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Preparation and Evaluation *in Vitro* and *in Vivo* of Fibrinogen Microspheres Containing Adriamycin[1)]

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Fibrinogen microspheres containing adriamycin were prepared and evaluated as a novel drug delivery system. The shape of the microspheres was invariably spherical. The average diameters were 2.4 and 1.7 μm, and the drug contents in the microspheres were 10.2 and 9.9%, for the microspheres prepared at 110 and 140 °C, respectively. Release of the drug from fibrinogen microspheres was slow, and the drug release continued over 7 d. These results indicated that drug-loaded fibrinogen microspheres could provide a sustained release property *in vitro*.

The antitumor activity of fibrinogen microspheres containing adriamycin was evaluated against Ehrlich ascites carcinoma in mice on the basis of animal survival data. Tumor cell injections were performed on day 0 and microsphere injections on day 1, both intraperitoneally. A prolongation of the life span of tumor-bearing mice following injection of therapeutic microspheres was noted, and the microspheres containing adriamycin were therapeutically more active than adriamycin alone. The high chemotherapeutic efficiency of fibrinogen–adriamycin microspheres was striking at high doses, which would be toxic in the case of the drug alone.

These results indicated that fibrinogen microspheres containing adriamycin may be effective in cancer chemotherapy. Microspheres composed of fibrinogen show good biocompatibility and could be useful as a novel drug carrier in injectable delivery systems for anticancer agents.

Keywords——fibrinogen microsphere; novel drug carrier; drug delivery system; adriamycin; sustained release; Ehrlich ascites carcinoma; mouse

In cancer chemotherapy, selective delivery of anticancer agents to the target tumor in a sufficient amount for a long period of time is one of the most promising approaches to enhance the therapeutic effects and to minimize severe side effects. From this viewpoint, many attempts have been made to deliver anticancer agents to target sites by means of drug delivery systems.[3–5)]

The use of microspheres as sustained-release targeting agents for anticancer drugs has received much attention during recent years. Natural polymers such as albumin[6,7)] and gelatin,[8)] and synthetic polymers such as polylactic acid[9,10)] and polycarbonates[11)] have been investigated for use in drug delivery systems for anticancer agents. The possible use of fibrinogen microspheres as a novel drug carrier for injectable delivery systems of 5-fluorouracil has been examined.[12)] Fibrinogen is the precursor of fibrin, the blood clot substance. It is a large, asymmetric molecule which is greatly elongated. The molecular weight is approximately 340000.[13)] Fibrinogen has commonly been used as a coagulant, but little work has been done on it as a carrier of anticancer agents.[14)] Injectable microspheres prepared from fibrinogen should yield a novel biodegradable system for drug delivery.

In the present report, the preparation, release characteristics *in vitro*, and evaluation *in vivo* of fibrinogen microspheres containing an anticancer antibiotic, adriamycin (doxorubicin), are described.

Experimental

Materials——Adriamycin hydrochloride was generously supplied by Kyowa Hakko Kogyo Co., Tokyo. Fibrinogen from bovine blood (type I-S) was purchased from Sigma Chemical Co., St. Louis, and used without further purification.

Preparation of Fibrinogen Microspheres——Fibrinogen microspheres containing adriamycin were prepared based on the same principle as used for preparation of albumin microspheres.[12,15] Adriamycin hydrochloride (30 mg) and bovine blood fibrinogen (100 mg) were each dissolved in 1 ml of distilled water. The drug and fibrinogen solutions were combined. The resulting solution was mixed with 100 ml of 10% Span 85 in cottonseed oil, and homogenized (Nihon Seiki Seisakusho, type HB) for 10 min at 4500 rpm. The emulsion was added to an additional 100 ml of cottonseed oil preheated to a desired temperature (110 or 140 °C) and after standing for 30 min with constant stirring, the mixture was cooled to room temperature. The microspheres formed were washed free of oil by adding 200 ml of ether, centrifuging for 10 min at 4500 rpm, and decanting the supernate. After the third wash, the microspheres were allowed to dry in a desiccator.

Microscopic Characterization of Microspheres——The dried microspheres were observed under a scanning electron microscope (Hitachi Seisakusho, type X-650). Photographs were taken after metal coating with an ion-coater (Eiko Seiki, model IB-3).

