by laboratory conditions (Sasaki and Makino[20]; Palmer and Funderburk[21]; Bruere and McLaren[22]; Beck and Mahan[23]). On the basis of the data presently available, the number of active NORs in *Bulinus* appears to be independent of the number of genomes present.

1 Reprint requests to M.A.G., Medical Genetics Section, Baylor College of Medicine, 1200 Moursund Avenue, Houston (Texas 77030, USA).
2 Present address: Department of Microbiology, State University of New York, Buffalo (New York 14214, USA).
3 Motara, M.A., and Rai, K.S., Chromosoma *64* (1977) 125.
4 Becak, W., and Goissis, G., Experientia *27* (1971) 345.
5 Becak, W., and Pueyo, M.T., Exp. Cell Res. *63* (1970) 448.
6 Ruiz, I.R.G., Soma, M., and Becak, W., Cytogenet. Cell Genet. *29* (1981) 84.
7 Tymowska, J., and Fischberg, M., Chromosoma *44* (1973) 335.
8 Muller, H.J., League, B.B., and Offerman, C.A., Anat. Rec. suppl. *51* (1931) 110.
9 Goldman, M.A., LoVerde, P.T., and Chrisman, C.L., Evolution *37* (1983) 592.
10 Bloom, S.E., and Goodpasture, C., Hum. Genet. *34* (1976) 199.
11 Munke, M., and Schmiady, H., Experientia *35* (1979) 602.
12 Wang, H.C., and Juurlink, B.H.J., Chromosoma *75* (1979) 327.
13 Howell, W.M., and Black, D.A., Experientia *36* (1980) 1014.
14 Miller, O.J., Miller, D.A., Dev, V.G., Tantravahi, R., and Croce, C.M., Proc. natl Acad. Sci., USA *73* (1976) 4531.
15 Miller, O.J., Chromosomes Today *7* (1981) 64.
16 Brown, W.V., Textbook of cytogenetics. C.V. Mosby Co., St. Louis, Mo. 1972.
17 Goldman, M.A., LoVerde, P.T., Chrisman, C.L., and Franklin, D.A., Malacol. int. J. Malacol. (1983) in press.
18 King, M., Chromosoma *80* (1980) 191.
19 Hsu, T.C., Brinkley, B.R., and Arrighi, F.E., Chromosoma *23* (1967) 137.
20 Sasaki, M.S., and Makino, S., Am. J. Hum. Genet. *15* (1963) 24.
21 Palmer, C.G., and Funderburk, S., Cytogenetics *4* (1965) 261.
22 Bruere, A.N., and McLaren, R.D., Can. J. Genet. Cytol. *9* (1967) 543.
23 Beck, M.L., and Mahan, J.T., Copeia *1979* (1971) 341.

0014-4754/83/080911-03$1.50 + 0.20/0
©Birkhäuser Verlag Basel, 1983

Continued in vitro and in vivo release of an antitumor drug from albumin microspheres[1]

S. Fujimoto, F. Endoh, Y. Kitsukawa, K. Okui, Y. Morimoto[2], K. Sugibayashi[2], A. Miyakawa[3] and H. Suzuki[3]

The First Department of Surgery, School of Medicine, Chiba University, 1-8-1, Inohana, Chiba 280 (Japan), July 29, 1982

Summary. Heated albumin microspheres with an average diameter of 45 ± 8 μm and containing mitomycin C, released, in vitro, about 20% of this antibiotic over a 3-day period. VX-2 tumors were implanted into the hind leg of rabbits and the drug-containing microspheres were injected into the femoral artery of these animals. High levels of the drug were maintained for several hours in the tumor and growth of the tumor was inhibited considerably, compared to findings in control rabbits given the conventional mitomycin C. Half the number of the rabbits treated with our new method are alive with no evidence of tumor.

Antitumor drugs are often prescribed to treat a malignancy when surgery is not feasible. When the tumor thrives on a blood supply from an end-artery, these anti-tumor drugs are either infused or perfused, in order to obtain high local concentrations of the drugs with relatively few side effects. Methods describing the local application of drugs to treat tumors have been reported[4–8]. To provide a depot for the accumulation of the anti-tumor drug at the tumor-occupied site, we evaluated the depot effect of albumin microspheres containing mitomycin C (MMC) embolized in arteries on VX-2 tumors implanted into the hind leg of rabbits.

