytocidal effect against metastatic VX2 cells.

On the basis of the evidence presented in this investigation, it is suggested that the application of S/O emulsion to a delivery system of bleomycin would be advantageous, because a sufficiently high concentration of bleomycin can be supplied in the injection site and to the regional lymph node for a considerably longer period. Reduction of peak plasma concentration is thought to be worthwhile since a decrease of adverse effect is expected. In addition, the possibility of biodegradation of the emulsion components should present no clinical inconvenience. The present preclinical trial using the rabbit

metastasis model offers further support for the utility of this treatment modality in surgical adjuvant chemotherapy.

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