STUDIES ON LIPOSOME-ENCAPSULATED CARBOQUONE. III.\textsuperscript{1)}
ENHANCEMENT OF LYMPHATIC TRANSPORT OF CARBOQUONE BY
ENCAPSULATION

MASAFUMI HISAOKA, KAZUHIRO TSUKADA AND TADASHI MORIOKA

Product Development Laboratories, Sankyo Company Ltd., Hiromachi 1-2-58, Shinagawa-ku, Tokyo, 140, Japan

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To examine the possibility of utilizing liposomes as drug carriers of carboquone (CQ), an antitumor agent, effects of encapsulation on transport into lymphatics have been studied.

Enhancement of lymphatic transport of CQ was obtained by the administration of liposome encapsulated CQ (CQ-liposome) into abdominal cavity in thoracic duct cannulated rats. And also, the enhancement of CQ transport into regional lymph nodes belong to gastrointestinal tract was also demonstrated in normal rats. When CQ-liposome was administered intravenously or intramuscularly, however, excellent transport into lymphatics was not recognized. Various pharmaceutical formations such as emulsions and oily solution failed to produce as high a lymph level as CQ-liposome.

These results show that liposome may represent an effective system to deliver CQ into lymphatics by intraperitoneal administration.

Keywords—drug delivery system; liposome; carboquone; emulsion; oily solution; transport; thoracic duct lymph; lymph node; rat

For effective cancer chemotherapy, an optimal concentration of antitumor agent must reach the tissues where the cancer cells prevail. Most of the antitumor agents developed previously, however, have the cytotoxicities against normal cells as against cancer cells. So, development of a drug delivery system, which can control the transfer of antitumor agents into the cancer tissues, is desirable.

Sezaki and his co-workers have been studying the utility of emulsion,\textsuperscript{2-6)} or other pharmaceutical forms\textsuperscript{7)} as drug carriers to deliver the drugs into lymphatics for the treatment of metastasis, and reported that the water-in-oil emulsion type was the most effective formulation. Takahashi \textit{et al.} have also reported on fat emulsions for chemotherapy of metastasis.\textsuperscript{8,9)} However, no detailed studies on liposomes in terms of the role of transferring drug to the lymphatics have been reported.

In the previous report, carboquone (CQ) was encapsulated in liposomes by reverse phase evaporation method using distearoyl phosphatidylcholine as wall material.\textsuperscript{10)} The pharmacokinetic behavior in rat was studied after intravenous administration of liposome encapsulated CQ (CQ-liposome).

In the present article, the effect of the encapsulation of CQ within liposomes on the lymphatic transport was studied and CQ-liposome was compared with other formulations such as emulsions or oily solution.

Materials—CQ and 2,5-diethyleneimino-3, 6-dimethylbenzoquinone (MEB), used as an internal standard, were synthesized in Sankyo Co., Ltd. Esquinon for injection\textsuperscript{®} (Sankyo Co., Ltd.) was used for animal experiments as unencapsulated CQ (free CQ). Distearoyl phosphatidylcholine was purchased from Sigma Chemical Co., Ltd. Glyceryl dioctyl dodecylate was purchased from Nishin Seiyu Co., Ltd. Castor oil was
purchased from Miyazawa Yakuhin Co., Ltd. All the other reagents used were of reagent grade or analytical grade.

Preparation of Formulations — CQ-Liposome was prepared by the method of reverse-phase evaporation vesicles reported previously. Briefly, 5 mg of CQ and 250 mg of distearoyl phosphatidylcholine were dissolved in the mixture of 35 ml of chloroform and 35 ml of isopropyl ether. After addition of 10 ml of 1/10 phosphate buffered saline solution (pH 7.2), the mixture was sonicated for 5 min at room temperature, and the chloroform and isopropyl ether were evaporated with a rotary evaporator. This crude liposomal suspension was subjected to gel chromatography on a Sephadex G-50 column. The fractions containing liposomes were pooled and centrifuged at 27000 × g for 20 min. Precipitated liposome were collected and resuspended in 1/10 phosphate buffered saline solution. CQ-liposome suspension contained 1400 μg of CQ and 130 mg of phospholipid in 4 ml liquid. Emulsions were prepared by the following method. Four mg of CQ and 250 mg of distearoyl phosphatidylcholine were dissolved in 34 ml of chloroform. To this solution, 34 ml of isopropyl ether, 0.5 ml of glyceryl dioctyl dodecylate and 10 ml of 1/10 phosphate buffered saline were added, and the mixture was sonicated by horn type sonicator (Choonpa Kogyo Co., Ltd.). Chloroform contained in this emulsified solution was evaporated with a rotary evaporator under reduced pressure. Oily solution was prepared by mixing of 10 mg of CQ dissolved in 20 ml of chloroform with 20 ml of castor oil, and chloroform contained in this solution was evaporated with a rotary evaporator under reduced pressure.

