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**Drug-carrier Property of Albumin Microspheres in Chemotherapy. IV.¹⁾
Antitumor Effect of Single-shot or Multiple-shot Administration of
Microsphere-entrapped 5-Fluorouracil on Ehrlich Ascites
or Solid Tumor in Mice²⁾**

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To examine the possibility of utilizing albumin microspheres containing 5-fluorouracil (5-FU) as a drug carrier, the antitumor activity of albumin microsphere-entrapped 5-FU against Ehrlich ascites carcinoma and solid tumor in mice was studied. After intraperitoneal injection of microsphere-entrapped 5-FU into Ehrlich ascites-bearing mice, the 5-FU level in the ascites was very high compared with that after injection of the free drug. The suppressive effect of microsphere-entrapped 5-FU on tumor growth in ascites-bearing mice was significantly higher than that of the free drug. After multiple-shot administration of microsphere-entrapped 5-FU to ascites-bearing mice, the increase in life-span was over 50% compared with a control. The therapeutic effect of microsphere-entrapped 5-FU on Ehrlich solid tumor after intratumoral injection was also studied. 5-FU level in the tumors of mice was significantly higher after intratumoral injection of microsphere-entrapped 5-FU than when the free drug was administered. The injection of microsphere-entrapped 5-FU, which was slowly released from the albumin microspheres in the solid tumor, caused a higher suppression of tumor growth at the inoculation site than administration of the free drug at the same dose. These results suggest that albumin microspheres containing 5-FU may represent an effective system for drug delivery, with prolonged action.

Keywords—albumin microsphere; drug carrier; 5-fluorouracil; Ehrlich ascites carcinoma; Ehrlich solid tumor; prolonged action; mouse

The effective use of pharmacologically active agents is often limited by side effects on the healthy tissue, incomplete transport to the desired site of action, and/or insufficiently sustained action after reaching the target tissue. This is particularly true in the chemotherapy of cancer. One of the methods used to overcome these shortcomings is to employ non-toxic and biodegradable drug carriers which direct drugs to the target tissues. From this point of view, various investigators have attempted to develop useful drug carriers, based on evaluation by *in vitro* and *in vivo* experiments.⁴⁾ The use of liposomes has been extensively investigated by Gregoriadis *et al.*⁵⁾ and their potential as carriers for pharmaceuticals *in vivo* seems clear. Kramer suggested that albumin microspheres, which have been used as a lung or liver scanning agent,⁶⁾ might be utilized as vehicles for achieving specific drug delivery,⁷⁾ and he

- 1) Preceding paper, Part III: K. Sugibayashi, M. Akimoto, Y. Morimoto, T. Nadai, and Y. Kato, *J. Pharm. Dyn.*, **2**, 350 (1979).
- 2) Part of this work was presented at the 99th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, Aug., 1979.
- 3) Location: 1-1 Keyakidai, Sakado, Saitama, 350-02, Japan.
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and his co-workers reported that human albumin microspheres containing 6-mercaptopurine were phagocytized by several tumor cell lines.⁸⁾

We also have been studying the utility of albumin microspheres as drug carriers which might localize antitumor agents in target tissues. In previous studies, an enhanced accumulation of 5-fluorouracil (5-FU) entrapped in bovine serum albumin (BSA) microspheres was shown in mice.⁹⁾ Further, it was found that the injection of 5-FU after its entrapment in microspheres prolonged the survival of Ehrlich ascites-bearing mice to a greater extent than did a similar amount of free 5-FU.¹⁾

The present study was undertaken to investigate the antitumor effects of 5-FU entrapped in albumin microspheres after single-shot or multiple-shot administration, using Ehrlich ascites and solid tumors as model tumors *in vivo*.