Materials and methods. Preparation of albumin microspheres. Bovine serum albumin microspheres containing MMC (Kyowa Hakko, Co., Ltd, Tokyo) were prepared by a modification of the method of Scheffel et al.[9]. In brief, an aliquot of MMC-albumin aqueous solution was emulsified in cottonseed oil containing Span 85, solidified at 150–170 °C and the solidified microspheres then immersed in ethyl ether to remove the oil. The drug concentrations were 8–10% and the average diameter 45 ± 8 μm, as shown in figure 1. Bovine serum albumin and Span 85 were purchased from Seikagaku Kogyo Co., Ltd (Tokyo) and Wako Pure Chemicals (Tokyo), respectively.

In vitro drug release. Drug release from microspheres was determined by a dynamic dialysis system with a cellulose tube (Visking Co., Chicago, Ill., USA). 100 mg of microspheres containing MMC were suspended in isotonic phosphate buffer (pH 7.2). To remove the MMC adhering to the microspheres, 10 min sonication and centrifugation at 50 × g were carried out; subsequently, the precipitate obtained was resuspended in 3.0 ml of isotonic phosphate buffer (pH 7.2) in a cellulose tube. The suspension in the cellulose tube was dialyzed at 37 °C against 47 ml of isotonic phosphate buffer. The inner (3.0 ml) and outer

Summary of experimental procedures (male albino rabbits)

Procedures performed	Control (n = 10)	Placebo microspheres (n = 10)	Conventional MMC (n = 20)	MMC microspheres (n = 25)
Drug administered	0.9% NaCl	Placebo microspheres (no MMC)	1.2 mg/kg of conventional MMC	MMC microspheres (1.2 mg/kg as MMC)
MMC levels in peripheral blood			Measured (3 animals)	Measured (3 animals)
MMC levels in VX-2 tumor tissues			Measured (10 animals)	Measured (12 animals)
Measurement of VX-2 tumor growth	yes	yes	yes (7 animals)	yes (10 animals)
Assessment of survival duration	yes	yes	yes (10 animals)	yes (10 animals)

(47 ml) solutions were mixed using an electric motor and an acrobat stirrer, respectively. 1 ml of the outer solution was taken as a sample at a determined time and then 1 ml of the buffer was added to keep the volume constant. The MMC concentration of the samples was assessed spectrophotometrically by measuring the OD at 360 nm.

Animals and tumors: 65 male albino rabbits weighing 2.5-3.0 kg were used. Four blocks (3×3×3 mm) of VX-2 carcinoma were transplanted into the right hind leg. About 1 week later when the VX-2 tumor was about 2 cm in diameter, these rabbits were separated at random into 4 groups consisting of the control (10 rabbits), the placebo microspheres (no MMC) group (10 rabbits), and the conventional MMC (20 rabbits) and MMC microsphere (25 rabbits) groups (table).

Under anesthesia with pentobarbital-Na (20-25 mg/kg), the right femoral artery was exposed and 0.9% NaCl, the placebo microspheres, conventional MMC (1.2 mg/kg) or MMC microspheres (1.2 mg/kg as MMC) were injected over a 5-min period. In both the conventional MMC and MMC microsphere groups, peripheral blood samples were taken at a determined time from the left femoral vein. To measure MMC levels in the VX-2 tumors, these tissues were excised at a determined time from another 22 rabbits given the above-mentioned conventional MMC or MMC microspheres (table).

The LD_{50} of MMC is internationally recognized to be 5.0 mg/kg in mice and 2.9 mg/kg in rats, but that for rabbits has not been determined. In preliminary experiments, rabbits died after either intra-arterial or i.v. administrations of MMC 2.0 mg/kg, and the safest dose was estimated to be 1.2 mg/kg.

