Communications to the Editor

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Timed-Release of Mitomycin C from Its Agarose Bead Conjugate

A new derivative of mitomycin C, mitomycin C-agarose bead conjugate, was synthesized by using cyanogen bromide method. Biologically active mitomycin C was released successively from the conjugate with a half-life of about 6 day *in vitro*, and also *in vivo* following subcutaneous injection of conjugate.

Keywords——mitomycin C; agarose bead conjugate; covalent linkage; cyanogen bromide method; *in vitro* sustained release; *in vivo* sustained release; injectable delivery system; cancer chemotherapy

The ideal dosage form in cancer chemotherapy is one that provides a tumor site with anticancer agents at a controlled rate over a long period of time without considerable interaction with normal tissues. In the past approaches, therefore, much efforts have been directed toward a development of a timed-release system which could be implanted in the closest possible proximity to a malignant tissue. Yolles, *et al.* incorporated anticancer agents in a polymeric matrix and demonstrated the sustained release of the drugs both *in vitro* and *in vivo*.[1] Segal, *et al.* injected phospholipid vesicles, liposome, containing actinomycin D and 5-fluorouracil, directly into the rat testicle and found the delayed release of entrapped substances.[2] Various types of emulsion were also used as a sustained release device for anticancer agents,[3] and an enhancement of an antitumor effect was demonstrated in patients.[4]

In the present investigation, we prepared mitomycin C-agarose bead conjugate by coupling the drug to gelled agarose beads and *in vitro* and *in vivo* timed-release characteristics were demonstrated. Mitomycin C was linked covalently to the agarose beads by modifying the method of Axén, *et al.*[5] To a stirred suspension of 100 mg agarose spheres (Sepharose 4B, Pharmacia Fine Chemicals Co., Sweden) in 5 ml water, 100 mg of cyanogen bromide was added in three portions at intervals of several minutes and pH maintained at 10.7 by addition of 2 M sodium hydroxide. After 30 min reaction, the agarose beads were rapidly washed on a glass filter with 300 ml of 0.1 M sodium bicarbonate–0.5 M sodium chloride solution under suction. The activated beads were resuspended in 10 ml of the same medium dissolving 20 mg of mitomycin C (Kyowa Hakko Co. Ltd., Tokyo, Japan), and then the coupling reaction was allowed to proceed for 24 hr. After completion of the coupling reaction, the products were washed on a glass filter with the coupling solution, and then washed with 0.1 M acetate buffer and 0.1 M borate buffer alternatively in order to remove unconjugated drug. All the procedures were performed at room temperature. The products were lyophilized and stored at 4°. One milligram conjugate was estimated to contain about 18 μg of mitomycin C by measuring the amount of the unconjugated in the eluate.

In order to examine *in vitro* release rate of mitomycin C, 12 mg of the agarose bead conjugate was suspended in 5 ml isotonic phosphate buffer (pH 7.4) maintained at 37° with moderate shaking, and the liberated mitomycin C during 24 hr was determined after separation by filtration (0.1 μm, Millipore filter, Millipore Co., U.S.A.). The residual beads were resus-

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4) T. Takahashi, S. Ueda, K. Kono, and S. Majima, *Cancer*, **38**, 1514, (1976).
5) R. Axén and S. Ernback, *Europ. J. Biochem.*, **18**, 351 (1971).

pended in a new buffer of the same composition for the following release experiment. These procedures were repeated each time for 40 days. The liberated compound showed similar pattern of ultraviolet absorption to that of original mitomycin C, so the amount of released mitomycin C was calculated based on $\varepsilon=22000$, at $\lambda_{max}=364$ nm.

Fig. 1 illustrates the time course for the release of mitomycin C from agarose bead conjugate. Mitomycin C was liberated successively and about 50% of the conjugated mitomycin C was released during 6 days although a small but significant amount of the drug was released even after 40 days. In Table I, the amount of released mitomycin C during the period of 24 hr of the first and the 16th day determined by spectrophotometric analysis are compared with the values of antimicrobial activity against *Escherichia coli* B measured by disc-plate method. The results obtained from these two different analyses were almost identical to each other, suggesting that the liberated mitomycin C had retained its original activity.