Experimental

Materials—5-Fluorouracil (5-FU) was obtained from Kyowa Hakko Co., Ltd. and bovine serum albumin (BSA) from Seikagaku Kogyo Co., Ltd. 5-Fluorouracil-6-³H (³H-5-FU) and ¹²⁵I-human serum albumin (¹²⁵I-HSA) were purchased from the Japan Radioisotope Association. Pronase (1.17 × 10⁶ trypsin unit/g, Kaken Kagaku Co., Ltd.) was selected as a protease.

Preparation of BSA Microspheres—BSA microspheres containing 5-FU were prepared by a modification of the method of Scheffel *et al.*^{6b)} The drug content of the final product was 0.033 mg/mg.

In Vitro Drug Release—Drug release from microspheres was determined by means of a dynamic dialysis system employing cellulose tubing. The procedure was described in detail elsewhere.¹⁾ The effects of hydrolytic enzymes on the *in vitro* drug release were determined by adding protease (1 or 15 mg) or lysosomal fraction (1 mg) to the inner solution (pH 7.4 phosphate buffer containing 150 mg of microspheres) from the cellulose tubing. Lysosomal fraction was isolated from freshly collected Ehrlich ascites cells in 0.25 M sucrose solution by a modification of the method of Lewis *et al.*¹⁰⁾

In Vivo Experiments—Male ICR mice, weighing approximately 30 g, were used, and the dose of 5-FU was 0.5 mg/mouse.

In the first series of experiments, mice were intraperitoneally inoculated with 2 × 10⁷ Ehrlich ascites cells. Five, 7 and 10 days after inoculation, the volume of the ascites and the number of tumor cells in the ascites were determined in mice treated with 0.25 ml of microsphere suspension, free 5-FU or 0.9% NaCl solution as a control 24 hr following tumor cell inoculation. Viable tumor cells in the ascites were counted microscopically with a Thoma counting chamber, with trypan blue staining. Furthermore, 0.25 ml of ¹²⁵I-labelled albumin microsphere suspension containing nonlabeled 5-FU, nonlabeled albumin microsphere suspension containing ³H-5-FU or non-entrapped (free) ³H-5-FU solution was injected intraperitoneally 7 days after inoculation. Animals were sacrificed at intervals and ascites fluid was collected to determine the 5-FU and microsphere levels in the ascites.

In the second series of experiments, 1, 5 and 9 days after intraperitoneal inoculation with 1 × 10⁶ ascites cells of the tumor, the mice were injected intraperitoneally with 0.25 ml of microsphere suspension, free 5-FU or 0.9% NaCl solution. Changes in body weight and survival times of treated, tumor-bearing mice were recorded.

The third series of experiments was carried out to assess the antitumor effect of 5-FU given in microsphere form on Ehrlich solid tumor. A suspension of 2 × 10⁷ ascites cells of the tumor was subcutaneously implanted into the scapular region of mice. Direct injection into the solid tumor of about 0.15 ml of 5-FU entrapped microsphere suspension, free 5-FU or 0.9% NaCl solution was done at 1 day for a single-shot administration or at 1, 5 and 9 days for multiple-shot administration after tumor implantation. Daily measurements of tumor size were taken with callipers. The maximal perpendicular dimensions (mm) of a tumor were averaged and the volume was evaluated on the base of a radial tumor cell distribution. Furthermore, the levels of 5-FU and microspheres in each tumor at various times following administration were determined. The procedures were as same in the first series of experiments on ascites cells.

Analytical Methods—To measure 5-FU and microsphere levels in the ascites or solid tumor, each tumor was collected. Each aliquot of tumor was mixed with the same amount of 0.9% NaCl solution, homogenized and centrifuged for 10 min to produce a supernatant. For measurement of tritium level, the supernatant was treated with a Protosol (New England Nuclear)-ethyl alcohol solution (1:2) and diluted directly with a scintillator (Aquasol II, New England Nuclear), then the radioactivity was determined with a liquid scintilla-

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tion counter (LSC-651, Aloka Co.). For measurement of microsphere level, the radioactivity of ^{125}I -labeled microspheres in the tumors was determined with an auto-gamma scintillation spectrometer (type 5110, Packard).