Drug Contents in Microspheres——The amounts of adriamycin embedded in the final products were determined based on the method of Widder *et al.*[16] A 1-mg portion of the drug-containing carrier was incubated in 5 ml of aqueous 75% ethanol–0.45 N HCl solution for 24 h at 4 °C. The suspension of microspheres was then centrifuged for 10 min at 7000 rpm, and the drug content was determined by spectrofluorometric analysis (Shimadzu, model RF-510) of the supernate as described.[17]

***In Vitro* Drug Release**——Drug release from the fibrinogen microspheres was determined as described previously[18] using plastic dialysis cells with a cellulose membrane (Visking Co., type 36/32). The capacity of each half cell was 4 ml and the surface area of the membranes was 3.14 cm^2. Fibrinogen microspheres containing 1 mg of adriamycin were suspended in 4 ml of 0.9% NaCl. The suspension was placed in the donor compartment and an equal volume of 0.9% NaCl was put in the receptor compartment. The assembled cell was shaken horizontally at the rate of 100 strokes/min in an incubator maintained at 37 °C. The total volume of the receptor solution was removed at certain intervals and replaced with 4 ml of fresh medium. The drug concentration of the samples was determined by absorption measurement at 233 nm using a spectrophotometer (Hitachi, model 100-20). Data shown in Fig. 3 are averages of three experimental runs and the results were satisfactorily reproducible.

Animal Experiment——Male ddY mice, 24—34 g, were used. For evaluating the antitumor effect of the drug-loaded fibrinogen microspheres, ddY mice were inoculated intraperitoneally with 2×10^6 Ehrlich ascites carcinoma (EAC) cells. One day after inoculation of the cells, the mice were injected with a suspension of the fibrinogen microspheres containing adriamycin or with adriamycin solution (free adriamycin) in 0.9% NaCl containing 0.2% (v/v) Tween 80. Changes in body weight and survival time of the tumor-bearing mice were recorded. The mice were observed for 60 d.

Toxicity Study——Toxicity of the fibrinogen microspheres containing adriamycin was evaluated on the basis of survivors on the 21st d after intraperitoneal single administration to normal ddY mice.

Results

Characteristics and Drug Contents of Fibrinogen Microspheres

Figures 1A and 1B show scanning electron micrographs of fibrinogen microspheres containing adriamycin prepared at 110 and 140 °C, respectively. The microspheres were invariably spherical with a smooth surface. From these photographs, the sizes of 400 spheres were estimated for each preparation and the size distributions were obtained as illustrated in Fig. 2. The average diameters of the microspheres prepared at 110 and 140 °C were 2.4 and 1.7 μm, respectively, as shown in Table I.

When fibrinogen microspheres were prepared at 110 and 140 °C, the average yields of fibrinogen microspheres were 75.1 and 71.6% and the percentages of adriamycin entrapped in the microspheres were 10.2 and 9.9%, respectively (Table I).

In Vitro Release of Adriamycin from Fibrinogen Microspheres

The amount of adriamycin which can be released from fibrinogen microspheres into 0.9% NaCl was determined with a dialysis cell as described earlier.[18] Figure 3 shows plots of the data, expressed as the cumulative amount of the drug released *versus* time. In contrast with

