STUDIES ON LIPOSOME-ENCAPSULATED CARBOQUONE. IV.\textsuperscript{1) ENHANCEMENT OF ANTITUMOR ACTIVITY OF CARBOQUONE AGAINST EHRlich ASCITES CARCINOMA BY ENCAPSULATION}

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The antitumor effects of liposome-encapsulated carboquone (CQ-liposome) were studied following intraperitoneal administration into mice bearing Ehrlich ascites carcinoma (EAC). CQ-liposome showed nearly 3 times superior efficiencies to parent CQ (free CQ) in the growth inhibitory effect on EAC cells inoculated in mice, and also showed almost the same toxicity as free CQ in normal mice. CQ-liposome prolonged the life span of EAC-bearing mice about 2 times that of free CQ. In the results of electrophoresis, it was found that CQ-liposome and EAC cells had positive and negative surface charges, respectively.

These observations suggest that CQ-liposome is a favorite delivery system to enhance the efficiency of CQ to EAC in mice. One of the reasons for its excellent effect may be explained by an easy contact of CQ-liposome with EAC cells because of its opposite surface charges.

Keywords — drug delivery system; liposome; carboquone; Ehrlich ascites carcinoma; growth inhibitory effect; increase in life span; mouse; electrophoresis

Liposomes used to be mainly a tool in the membrane research where they were used to study the properties of lipid bilayers. However, recently, liposomes have also been suggested as drug carriers for delivery system of antitumor agents to afflicted tissues of cancer cells\textsuperscript{2-7)}. We have been investigating the drug delivery system of carboquone (CQ) which is an antitumor alkylating agent developed by Arakawa et al.\textsuperscript{8)}, and we have studied liposomes to examine whether they were suitable for CQ. The method of encapsulation of CQ within liposomes and physical properties of liposome encapsulated CQ (Q-liposome) were described previously\textsuperscript{9)}. The characteristics of tissue distribution after intravenous administration\textsuperscript{10)} and transfer to the lymphatics after intraperitoneal administration\textsuperscript{11)} were also reported. It was recognized that CQ-liposome selectively distributes into lung, liver and spleen after intravenous administration and is also excellently transferred to the lymphatics after intraperitoneal administration. Based on these results, we can consider that liposomes are suitable for use as carriers to deliver CQ into specific sites of the body. In order to study the effects of these characteristics of CQ-liposome on antitumor efficiencies, especially after intraperitoneal administration, the effect of CQ-liposome on Ehrlich ascites carcinoma (EAC) in mice was examined. It is said that surface charge of liposomes is an important factor on tissue distribution, body clearance\textsuperscript{11)} and efficiencies on tumor cells. In this report, we described the effects of CQ-liposome on EAC in mice and the results of studies on surface charge of CQ-liposome.

MATERIALS AND METHODS

Materials — CQ and 2,5-diethylideneimino-3,6-dimethylbenzo-quinone (MEB), used as an internal standard, were synthesized in Sankyo Co., Ltd. Esquinon for injection\textsuperscript{©} (1 mg of CQ was contained in a vial, Sankyo Co., Ltd.) was used for
animal experiments as Free CQ. Distearoyl phosphatidylcholine was purchased from Sigma Chemical Co., Ltd. Trypan blue was purchased from Iwai Chemical Co., Ltd. Ampholine carrier ampholites (pH 3.5–10) for electrophoresis was purchased from LKB Producer AB, Sweden. All the other reagents and solvents used were of analytical or reagent grade.

Preparation of Test Samples — CQ-Liposome was prepared in the same way as reported previously by using distearoyl phosphatidylcholine, and suspended in 1/10 phosphate buffered saline (pH 7.2) for uses. The mean CQ concentration in these suspensions was 465 μg/40 mg of distearoyl phosphatidylcholine/ml. Free CQ was prepared by dissolving Esquinor in injection in adequate volume of saline solution. The dose of CQ-liposome was expressed as an amount corresponding to parent CQ.

Animals and Tumors — Female or male ICR/CD-1 mice, weighing 25–35 g, were purchased from Charles River Japan Inc., Tokyo. The animals were maintained in plastic cages under constant temperature and humidity provided with food and water freely for a week before experiment. Ehrlich ascites carcinoma (EAC) was originally obtained from the National Institute of Genetics, Mishima, in 1955 and has been passaged in ICR/jcl mice. For antitumor tests, EAC cells (1 × 10⁶) were inoculated intraperitoneally.

Growth Inhibitory Effect on EAC Cells — CQ or CQ-liposome was injected intraperitoneally to mice 1 d after tumor inoculation and abdominal ascites were withdrawn on 7th day after inoculation. The total number of harvested viable cells, not being dyed with trypan blue, was counted using a haemocytometer. In the same way, the

![Graphs showing time-courses of Ehrlich ascites carcinoma cells and abdominal ascites volume.](image)

**FIG. 1.** Time-Courses of Ehrlich Ascites Carcinoma Cells (A) and Abdominal Ascites Volume (B) after Treatment with Free CQ and CQ-Liposome in ICR/CD-1 Mice

Δ: control, ●: free CQ, ○: CQ-liposome.