Results and Discussion

(1) Effect of Hydrolytic Enzymes on the Release of 5-FU from Microspheres

The *in vitro* release of 5-FU from albumin microspheres continued for over one week, as shown in a preceding paper.¹⁾ The effect of hydrolytic enzymes on the release of 5-FU from microspheres is shown in Fig. 1. The addition of lysosomal fraction to a microsphere suspension resulted in a small increase in the amount of drug release, and the addition of a protease (Pronase) resulted in a large increase. This increase may be due to digestion of albumin microspheres by the hydrolytic enzymes. This suggests that microspheres taken up into tumor cells by endocytosis could be digested by a lysosomal enzyme.

We have already suggested that 5-FU entrapped in albumin microspheres shows prolonged action against Ehrlich ascites carcinoma in mice,¹⁾ and this may therefore be due to sustained release of 5-FU at the ascites and an increase in the intracellular concentration of the drug due to endocytosis.

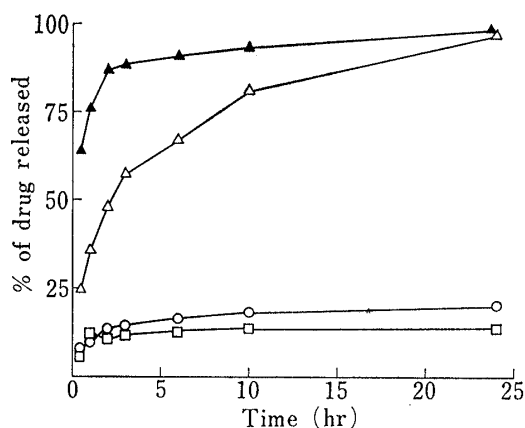


Fig. 1. Effect of Hydrolytic Enzymes on the Release of 5-FU from Albumin Microspheres

Treatment with 1 mg (Δ) or 15 mg (\blacktriangle) of protease, 1 mg of lysosomal fraction (\circ) or buffer only (\square). Each point represents the average of three experiments.

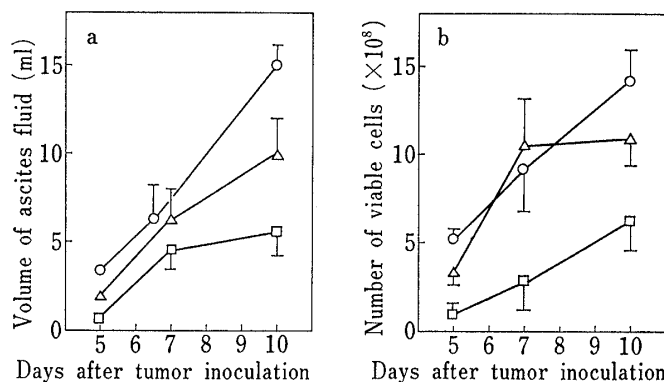


Fig. 2. Effects of Free and Albumin Microsphere-entrapped 5-FU on the Ascites Volume and Ehrlich Ascites Tumor Cell Count in Mice

Treatment with 0.9% NaCl solution (\circ), free 5-FU (Δ) and microsphere-entrapped 5-FU (\square). Results are expressed as the means \pm S.E. of 5 mice.

(2) Effect of Microsphere-entrapped 5-FU on the Growth of Ehrlich Ascites Cells

Upon intraperitoneal administration of free and microsphere-entrapped 5-FU to mice inoculated with Ehrlich ascites cells, the survival time of mice treated with the microsphere-entrapped drug is longer.¹⁾ In this work, we studied the effect of microsphere-entrapped 5-FU on the production of ascites fluid and cell counts to confirm the therapeutic effect of the drug entrapped in albumin microspheres. The elimination rates of free and microsphere-entrapped 5-FU from the injection site, and the biodegradation of albumin microspheres after injection were also estimated.