MMC concentrations in serum and tumor tissue homogenate were determined by the bioassay method using *Escherichia coli* B[10]. The diameter of the VX-2 tumor was measured 2-dimensionally using a sliding calliper and the tumor weight was estimated using the protocol of Battelle's Columbus Laboratories[11]. All of the animals were cared for until death except for 12 animals in the MMC microsphere group and 10 animals in the conventional MMC group from which the tumors were removed for study. The statistical differences were assessed using Student's t-test.

Results. In vitro drug release. In vitro MMC release from the MMC microspheres was slow, as shown in figure 2. The

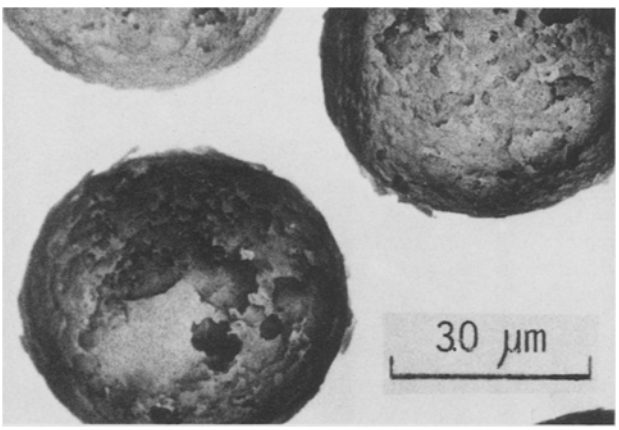


Figure 1. Scanning electron micrograph of the heated albumin-mitomycin C microspheres.

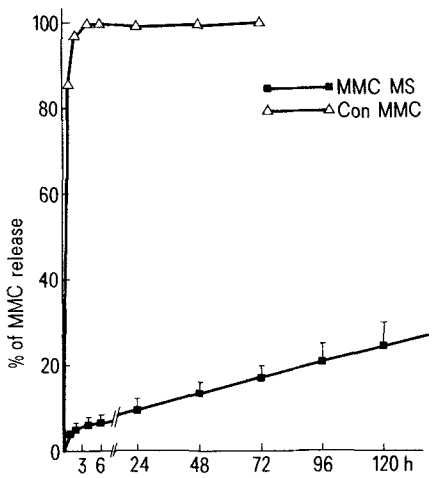


Figure 2. Time course of mitomycin C levels in VX-2 tumor of mitomycin C microsphere and conventional mitomycin C groups. Each plot represents an average of 2 separate determinations of the excised tumors from 2 rabbits. Abbreviations are as in the legend to figure 6.

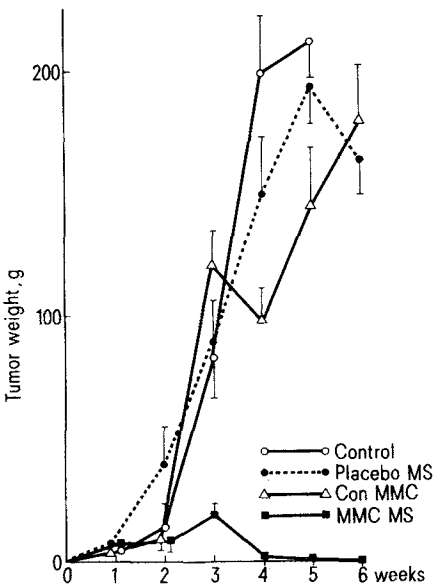


Figure 3. Time course of growth of VX-2 tumor implanted into the right hind leg of rabbits. Control group (10); Placebo microsphere group (10); Conventional MMC group (7); MMC microsphere group (10). Abbreviations: Placebo MS, placebo microsphere.

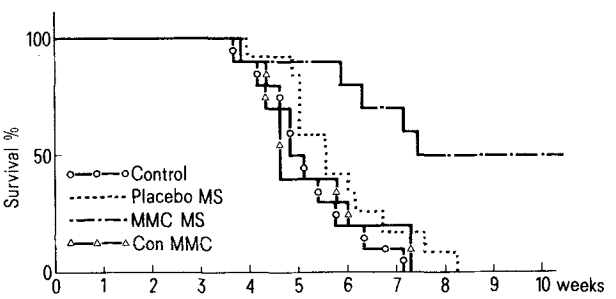


Figure 4. Survival of rabbits with an implanted VX-2 tumor. Abbreviations as in legends to figures 3 and 6.

amount of MMC released from the microspheres was about 10% after 24 h and about 20% after 3 days. In contrast, the conventional MMC was completely soluble after 2 h.