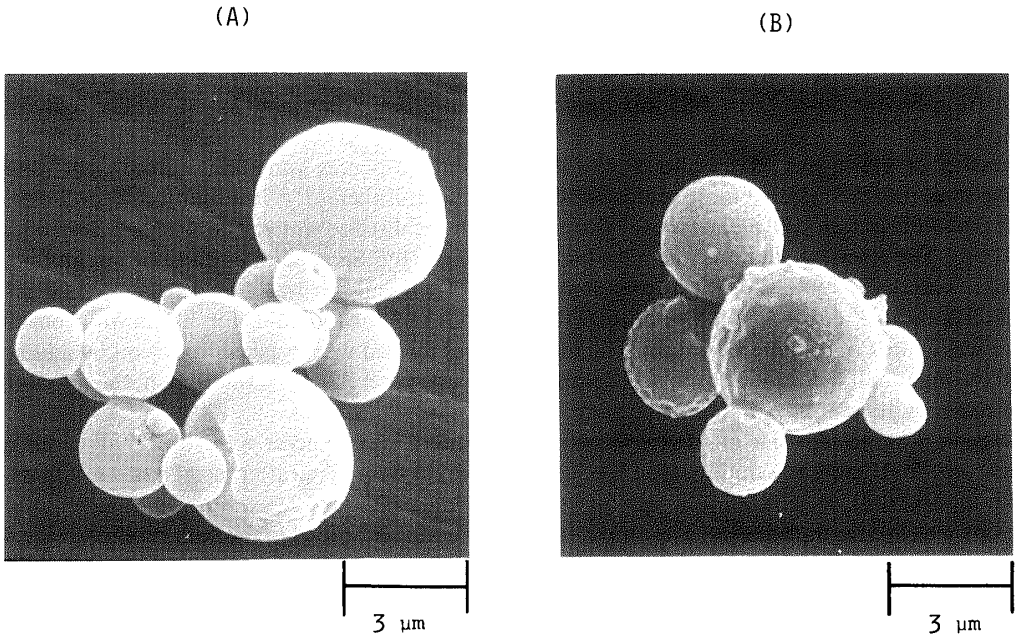

Fig. 1. Scanning Electron Micrographs of Fibrinogen Microspheres Containing Adriamycin

The temperature of microsphere preparation was 110 °C (A) or 140 °C (B).

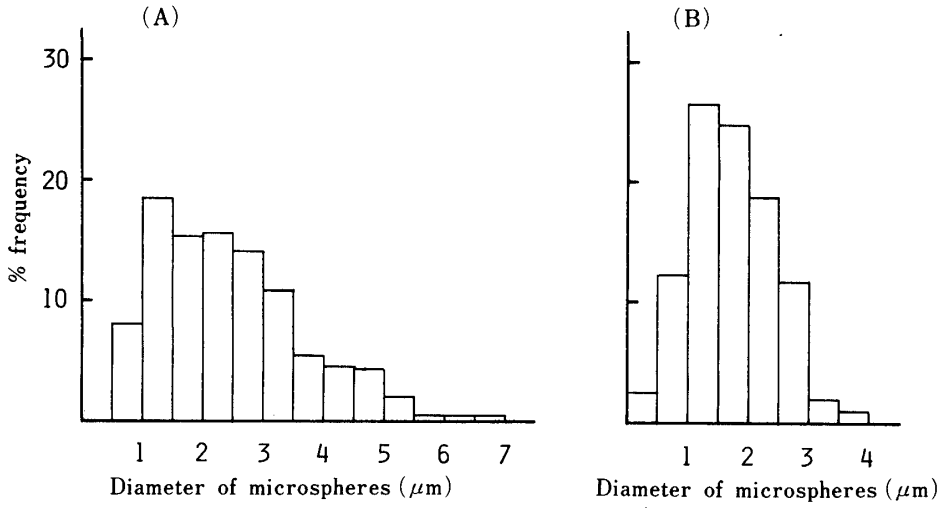

Fig. 2. Particle Size Distribution of Fibrinogen Microspheres Containing Adriamycin

The temperature of microsphere preparation was 110 °C (A) or 140 °C (B).

TABLE I. Characteristics of Fibrinogen Microspheres Containing Adriamycin

Temperature of prep. (°C)	Yield[a] (%)	Diameter (μm)	Drug content (%)
110	75.1 ± 5.7[b]	2.4 ± 0.06[c]	10.2 ± 0.2[b]
140	71.6 ± 3.3[d]	1.7 ± 0.04[c]	9.9 ± 0.6[d]

a) Yield (%) = $\frac{\text{total weight of microspheres obtained}}{\text{total weight of polymer and drug used}} \times 100$. *b*) Mean ± standard error of the mean (SEM) ($n=5$). *c*) Mean ± SEM ($n=400$). *d*) Mean ± SEM ($n=4$).

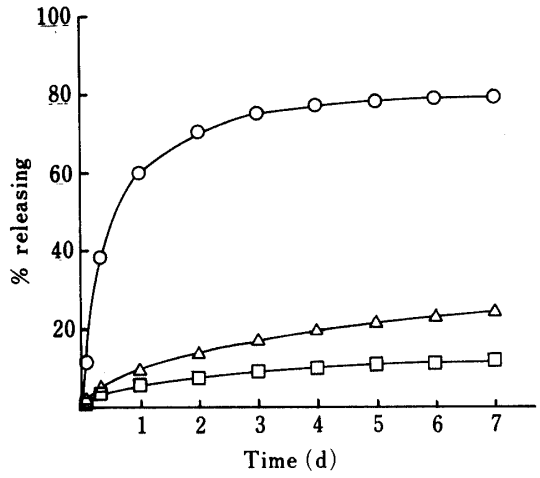

Fig. 3. Release Patterns of Adriamycin from Fibrinogen Microspheres at 37 °C

○, free adriamycin; △, microspheres prepared at 110 °C; □, microspheres prepared at 140 °C.