Following inoculation, the viable cell counts and the ascites volume increased proportionally (Fig. 2). The viable cell counts after treatment with microspheres were significantly different from that for 0.9% NaCl solution (Student's *t*-test: 5 days, $p < 0.001$; 7 days, $p < 0.05$; 10 days, $p < 0.02$). However, a group treated with free 5-FU was not different from NaCl solution alone (5 days, $p < 0.01$; 7 and 10 days, no significance). These results suggest that, in the

treatment of tumor-bearing mice, 5-FU entrapped in microspheres may, under certain conditions, be superior to non-entrapped 5-FU because of the sustained release of 5-FU from the albumin microspheres.

The time course of microsphere and 5-FU levels in the ascites are presented in Fig. 3, which shows the elimination of radioactivity due to ^{125}I -labeled microspheres and ^3H -5-FU in the ascites. The metabolism and elimination of non-entrapped 5-FU were fast, and the percentage of tritium radioactivity remaining at 24 hr after injection was only 0.93% of the dose. The percentage of tritium radioactivity released from microspheres and remaining in the ascites was 2.46% at 24 hr and 1.31% at 48 hr after injection of microsphere-entrapped 5-FU. In contrast, the loss of albumin microspheres from the injection site at 24 hr after injection was extremely small. These data suggest that the biodegradation of albumin microspheres in the ascites is very slow. 5-FU which is slowly released from the microspheres is more effective against the Ehrlich ascites cells; the rate of multiplication of Ehrlich ascites cells was clearly lower in the presence of microsphere-entrapped 5-FU than non-entrapped 5-FU.

(3) Effect of Multiple-shot Administration of Microsphere-entrapped 5-FU on Ehrlich Ascites Carcinoma

When microsphere-entrapped 5-FU was intraperitoneally injected 24 hr after inoculation with Ehrlich ascites cells, the life span was increased by 20.5% compared with a control (0.9% NaCl solution).¹¹⁾ In the above experiments, however, suppression of tumor growth ceased after one week, presumably because the amount of 5-FU delivered by single-shot administration of microspheres was therapeutically insufficient by one week after administration. We therefore examined the effects of multiple-shot administration of microsphere-entrapped 5-FU on the tumor growth. Figs. 4 and 5 show the changes in body weight and survival of mice after injection with the microspheres 1, 5 and 9 days after inoculation with Ehrlich ascites carcinoma. The increase in body weight after administration of the microspheres was significantly smaller than that in another group receiving 0.9% NaCl solution or free 5-FU.¹¹⁾ The

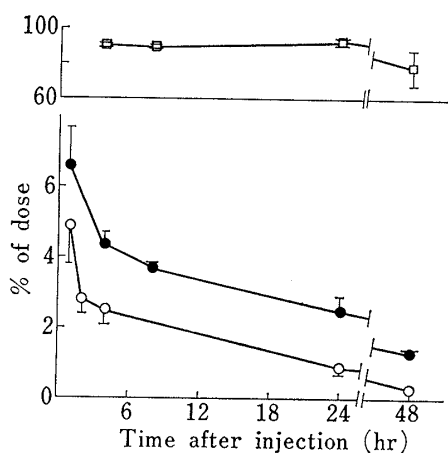


Fig. 3. 5-FU and Albumin Microsphere Levels in Ascites Fluid after Administration of Free or Microsphere-entrapped 5-FU

Free 5-FU (○), 5-FU released from microspheres (●), microsphere level (□).

Results are expressed as the means \pm S.E. of 3 mice.