The time course of tumor growth. Figure 3 demonstrates the time course for the right hind leg tumor. The tumor growth in the MMC microsphere group was markedly inhibited and 3 weeks later the tumor disappeared, while that in the placebo microsphere and conventional MMC groups was much the same as in the control.

Survival. The length of survival in the 4 groups is shown in figure 4. In all 3 groups, excluding the MMC microsphere group, all of the rabbits died within 9 weeks, while 5/10 of the MMC microsphere group are still living, with no evidence of tumor.

Comparison of MMC levels in the peripheral blood and VX-2 tumors. MMC serum levels in the conventional MMC and MMC microsphere groups are illustrated in figure 5. The initial half-reduction time in the conventional MMC group was approximately 7 min and 2 h later the level dropped below the limiting level for bioassay. The initial reduction half time in the MMC microsphere group was about 20 min and the peripheral serum concentration could be estimated up to 4 h later.

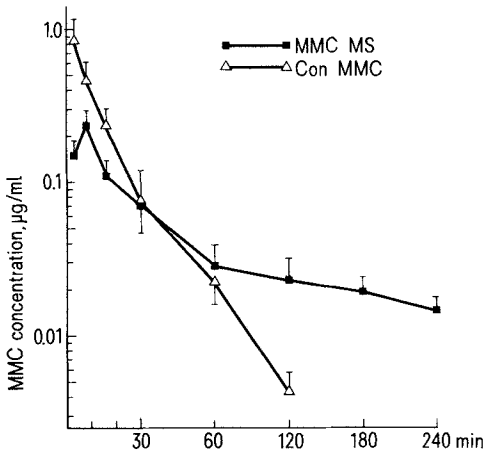


Figure 5. Comparison of peripheral serum levels of mitomycin C between the mitomycin C microsphere and conventional mitomycin C groups. Three rabbits per group. Each plot and vertical bar represents a mean and SD, respectively. The used abbreviations are given in the legend to figure 6.

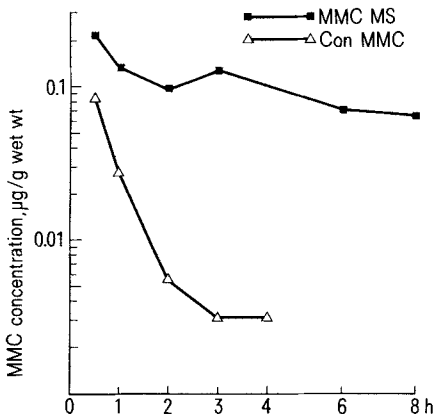


Figure 6. In vitro release of mitomycin C from heated albumin microspheres containing mitomycin C. Each plot and vertical bar represents an average and SD of triplicate determinations, respectively. Abbreviations: MMCMS, mitomycin C microsphere; ConMMC, conventional mitomycin C.

MMC concentration in the VX-2 tumors are shown in figure 6. VX-2 tumors in the MMC microsphere group contained a markedly high level of MMC, for up to 8 h. In contrast, in the conventional MMC group, the time course for MMC tumor levels was similar to that in the case of peripheral blood levels.

Discussion. The effect of an antitumor drug depends on the concentration and the period of effectiveness. As antitumor drugs have side effects, intra-arterial infusion chemotherapy has been performed in the hope of producing the maximum of antitumor effects, with minimal side effects. A large majority of these infused drugs, however, disperse rapidly through the target tumor (see figs 3-6). MMC microspheres were prepared for intra-arterial use in such a manner that the thickened MMC would be released slowly into the tissues surrounding the target tumor, thus obstructing the runoff of the drainage vein. Figure 5 shows that the MMC microspheres infused intra-arterially sustained a relatively high concentration for a few hours 60 min after infusion, while the conventional MMC was eliminated rapidly from the peripheral blood. After conventional MMC is injected, the circulating blood contains roughly the same density of MMC, in each part of the body. In contrast, the drug level in the MMC microsphere group appeared to be high in the right hind leg, and as shown in figure 6, was much higher in the tumor.