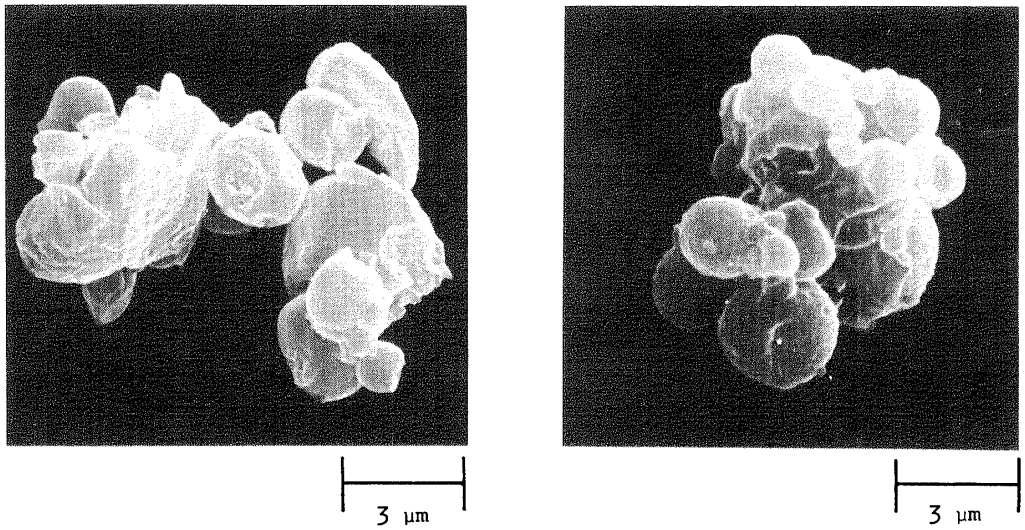

Fig. 4. Scanning Electron Micrographs of Fibrinogen Microspheres Containing Adriamycin after Release for 7 d

The temperature of microsphere preparation was 110 °C (A) or 140 °C (B).

the rapid release of free adriamycin from cellulose membranes, the release of the drug from microspheres through the membrane was slow, indicating that sustained release occurs. Entrapment in the fibrinogen microspheres resulted in a retarded release of adriamycin.

The scanning electron micrographs showed that fibrinogen microspheres after drug release for 7 d were aggregated (Fig. 4). Such aggregation of the fibrinogen microspheres was considered to be largely a result of the temperature of incubation (37 °C). Adriamycin was considered to have been released primarily by diffusion through the polymer matrix, because the surfaces of the microspheres after the release experiments had not changed significantly.

Antitumor Activity of Fibrinogen Microspheres Containing Adriamycin against Ehrlich Ascites Carcinoma in Mice

The antitumor activity of fibrinogen microspheres containing adriamycin was evaluated against EAC in ddY mice. Tumor cell injections were done on day 0 and microsphere injections on day 1, both intraperitoneally.

First, the antitumor effect of the drug-loaded fibrinogen microspheres prepared at 110 °C was evaluated by following the change in body weight. Figure 5 shows the results for mice