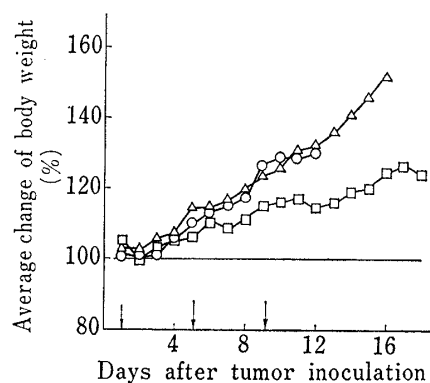


Fig. 4. Change in Body Weight of Ehrlich Ascites Carcinoma-bearing Mice after Multiple-shot Administration of Free or Albumin Microsphere-entrapped 5-FU

Treatment with 0.9% NaCl solution (○), free 5-FU (△) and microsphere-entrapped 5-FU (□).

Each point represents the average of 10 mice. Arrows indicate the administration of free or albumin microsphere-entrapped 5-FU.

11) Microspheres/0.9% NaCl solution, $p < 0.05$, 9 days following inoculation; microspheres/free 5-FU, $p < 0.05$, 12 days following inoculation.

effect of multiple-shot administration of microsphere-entrapped 5-FU was superior to that of multiple-shot administration of free 5-FU. Survival time was also improved from 20.5% for single-shot administration of the microspheres to 52.0% for multiple-shot administration. These results indicate that multiple-shot administration of albumin microsphere-entrapped 5-FU may be effective in cancer chemotherapy.

(4) Effect of Single-shot or Multiple-shot Administration of Microsphere-entrapped 5-FU on Ehrlich Solid Tumor

The effect of entrapped 5-FU on Ehrlich solid tumor was studied. Fig. 6 shows 5-FU levels and radioactivity due to ^{125}I -labeled microspheres in the solid tumors after injection of free or microsphere-entrapped 5-FU into solid tumor-bearing mice. Solid tumor cells are not mobile and the neoplastic tissue is rigid, so the disappearance of 5-FU from the solid tumor should be slower than that from the ascites. In the solid form, the elimination of tritium radioactivity of 5-FU was apparently retarded by slow release of 5-FU from albumin microspheres compared with that of free 5-FU. The results indicate the utility of intratumoral injection of the microspheres in the therapy of solid tumors.

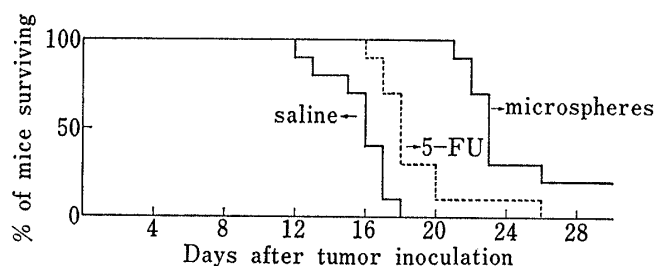


Fig. 5. Effect of Multiple-shot Administration of Free or Albumin Microsphere-entrapped 5-FU on the Survival of Mice Inoculated with Ehrlich Ascites Carcinoma

Two out of 10 mice given albumin microsphere-entrapped 5-FU survived over 60 days.

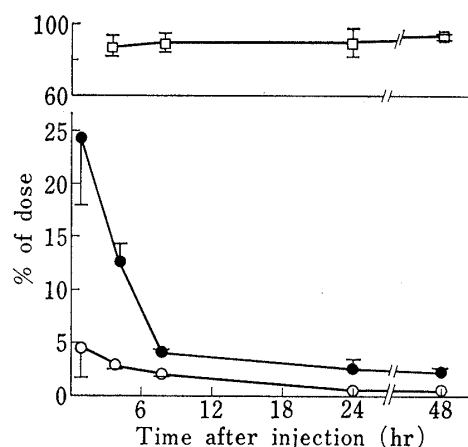


Fig. 6. 5-FU and Albumin Microsphere Levels in the Tumor after Administration of Free or Albumin Microsphere-entrapped 5-FU

Symbols: as in Fig. 3.

Results are expressed as the means \pm S.E. of 3 mice.