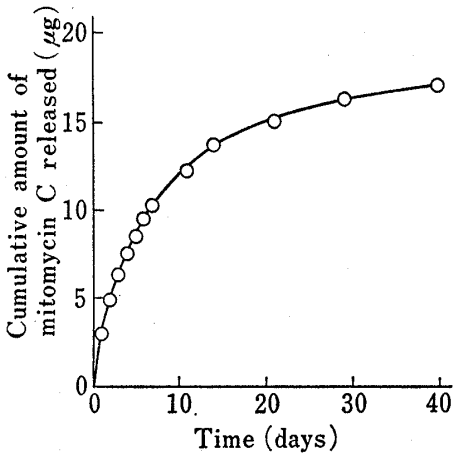

Fig. 1. Cumulative Amount of Mitomycin C released from 1 mg Agarose Bead Conjugate *in Vitro*

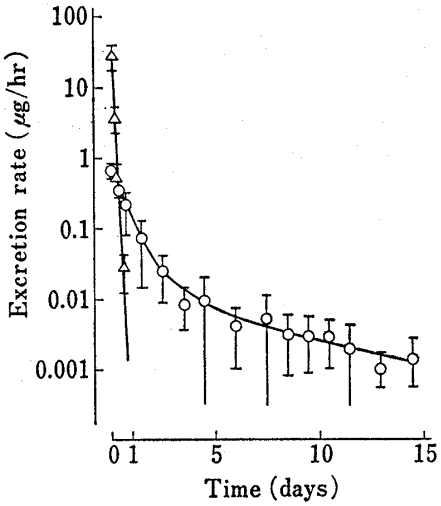

Fig. 2. Urinary Excretion Rate of Mitomycin C after Subcutaneous Injection of Mitomycin C-Agarose Bead Conjugate or Free Mitomycin C

○, mitomycin C-agarose bead conjugate; △, free mitomycin C.
Results are expressed as the mean±S.D. of three rats.

TABLE I. Amount of Mitomycin C released from 1 mg Agarose Bead Conjugate during 24 Hours Incubation *in Vitro*

Incubation period	Amount of released mitomycin C (μg)[a]	
	Spectrophotometric analysis (364 nm)	Microbial analysis (*E. coli* B)
First day	3.02±0.004	2.81±0.27
16th day[b]	0.33±0.02	0.33±0.04

a) Results are expressed as the mean±S.D. of three experiments.
b) This experiment was undertaken following 15 days incubation.

In experiments *in vivo*, the release rates of the drug were examined by measuring the amount of mitomycin C excreted in urine at fixed intervals of time, following subcutaneous injection of the conjugate as suspension to male Wistar albino rats. The dose of mitomycin C was 0.9 mg (50 mg conjugate) per rat (90 g). Mitomycin C was determined by microbiological

analysis. The mean values of three rat experiments are shown in Fig. 2, comparing with those following injection of the same amount of unconjugated (free) mitomycin C. The excretion rate of mitomycin C following injection of conjugate was slow and gradually decreased to a small amount, whereas mitomycin C administered as a free form was excreted rapidly and no antimicrobial activity could be detected in the urine samples after 24 hr. These results indicate the sustained release of mitomycin C from agarose bead conjugate in the hypodermic spaces of the rat. In this case, the average amount of mitomycin C recovered in urine within 15 days was only 12 μg, owing to a metabolic inactivation of the liberated drug.

At the end of the *in vivo* experiment, the conjugate was collected from the hypodermic spaces of the sacrificed animals and the amount of remaining drug was determined also from the successive *in vitro* release experiment. One milligram conjugate obtained from the animal at the 16th day after injection liberated 0.34 μg of antimicrobially active mitomycin C during 24 hr incubation in buffer solution. This is almost identical to the results of *in vitro* release experiment of the 16th day shown in Table I. These results suggest that mitomycin C was released from the conjugate in the body at almost similar rate to the *in vitro* experiment.

Present results prove the feasibility of mitomycin C-agarose bead conjugate as an injectable delivery system for supplying mitomycin C to a tumor site at a controlled rate over a long period. Further examination concerning the antitumor activity of this system is now in progress.

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Synthesis of D-Glucuronic Acid Derivatives of 5-Fluorouracil having O-Glycosidic Linkage

5-Fluorouracil-4-O-glucuronic acid derivatives (**V**, **VII**) were synthesized by a condensation reaction of the silver salt of 2-benzyloxy-5-fluoro-4-pyrimidone with methyl 1-bromo-1-deoxy-2,3,4-tri-O-acetyl-α-D-glucopyranuronate and subsequent careful removal of the protecting group.

Keywords——5-fluorouracil; D-glucuronic acid; 5-fluorouracil-O-glucuronide; anticancer activity; ultraviolet spectra; nuclear magnetic resonance spectra

Since Duschinsky, *et al.* reported the first synthesis of 5-fluorouracil (5-FU) as a potential anticancer agent in 1957,[1] a number of its derivatives have been synthesized and their anticancer activities have been investigated.[2]

It is interesting from the viewpoint of drug design of anticancer agents that the pH value in normal tissue is approximately 7.3, whereas in tumour tissue it is relatively lower

1) R. Duschinsky, E. Pleven, and C. Heidelberger, *J. Am. Chem. Soc.*, **79**, 4559 (1957).
2) C. Heidelberger, "Progress in Nucleic Acid Research and Molecular Biology," vol. 4 Academic Press, New York and London, 1965, p. 1.