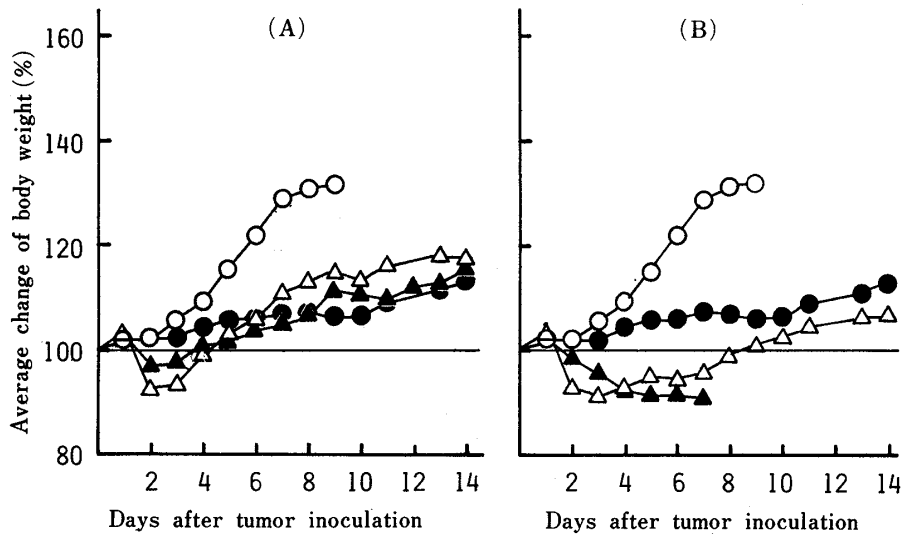

Fig. 5. Changes in Body Weight of Ehrlich Ascites Tumor-Bearing Mice after Injection of Fibrinogen Microspheres Containing 6.9 (A) and 13.7 (B) mg/kg of Adriamycin Prepared at 110 °C

●, control normal mice; ○, control tumor-bearing mice; ▲, treated with free adriamycin; △, treated with fibrinogen microspheres containing adriamycin.

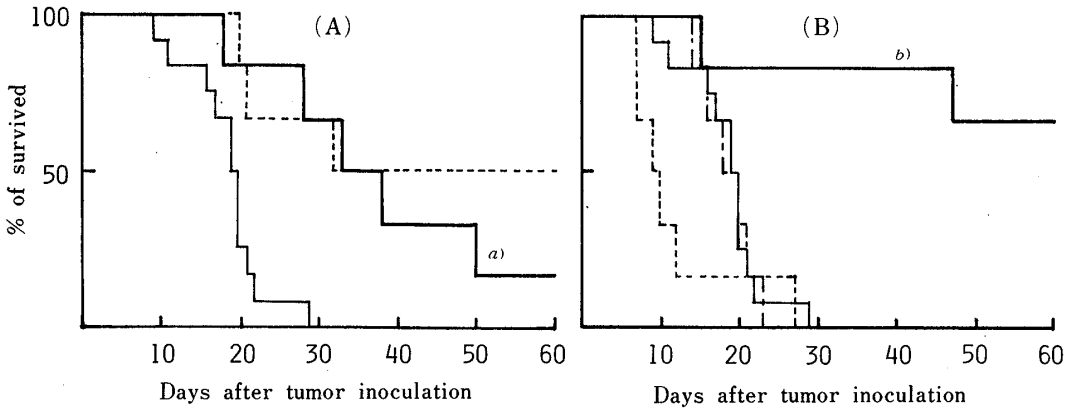

Fig. 6. Effect of Fibrinogen Microspheres Containing 6.9 (A) and 13.7 (B) mg/kg of Adriamycin Prepared at 110 °C and of Free Adriamycin on Survival Time of Mice Bearing Ehrlich Ascites Carcinoma

——, control; ------, free adriamycin; — - —, microspheres without drug; ——, microspheres containing adriamycin.

a) and *b*) denote a significant difference at $p<0.05$ and $p<0.01$ from the control, respectively.

treated with free adriamycin and with fibrinogen microspheres containing adriamycin, as well as those in normal and tumor-bearing mice. At a dose of 6.9 mg/kg (Fig. 5A), the body weight curve of the mice treated with the microspheres was identical with that of the mice receiving free adriamycin or the control tumor-bearing group. At a dose of 13.7 mg/kg (Fig. 5B), loss of body weight was observed after administration of the microspheres, but the weight of the mice recovered to that of the control group at 10 d after administration.

Next, the antitumor effect of the microspheres was evaluated on the basis of animal survival data. Figure 6 shows the antitumor activity of fibrinogen microspheres containing adriamycin in comparison with that of free adriamycin. When 2×10^6 cells of Ehrlich ascites were inoculated intraperitoneally into mice, the animals died between 9 and 29 d later due to the carcinoma (control in Fig. 6), and the mean survival time was 18.6 ± 1.5 (mean $\pm$ S.E.,