Drugs were injected intraperitoneally at 24 h after tumor inoculation at the dose of 1.25 mg/kg. Results are expressed as the mean value of 5 mice.
time-courses of viable cells after treatment with free CQ and CQ-liposome were obtained. Pharmaceutics, at the dose of 1.25 mg/kg, were injected intraperitoneally at 24 h after tumor inoculation, and the number of viable cells was determined on 2, 3, 4, 6, 8, 11 and 14 d after inoculation.

Toxicity to Mice — Toxicities of free CQ and CQ-liposome were evaluated based on the mortality of mice 35 d after single intraperitoneal administration to male ICR/CD-1 mice.

Therapeutic Efficiencies on Tumor-Bearing Mice — Free CQ and CQ-liposome were administered to mice intraperitoneally at 24 h after tumor inoculation. The antitumor activity was evaluated by comparing the mean survival time of the treated animals (T) with that of the control animals (C), i.e. by calculating the percentage increase in life span (ILS); $(T/C - 1) \times 100\%$. The observation period was 60 d.

Electrophoresis — Isoelectric focusing: Linear sucrose density gradients, 0—50% (w/v), containing 1% (w/v) Ampholine (pH 3.5—10) were prepared in LKB Ampholine column (110 ml), and electrophoresis was carried out for 24 h at 1100 volts (constant) under cooling with running water. After electrophoresis, the fractions were collected from the column, and pH and optical densities at 600 nm were determined. Zeta potential measurement: CQ-liposome were diluted with distilled water to be 10% (v/v) and transferred to the electrophoresis cell. Electrophoresis was carried out using Zeta-Meter (Zeta-Meter Inc.,

**Fig. 2. Growth Inhibitory Effect of Free CQ and CQ-Liposome on Ehrlich Ascites Carcinoma Cells in ICR/CD-1 Mice**

- : free CQ, O : CQ-liposome.

Drugs were injected intraperitoneally at 24 h after tumor inoculation and cell number was counted on 7 d. Each point represents the mean value of 6 mice.

**Fig. 3. Lethal Toxicity of Free CQ and CQ-Liposome to ICR/CD-1 Mice**

- : free CQ, O : CQ-liposome.

Each point represents the result of 10 mice after 35 d following the intraperitoneal administration of the drugs.
Antitumor Activity of CQ-Liposome

New York) under microscope at 200 volts. The time required for a particle to traverse one micrometer was determined. Preparation of EAC cells: EAC cells used were obtained from mice 5 days after inoculation and centrifuged for 5 min at 1400 x g. The resulting cell pellet was washed 3 times by resuspension and pelleting in adequate saline solution, and used within 1 h after removal from mice.

RESULTS

Growth Inhibitory Effects on EAC Cells

Fig. 1 shows the in vivo growth inhibitory effects of free CQ and CQ-liposome in mice. Drugs were injected intraperitoneally at 24 h after EAC cells inoculation. Time-courses of total viable cell number and abdominal ascites volume in abdominal cavity were shown. As shown in Fig. 1 (A), there are much differences in viable cell counts between drug treated groups and control group. In the case of control group, remarkable growth of the cells was shown for a week after inoculation, and the cell number reached a plateau level on around 11th day. On the contrary, in the cases of drug treated groups, the growth of cells was suppressed beginning 2 d after injection of free CQ or CQ-liposome. The growth inhibitory effect of CQ-liposome appeared to be stronger than that of free CQ, and the abdominal ascites volume in mice treated with CQ-liposome was the smallest in these three groups, as shown in Fig. 1 (B).

In order to study the dose-responses of the growth inhibitory effect of free CQ and CQ-liposome, viable cells were counted 7 d after inoculation with various doses (Fig. 2). Drugs were injected intraperitoneally at 24 h after cell inoculation. Both free CQ-liposome showed dose-dependent activities. The dose of drug required to inhibit the cell growth by 90% (ED90) was calculated from the regression lines in Fig. 2. The ED90 values of free CQ and CQ-liposome were 1.5 mg/kg and 0.6 mg/kg, respectively. These results demonstrated that the growth inhibitory effect of CQ-liposome was approximately 3 times stronger than that of free CQ.

Toxicity to Mice

Fig. 3 shows the dose-toxicity relationship in normal mice receiving a single injection of free CQ or CQ-liposome. From these results, LD50 value of free CQ and CQ-liposome were estimated to be 2.2 and 2.5 mg/kg, respectively. There were not much differences between the toxicities of free CQ and CQ-liposome.