Animal Experiments — For the experiments of drug transfer into thoracic duct lymph, male Wister-Iamamichi rats weighing 328—364 g were

![Graph A](A free CQ)

![Graph B](B CQ-liposome)

**FIG. 1. Concentrations of CQ in Thoracic Duct Lymph and Plasma after Intraperitoneal Administration of Free CQ and CQ-Liposome**

*Dose: 1 mg/kg. --- ○ ---: thoracic duct lymph, —●—: plasma. Results are expressed as the mean ± S.E. of 4 rats.*
Lymphatic Transport of CQ-Liposome

ued. The procedure employed for the collection of lymph was modified from the method of Bollman et al. Rats were anesthetized intraperitoneally with pentobarbital sodium (30 mg per kg of body weight) during cannulation surgery into thoracic duct. A flexible polyethylene tube (i.d. 0.7 mm, o.d. 1.3 mm, Hibiki Honpo) was cannulated into the thoracic duct and fixed by binding. This cannula allowed a continuous drainage of lymph throughout the experiments. The operated rats were selected for the experiments according to the excellence in drainage of lymph after standing for about 20 h in Bollman cages with foods and saline solution. At various time intervals after drug administration, 0.6 ml of blood was obtained from tail blood vessel, and 0.3 ml of thoracic duct lymph was obtained during about 10 min interval including before and after the blood sampling time. One tenth ml of each plasma and thoracic duct lymph was pipetted with Eppendorf Pipette (Iatron Laboratories Inc.) and used for analysis.

For the experiments of drug transfer into regional lymph nodes of gastrointestinal tract, male Wister-Imamichi rats weighing 147–164 g were used. At various times after administration, three rats were killed with blood letting, and serum and lymph nodes were obtained. The lymph nodes of each rat were weighed, and 0.2 g of lymph nodes was homogenized with 4 ml of water using Polytron® homogenizer (Kinematica Co.) and this homogenate was used for assay.

In animal experiments, the doses of formulations were expressed as the amount corresponding to parent CQ.

Analytical Method — Concentrations of CQ in plasma, serum and regional lymph nodes were determined by high performance liquid chromatography (HPLC) method reported previously. Each sample solutions such as serum, plasma, thoracic duct lymph and homogenate of lymph nodes, was adjusted to 3 ml with distilled water and to this solution 10 ml of chloroform

![Graph](A) free CQ

![Graph](B) CQ-liposome

**FIG. 2. Concentrations of CQ in Regional Lymph Nodes of Gastrointestinal Tract and Serum after Intraperitoneal Administration of Free CQ and CQ-Liposome**

Dose: 2 mg/kg. --- ○ ---: regional lymph nodes, -●- : serum. Results are expressed as the mean ± S.E. of 3 rats.
and 1.0 ml of the internal standard solution containing adequate MEB were added. The mixture was mechanically shaken for 10 min and centrifuged for 5 min at 900 × g. Chloroform layer was separated and evaporated following addition of 2 drops of ethylene glycol. The residue was redissolved in 100—200 μl of methyl alcohol and 10—20 μl of the solution was injected in HPLC system for analysis.

RESULTS
CQ Levels in Thoracic Duct Lymph and Plasma after Intraperitoneal Administration

Fig. 1 shows the lymph and plasma concentrations of CQ after intraperitoneal administration of free CQ and CQ-liposome at a dose of 1 mg/kg. When the drugs were administered intraperitoneally, absorption was rapid with peak lymph and plasma concentrations reached within 15 min. In free CQ administration, not much difference can be detected between lymph and plasma concentrations at any time. However, when CQ-liposome was administered intraperitoneally, lymph concentrations of CQ were higher by about two times than plasma concentrations at peak level, and the areas under CQ concentration-time curves (AUC) were also two times larger (Table 1). Plasma concentrations remained at the same levels in both administered forms.