Fig. 7 shows the effects of free and microsphere-entrapped 5-FU on the growth of Ehrlich solid tumor. Nearly all mice in the groups administered 0.9% NaCl solution or free 5-FU had gross solid tumor involvement. Microsphere-entrapped 5-FU, however, was rather active against the solid tumor, and the tumor size became about 1/5 at 10 days and about 1/10 at 20 days after tumor inoculation compared with other groups receiving 0.9% NaCl solution or free 5-FU. This suppression of tumor growth at the inoculation site may be dependent on the prolonged release of 5-FU from the albumin microspheres.

Furthermore, we studied the effect of multiple-shot administration of microsphere-entrapped 5-FU on the Ehrlich solid tumor. Marked suppression of tumor growth at the inoculation site was observed on administering microsphere-entrapped 5-FU. This antitumor activity was surprisingly strong compared with single-shot administration of microsphere-entrapped 5-FU to the solid tumor (Fig. 8).

The experimental results in this paper show the antitumor effect of albumin microsphere-entrapped 5-FU to be greater than that of free 5-FU in two tumor systems, Ehrlich ascites

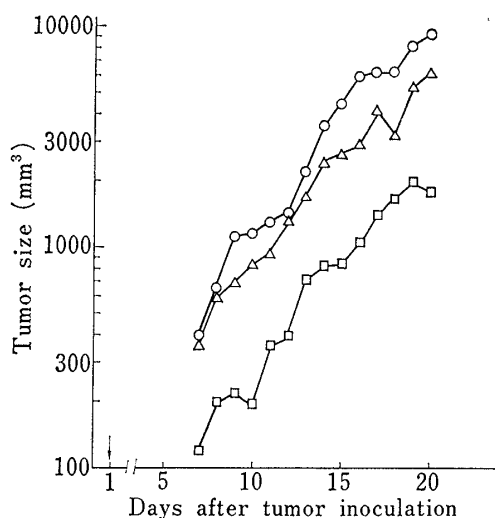


Fig. 7. Effect of Single-shot Administration of Free or Microsphere-entrapped 5-FU on the Growth of Ehrlich Solid Tumor

Symbols: as in Fig. 4.
Each point represents the average of 10 mice.
The arrow indicates the administration of free or albumin microsphere-entrapped 5-FU.

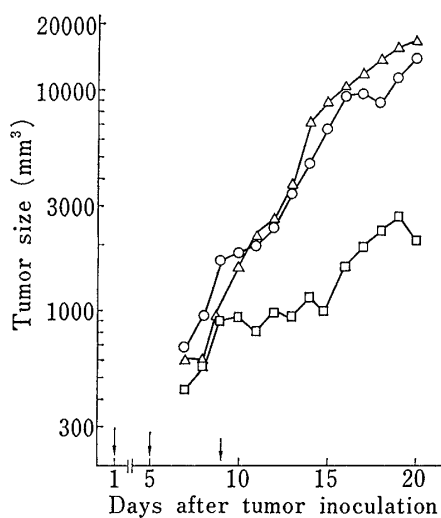


Fig. 8. Effect of Multiple-shot Administration of Free or Microsphere-entrapped 5-FU on the Growth of Ehrlich Solid Tumor

Symbols: as in Fig. 4.
Each point represents the average of 10 mice.
Arrows indicate the administration of free or albumin microsphere-entrapped 5-FU.

carcinoma and solid tumor. Further, treatment by multiple-shot administration of microsphere-entrapped 5-FU was particularly effective.

This result may be explained in terms of the sustained release of 5-FU from albumin microspheres *in vivo*. 5-FU has been used clinically for the treatment of various tumors by parenteral administration,¹²⁾ because oral administration of this drug shows higher toxicity than parenteral administration.¹³⁾ It seems likely that 5-FU entrapped in albumin microspheres shows pronounced antitumor activity and low toxicity.

Studies are presently under way in our laboratory to examine the site specificity of the drug and its prolonged action against experimental liver tumor after intravenous injection of albumin microsphere-entrapped 5-FU.

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