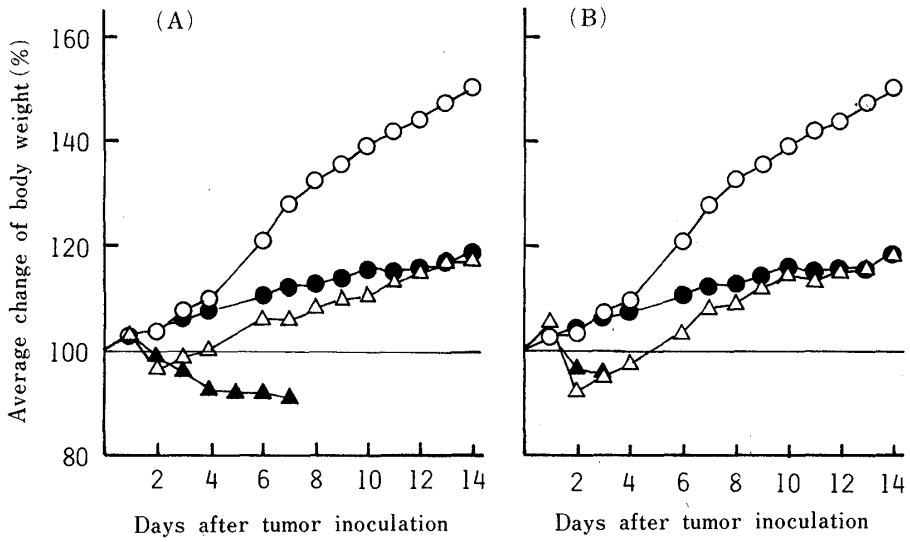

Fig. 7. Changes in Body Weight of Ehrlich Ascites Tumor-Bearing Mice after Injection of Fibrinogen Microspheres Containing 13.7 (A) and 34.3 (B) mg/kg of Adriamycin Prepared at 140 °C

●, control normal mice; ○, control tumor-bearing mice; ▲, treated with free adriamycin; △, treated with fibrinogen microspheres containing adriamycin.

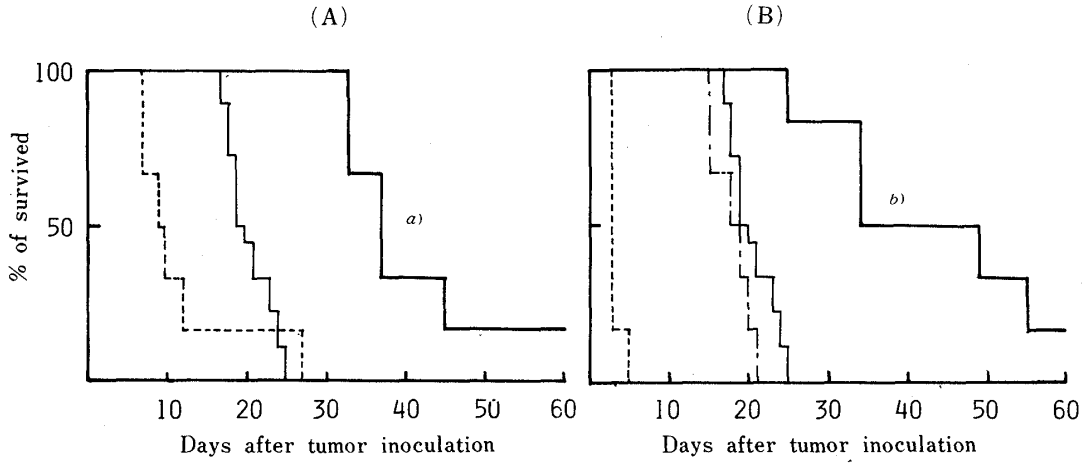

Fig. 8. Effects of Fibrinogen Microspheres Containing 13.7 (A) and 34.3 (B) mg/kg of Adriamycin Prepared at 140 °C and of Free Adriamycin on Survival Time of Mice Bearing Ehrlich Ascites Carcinima

——, control; ------, free adriamycin; —·—, microspheres without drug; ——, microspheres containing adriamycin.
a) and *b*) denote a significant difference at $p<0.01$ and $p<0.02$ from the control, respectively.

$n=12$). Injection of fibrinogen microspheres without the drug did not produce any significant prolongation of survival time (Fig. 6B). As is apparent in Fig. 6(A), fibrinogen microspheres had almost the same activity as free adriamycin at a dose of 6.9 mg/kg so that the antitumor activity of adriamycin remained mostly intact even after incorporation into these preparations. Above this dose, on the other hand, microspheres containing adriamycin were therapeutically more active than adriamycin alone (Fig. 6B). Four of six mice survived over 60 d after injection of fibrinogen microspheres containing adriamycin.