The ratio of lymph concentration to plasma concentration at each sampling time was obtained from the results in Fig. 1. In case of CQ-liposome, the ratios at all the sampling times, except for 5 min after administration, were higher than the value of 1.0 (1.8—3.8). On the contrary, the ratio at 5 min was 0.5 and at the other points the ratios were about 1.0 for free CQ. Based on these results, excellent transport of CQ-liposome into thoracic

![Graphs showing concentrations of CQ in lymph and plasma](image)

**FIG. 3.** Concentrations of CQ in Thoracic Duct Lymph and Plasma after Intravenous and Intramuscular Administration of Free CQ and CQ-Liposome

Dose: 1 mg/kg, i.v; --- Δ ---: thoracic duct lymph, --- ▲ ---: plasma, i.m; --- ○ ---: thoracic duct lymph, --- ● ---: plasma. Results are expressed as the mean ± S.E. of 3 rats.
duct lymph after intraperitoneal administration was evident.

**CQ Levels in Regional Lymph Nodes of Gastrointestinal Tract and Serum after Intraperitoneal Administration**

Fig. 2 shows the CQ concentrations in regional lymph nodes of gastrointestinal tract and serum after intraperitoneal administration of free CQ and CQ-liposome to normal rat at the dose of 2 mg/kg. When free CQ was administered, there was no difference between serum levels and lymph node levels at any time. However, when CQ-liposome was administered, lymph node levels were approximately 3 times higher than serum levels, and AUC of lymph nodes was 2.5 times larger than that of serum (Table I). Besides, the transport of CQ-liposome to lymph nodes was so rapid that the peak appeared at 5 min after administration.

**CQ Levels in Thoracic Duct Lymph and Plasma after Intravenous and Intramuscular Administration**

Fig. 3 shows the lymph and plasma concentrations of CQ after intravenous and intramuscular administration of free CQ and CQ-liposome. The dose of each study was 1.0 mg/kg. There was not much difference between lymph and plasma levels after intravenous administration of free CQ. However, in the case of CQ-liposome, plasma concentrations showed very high levels at the initial stage after intravenous administration and they decreased to undetectable level at 2 h. Lymph levels, on the contrary, were extremely lower than plasma levels that AUC of lymph was approximately 1/4 times smaller than that of plasma (Table I).

When the drugs were administered intramuscularly, there were some difference between lymph and plasma levels. In the case of free CQ, lymph levels were a little higher than plasma levels. The results were opposite in the case of CQ-liposome and CQ levels were higher in plasma.

Based on these results, excellent lymphatic transport of CQ-liposome was not observed after intravenous or intramuscular administration and it is suggested that the penetration of CQ into thoracic duct lymph was limited by encapsulation within liposomes.

**CQ Levels in Thoracic Duct Lymph and Plasma after Intraperitoneal Administration as Emulsions**

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**TABLE I. Area under the CQ Concentration–Time Curve in Each Experiments**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Administration</th>
<th>Drug</th>
<th>Number</th>
<th>Dose</th>
<th>[AUC]₆₈ (ng.h/0.1 ml or 0.1 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lymph</td>
</tr>
<tr>
<td>Penetration into thoracic</td>
<td>i.p.</td>
<td>Liposome</td>
<td>4</td>
<td>1 mg/kg</td>
<td>75±10</td>
</tr>
<tr>
<td>duct lymph⁴</td>
<td>Free CQ</td>
<td>4</td>
<td>1 mg/kg</td>
<td>23±6</td>
<td>23±3</td>
</tr>
<tr>
<td></td>
<td>Emulsion</td>
<td>3</td>
<td>2 mg/kg</td>
<td>40±4</td>
<td>42±6</td>
</tr>
<tr>
<td></td>
<td>Oily Solution</td>
<td>3</td>
<td>2 mg/kg</td>
<td>37±2</td>
<td>26±4</td>
</tr>
<tr>
<td></td>
<td>i.v.</td>
<td>Liposome</td>
<td>3</td>
<td>1 mg/kg</td>
<td>18±5</td>
</tr>
<tr>
<td></td>
<td>Free CQ</td>
<td>3</td>
<td>1 mg/kg</td>
<td>35±3</td>
<td>39±5</td>
</tr>
<tr>
<td></td>
<td>i.m.</td>
<td>Liposome</td>
<td>3</td>
<td>1 mg/kg</td>
<td>28±6</td>
</tr>
<tr>
<td></td>
<td>Free CQ</td>
<td>3</td>
<td>1 mg/kg</td>
<td>29±3</td>
<td>25±0</td>
</tr>
<tr>
<td>Penetration into regional</td>
<td>i.p.</td>
<td>Liposome</td>
<td>3</td>
<td>2 mg/kg</td>
<td>70</td>
</tr>
<tr>
<td>lymph node⁵</td>
<td>Free CQ</td>
<td>3</td>
<td>2 mg/kg</td>
<td>28</td>
<td>36</td>
</tr>
</tbody>
</table>

a) Areas are expressed as the mean ± S.E. b) Areas are expressed as the values calculated from the mean concentration–time curve.
and Oily Solution