The peripheral blood level in the MMC microsphere group seemed to be related to drug leakage from the MMC microspheres entrapped in the small arteries, and which were distributed throughout the VX-2 tumor involved in the surrounding tissues. MMC blood concentrations at time '0', as estimated by extrapolation, were approximately 1.0 µg/ml in the conventional MMC group, while in the MMC microsphere group the concentration was about 0.2 µg/ml. Since both groups were given exactly the same dose, about $^4/_5$ of the infused MMC microspheres were probably caught in the small arteries in the right hind leg and the remaining $^1/_5$, consisting of microspheres cracked because of arterial pulsation and because of swelling of the microsphere, were cleared at a reduced speed, that is, at a half reduction time of about 20 min.

Human cardiovascular studies revealed that blood flow towards the muscle, skin, and skeleton including the bone marrow, fat and connective tissue is about 36% of total blood flow[12]. When this 36% is assumed to be all of the blood flow toward the extremities, the unilateral lower extremity probably receives about 15% of the total blood flow. On the basis of this assumption, it may be inferred that, despite differences in species, the drug level in the tumor-bearing leg can be calculated as $1/15 \times 100$, namely 6.7 times higher than the peripheral drug level. This assumption accords substantially with MMC levels in VX-2 tumors in figure 6. Moreover, from the time course of MMC levels in the tumor, tumor disappearance in the MMC microsphere group would follow, as a matter of course.

The mechanical occlusion of tumor vessels by arterial ligation or arterial embolization, has been attempted[13]. Since MMC microspheres have an average diameter of 45 ± 8 µm, intra-arterial administration appears to occlude small arteries in the target site for several hours. However, as shown in figures 3 and 4, the placebo microspheres neither retarded tumor growth nor prolonged survival, compared to the control. Our findings suggest that the effects of MMC microspheres are due to the prolonged drug action rather than to an influence on the tumor vessels, per se.

1 We thank M. Ohara for reading the manuscript.
2 Present address: Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama (Japan).
3 Present address: Professional Relations Department, Kyowa Hakko Co. Ltd, Tokyo (Japan).
4 Sugibayashi, K., Morimoto, Y., Nadai, T., Kato, Y., Hasegawa, A., and Arita, T., Chem. Pharm. Bull. *27* (1979) 204.
5 Sugibayashi, K., Akimoto, M., Morimoto, Y., Nadai, T., and Kato, Y., J. Pharm. Dyn. *2* (1979) 350.
6 Morimoto, Y., Sugibayashi, K., Okumura, M., and Kato, Y., J. Pharm. Dyn. *3* (1980) 264.
7 Morimoto, Y., Sugibayashi, K., and Kato, Y., Chem. Pharm. Bull. *29* (1981) 1433.
8 Kato, T., Nemoto, R., Mori, H., and Kumagai, I., Cancer *46* (1980) 14.
9 Scheffel, U., Rhodes, B.A., Natarajan, T.K., and Wagner, Jr, H.N., J. nucl. Med. *13* (1972) 498.
10 Fujita, H., Cancer Chemother. *1* (1974) 497.
11 Ovejera, A.A., Houchens, D.P., and Barker, A.D., Ann. clin. Lab. Sci. *8* (1978) 50.
12 Abboud, F.M., Schmid, P.G., Heistad, D.D., and Mark, A.L., in: Clinical cardiovascular physiology, p. 143. Ed. H.J. Levine. Grune & Stratton, Inc., New York/San Francisco/London 1976.
13 Wallace, S., Granmayeh, M., DeSantos, L.A., Murray, J.A., Romsdahl, M.M., Bracken, R.B., and Jonsson, K., Cancer *43* (1979) 322.