Figure 7 shows the changes in body weight in mice treated with free adriamycin and fibrinogen microspheres containing adriamycin prepared at 140 °C. Two dose levels, 13.7 and

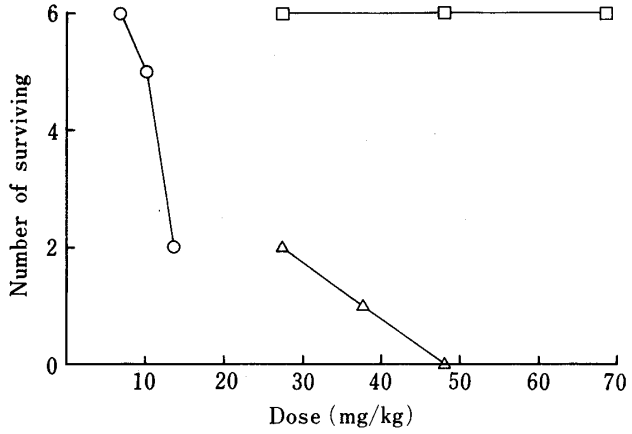

Fig. 9. Toxicity of Free Adriamycin and Fibrinogen Microspheres Containing Adriamycin in Normal Mice

○, free adriamycin; △, microspheres prepared at 110 °C; □, microspheres prepared at 140 °C.

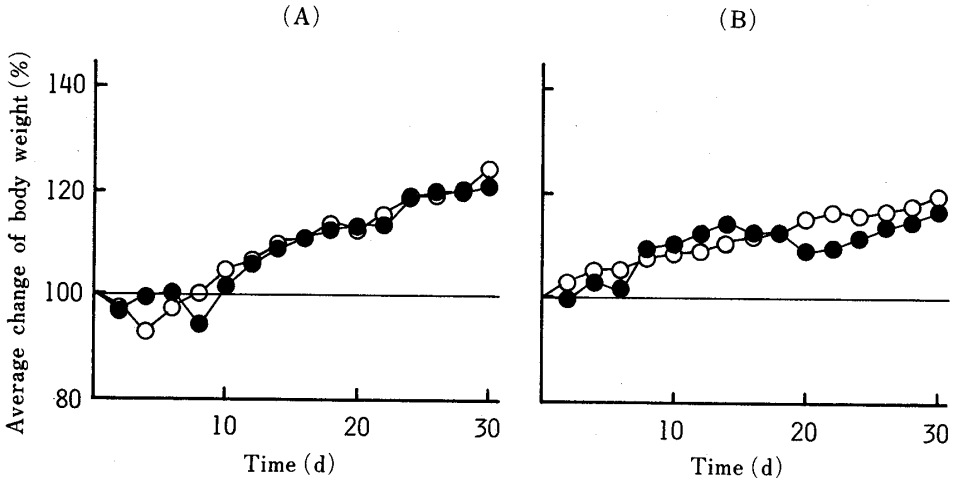

Fig. 10. Changes in Body Weight of Normal Mice after Injection of 8.2 (A) and 9.4 (B) mg per Mouse of Fibrinogen Microspheres without Drug

○, normal untreated mice; ●, treated with fibrinogen microspheres without drug. The temperature of microsphere preparation was 110 °C (A) or 140 °C (B).

34.3 mg/kg, were used in this study. At both doses, the increase in body weight after injection of the microspheres was smaller than that in tumor-bearing mice (Fig. 7). In the groups receiving free adriamycin, a decrease in body weight due to its toxicity was observed at both doses. The increase in life-span effected by microsphere treatment is shown in Fig. 8; free adriamycin showed marked toxicity at both doses.

Toxicity to Mice

Figure 9 shows the 21 d survival data after a single injection of free adriamycin or adriamycin–fibrinogen microspheres into normal ddY mice. For the same amount of adriamycin, survival was greatly increased in the microsphere groups, and this increase was found to be more pronounced in the microspheres prepared at 140 °C. This increased survival was interpreted as a sign of reduced toxicity. This difference in toxicity between the free- and microsphere-adriamyin is reflected in an increased therapeutic effectiveness as evaluated from the survival data (Figs. 7 and 8).