Effect on the Life Span of Tumor-Bearing Mice

In Table I, the antitumor activities of free CQ and CQ-liposome on the life span of tumor-bearing mice are summarized. Both free CQ and CQ-liposome exhibited their maximum antitumor activities on EAC at the dose of 1.25 mg/kg. The percentage of increase in life span (ILS) of free CQ was 52% at this dose. Over this dose, free CQ

TABLE I. Effect of Free CQ and CQ-Liposome on Increase in Life Span of ICR/CD-1 Mice bearing Ehrlich Ascites Carcinoma Cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Mean survival (d)</th>
<th>ILS (%)</th>
<th>Survivors on 60 d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>16.5</td>
<td>0</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>0.63</td>
<td>24.8</td>
<td>50</td>
<td>0/6</td>
</tr>
<tr>
<td>Free CQ</td>
<td>1.25</td>
<td>25.0</td>
<td>52</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>14.8</td>
<td>-10</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>0.63</td>
<td>29.6</td>
<td>79</td>
<td>1/6</td>
</tr>
<tr>
<td>CQ-Liposome</td>
<td>1.25</td>
<td>34.6</td>
<td>110</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>34.0</td>
<td>106</td>
<td>1/6</td>
</tr>
</tbody>
</table>

a) Calculated from survival time of dead animals except 60-d survivors.
exhibited a toxicity. On the contrary, CQ-liposome had a superior activity to free CQ and its maximum ILS value was 110% at the dose of 1.25 mg/kg. Over this dose, CQ-liposome exhibited a slight toxicity but still showed almost the same ILS value of 106% as maximum ILS even at a dose of 2.5 mg/kg. Besides, in case of CQ-liposome treated groups, one of six mice bearing EAC was still surviving 60 d after inoculation at each dose level. In case of free CQ, a 60-day survivor was found only at the dose of 2.5 mg/kg, exhibiting its toxicity.

*Electrophoresis of CQ-Liposome and EAC Cells*

Fig. 4 shows the result of isoelectric focusing of CQ-liposome. The isoelectric point of CQ-liposome was 9.5. From this result, it is suggested that CQ-liposome would be positively charged. A similar observation was also made about EAC cells. Negative charge of cells was clarified, but correct isoelectric point could not be obtained if the electrophoresis was conducted under more restrictive conditions. Same results were obtained from the electrophoresis by using the Zeta-Meter, that is, CQ-liposome and EAC cells had the positive and negative surface charge respectively.

**DISCUSSION**

We have previously reported\(^1\,\,^{10}\) that CQ-liposome had some characteristics for distribution in the body. It is suggested that CQ-liposome would be a favorite pharmaceutical formulation for treatment of cancer. In order to ascertain this suggestion, we have investigated the efficiencies of CQ-liposome on EAC in mice after intraperitoneal injection. CQ-liposome showed more excellent efficiencies than free CQ in growth inhibitory effect on EAC cells in mice (Figs. 1, 2) and on survival of mice bearing EAC cells (Table 1). There were not much differences in toxicity to mice between CQ-liposome and free CQ, but CQ-liposome inclined to show a less toxicity than free CQ (Fig. 3). From these results with toxicity, the dose of drugs which caused 10% of death to mice (LD\(_{10}\)) was calculated. The LD\(_{10}\) of free CQ and CQ-liposome were 1.9 and 2.2 mg/kg, respectively. It was previously descrived that the ED\(_{90}\) of free CQ and CQ-liposome were 1.5 and 0.6 mg/kg, respectively. Therefore, the therapeutic index\(^12\) (LD\(_{10}/ED_{90}\)) of CQ-liposome (index value 3.7) was 2.9 times higher than that of free CQ (index value 1.3). Actually, CQ-liposome showed excellent efficiency on survival of mice bearing EAC cells (Table 1). The mechanism of this cytotoxicity of CQ-liposome to cancer cells is not clear, however, many possibilities such as exchange,\(^13\) fusion, adsorption,\(^14\) and phagocytosis have been reported concerning the interaction of liposomes with various cells. These biological effects of liposomes were reviewed by Kimelberg and Mayhew.\(^15\) In case of CQ-liposome these phenomena will be imperative in contacting with EAC cells in abdominal cavity.

From the results of electrophoresis using the methods of isoelectric focusing and Zeta potential measurement, it is cleared that the surface of CQ-liposome has a positive charge and on the contrary EAC cells has a negative charge. In regard to surface charge of EAC cells, some results of electrophoresis have been reported\(^16,\,\,^{17}\) and also showed them to be negatively charged. These

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**FIG. 4. Isoelectric Focusing of CQ-Liposome Focusing** was carried out in 1% (w/v) Ampholine at pH 3.5—10, for 24 h at 1100 V. Approximately 3 mg of liposomes by dry weight was used.
opposite charges of CQ-liposome and EAC cells will give a favorable influence on familiar contact with each other.

Release rate of drug from liposomes has been discussed in respect to drug efficiencies. Some kinds of liposomes reported previously were mainly aimed at prolonged release for retaining high concentration in active sites, however, the release of CQ from CQ-liposome was very rapid, therefore, we can not expect the results of a prolonged lifetime of CQ in injected sites. That is, the excellent efficiency of CQ-liposome will be due to its characteristics to contact with EAC cells selectively. We are currently investigating the mechanism of exhibition of cytotoxicity to cancer cells by CQ-liposome.

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