Lymph and plasma concentrations of CQ after intraperitoneal administration of emulsions and oily solution were shown in Fig. 4. Emulsions, which were prepared to have almost the same compositions as CQ-liposome and administered intraperitoneally, showed the same lymph concentrations as plasma concentrations. Whereas, when CQ oily solution made of castor oil was administered intraperitoneally, lymph concentrations seemed to be a little higher than plasma concentration. However, lymph and plasma levels, after intraperitoneal administration of oily solution were relatively lower than any of those after administration of CQ-liposome, free CQ or emulsions.

DISCUSSION

Recently, it has been said that simultaneous treatment of local tumor site and systemic use of drug was necessary for effective cancer chemotherapy.\textsuperscript{13} We have reported on the local treatment of peritonitis carcinomatosa by intraperitoneal administration of CQ,\textsuperscript{11} showing excellent effects of CQ in clinical uses. However, more potential effects would be expected if the optimal drug delivery systems were developed to treat metastasis along lymphatic pathway in the abdominal cavity. From this point of view, the formulation to enhance the lymphatic transport of CQ after intraperitoneal administration was contemplated. Therefore, we have examined the lymphatic transports of CQ-liposome after intraperitoneal, intravenous, and intramuscular administration. Excellent lymphatic transport was obtained only after intraperitoneal administration (Fig. 1—4). This result was obtained from the experiments in thoracic duct cannulated rat. In order to remove the effects of operation, we conducted the same experiment in normal rat to see whether CQ-liposome was transported into regional lymph nodes of gastrointestinal tract (Fig. 2). Even in normal rat, excellent transport of CQ-liposome to lymphatics was obtained. Hashida et al.\textsuperscript{9,4} have shown that some kinds of emulsions containing drugs could select their pathway to lymphatics in the injected site when they were administered intramuscularly. However, it is suggested from our results that CQ-liposome differs from the emulsions in transport, namely, it cannot select the pathway to lymphatics after intramuscular administration. The reason is not clearly understood as to why CQ-liposome selects lymphatic pathway when administered into the abdominal cavity but does not select it after intravenous or intramuscular administration. But it seems to be necessary for lymphatic transport of CQ-liposome after intravenous or intramuscular administration, to permeate through the vessel or muscle tissues, and CQ-liposome itself will not be able to permeate through these tissues. Fig. 4 shows the same lymph levels as plasma levels after intravenous administration. It is suggested that CQ is a drug which shows an excellent lymphatic transport, besides, a solution form seems to be more advan-

![Graph showing concentrations of CQ in thoracic duct lymph and plasma after intraperitoneal administration of emulsions and oily solution.](image)

**FIG. 4. Concentrations of CQ in Thoracic Duct Lymph and Plasma after Intraperitoneal Administration of Emulsions and Oily Solution**

*Dose: 2 mg/kg. Emulsion: --- △ ---: thoracic duct lymph, - △ - : plasma. Oily Solution: --- ○ ---: thoracic duct lymph, - ○ - : plasma. Results are expressed as the mean ± S.E. of 3 rats.*
Lymphatic Transport of CQ-Liposome

tageous for diffusion of CQ to lymphatic system in the blood or in muscle than liposomal form. It is speculated that CQ-liposome move more freely to lymphatic system in abdominal cavity than in the blood or in muscles, in addition, it has more chances to be phagocytized, thereby CQ-liposome shows the excellent lymphatic transport after intraperitoneal administration. It is also suggested that such excellent lymphatic transport occurs by the affinity characteristics of CQ-liposome to the lymphatic system in the abdominal cavity. Those characteristics for lymphatic transport were shown neither in the case of emulsions nor oily solution.

From these results, we can expect the utility of CQ-liposome for the treatment of metastasis along lymphatic pathway.

Free CQ, if it is administered intravenously, can produce the same lymph levels as CQ-liposome administered intraperitoneally. However, intraperitoneal administration of CQ-liposome will show lower toxicities than intravenous administration of free CQ, because plasma CQ levels in the case of CQ-liposome were 1/2 times lower than in the case of free CQ. Therefore, it is considered that the effect of CQ-liposome on prolongation of the survival time in mice inoculated with Ehrlich ascites carcinoma is superior to that of free CQ. We are currently investigating with the efficacy of CQ-liposome to Ehrlich ascites carcinoma in mice.

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