Fibrinogen microspheres without drug were prepared and evaluated in terms of the number of survivors and evidence of rejection 30 d after intraperitoneal injection into normal ddY mice. None of the 6 mice receiving 8.2 (Fig. 10A) or 9.4 (Fig. 10B) mg per mouse died and no localized inflammation or foreign reaction in the peritoneum was observed. The changes in body weight after injection of the plain fibrinogen microspheres were similar to those of

normal untreated mice (Fig. 10). Thus, fibrinogen microspheres showed good biocompatibility as an injectable carrier for drug delivery.

Discussion

Recently, much effort has been made to enhance the antitumor effect of anticancer agents in cancer chemotherapy. One possible approach is the topical administration of a sustained-release preparation on cancerous lesions. In our previous papers, matrices composed of ethylene-vinyl acetate[19] and ethylene-vinyl alcohol[20] copolymers were suggested to be useful vehicles for implanted delivery systems for anticancer agents.[21] The Pluronic® F-127 gel, in addition, appears to have potential application as a topical drug delivery system since it exhibits reverse thermal gelation behavior and has a sustained-release property and low toxicity.[22]

The use of microspheres as sustained-release targeting agents for anticancer agents has received much attention. Microspheres have been suggested as a means of attaining high local concentrations of a drug in specific tissues following introduction into the vascular system, by virtue of their diameter. Microspheres larger than 10 μm are deposited in the lung, while those of about 1 μm in size are taken up by the liver.[23] It has been reported that adriamycin entrapped in albumin microspheres (1.44 μm in size) is localized mainly in the liver after intravenous injection in rats.[24] The use of microspheres in intra-arterial chemoembolization[10] is a reasonable approach to the treatment of solid tumors in organs.

The possible use of fibrin film, a bioplastic prepared from human plasma, as a novel biodegradable vehicle for drug delivery systems has been examined.[18,25] In addition to fibrin, the drug-carrier properties of fibrinogen in chemotherapy also appeared to be of interest. Fibrinogen is the precursor of fibrin; it is a large, highly elongated asymmetric molecule with a molecular weight of 340000.[13] An interesting and outstanding feature of the fibrionogen is that intravenously administered, labelled fibrinogen is taken up in different human malignant and experimental transplantable tumors.[26-28] This suggests that fibrinogen containing anticancer agents can be localized at the tumor site with high specificity, thereby increasing the therapeutic index of anticancer agents.

In this work, the feasibility of using fibrinogen microspheres as a polymer matrix for the sustained release of an anticancer agent, adriamycin, was examined, as a part of an investigation into the pharmaceutical application of fibrinogen. Adriamycin has been used extensively in the treatment of a variety of malignant diseases. However, its clinical usefulness is severely restricted by its high toxicity.[29] Any dosage form or derivative of adriamycin with lower toxicity or higher chemotherapeutic efficiency should be of great interest in the treatment of various forms of leukemia and solid tumors.

Based on the evidence presented in this report, drug-loaded fibrinogen microspheres could be successfully prepared. The release rate of adriamycin from the microspheres *in vitro* was found to be small, indicating that sustained release occurs (Fig. 3). The level of drug release seems to depend on the temperature of the microsphere preparation. An increase in the temperature of the microsphere preparation increased the hardness of the microspheres and decreased the swelling of the microspheres suspended in a solution, leading to decreased drug release.

Reflecting the slow release *in vitro*, fibrinogen microspheres containing adriamycin limited the increase in body weight due to tumor growth and prolonged the life of the carcinoma-bearing mice (Figs. 5—8). This indicates that sustained release occurs in the peritoneum and that effective drug concentrations may be maintained by injection of the fibrinogen microspheres. The high chemotherapeutic efficiency of fibrinogen–adriamycin microspheres is striking at high doses which would be toxic if given as the free drug. This

increased survival can be interpreted as a sign of reduced toxicity. The LD_{50} of free adriamycin in male mice is estimated to be 13.7 mg/kg,[30] but may be much higher when adriamycin is administered in the fibrinogen microspheres containing the drug. It is possible, therefore, that the required total doses as well as systemic side effects of adriamycin might be significantly reduced by using the present system.

These results suggest that injection of sustained-release fibrinogen microspheres containing adriamycin has considerable potential as a means of cancer chemotherapy, providing a convenient method of drug delivery while minimizing drug toxicity and maximizing drug effectiveness. Further studies are in progress to determine the fate of intravenously administered fibrinogen microspheres containing adriamycin, as well as the degradation properties of the microspheres *in vivo*.

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