0014-4754/83/080913-04$1.50 + 0.20/0
©Birkhäuser Verlag Basel, 1983

Density gradient centrifugation of tumor cells from needle biopsies and their respective source tumors: a comparison of density distributions[1]

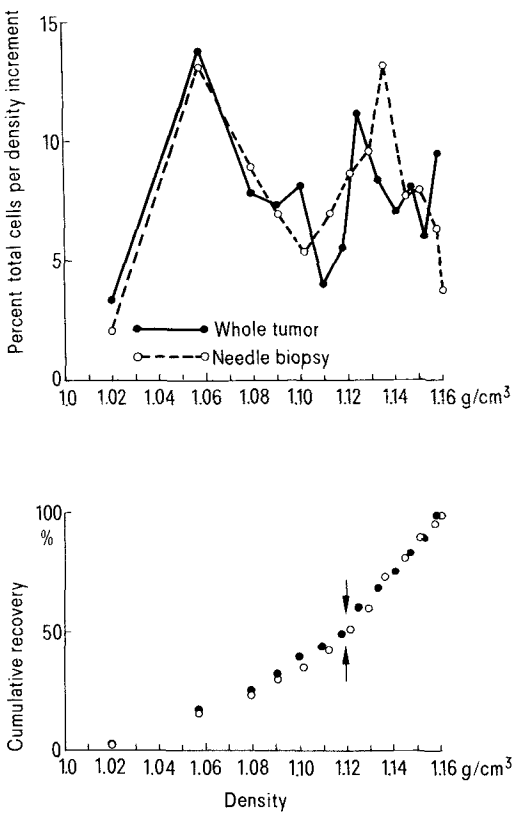
D.J. Grdina and G. Zin

Department of Experimental Radiotherapy, The University of Texas System Cancer Center M.D. Anderson Hospital and Tumor Institute, 6723 Bertner Avenue, Houston (Texas 77030, USA), December 10, 1982

Summary. A needle biopsy technique was applied to a murine fibrosarcoma (FSa) tumor system. FSa tumors of 8 mm diameter in size were biopsied and then made into single cell suspensions. Resulting density profiles of cells from both sources were compared following centrifugation in Renografin gradients. In all cases, there was excellent agreement between the density profiles of the material from each of the biopsies and the corresponding solid tumors.

Following centrifugation of fibrosarcoma (FSa) cells in linear continuous density gradients of Renografin, at least 5 subpopulations of tumors cells are obtained, each of which differs in clonogenic ability[2], proliferative capacity[3,4], and radiation sensitivity[5,6]. The relative contribution of normal cells to each of these bands can be identified and quantitated by immunological and/or cytometric methods[7,8]. These data suggest that density distributions of tumor cells may reflect the relative composition in the tumor of relatively resistant vs sensitive, proliferating vs quiescent, and/or oxic vs hypoxic cells. Before such data can be utilized in a predictive manner to describe tumor response, it is required that a biopsy system be developed in which biopsy material obtained is sufficiently representative of the tumor and that any density profiles observed are reflective of those found when the entire tumor is dissociated and made into suspension.

Materials and methods. FSa tumors were grown to 8 mm in diameter in the hind legs of C_3Hf/Kam specific pathogen-free mice. Prior to obtaining a biopsy, each animal was anesthetized with Nembutal sodium (0.06 mg/g b.wt; Abbot Lab, Chicago, IL). Using a Tru Cut disposable biopsy needle (Travenol Laboratories, Inc., Deerfield, IL), tumor biopsy samples containing 2×10^6 cells were routinely obtained. These samples, as well as their respective solid tumors, were removed aseptically. Tumor tissue was finely minced and then digested for 20 min at room temperature with trypsin, made up to 0.025% in solution A (8.0 g NaCl, 0.4 g KCl, 1.0 g glucose, and 0.35 g $NaHCO_3$ in 1 l of water). The suspension was then passed through a stainless steel mesh (200 wires/2.54 cm) and then centrifuged at $225 \times g$ for 5 min. The resulting single cells were counted by hemocytometer and viability was determined by trypan dye exclusion and phase contrast microscopy. Viability was routinely greater than 95% and the yield of viable cells was about 10^8 per g of tumor tissue[5].



Comparison of density banding profiles obtained from an individual FSa solid tumor made into single cell suspension and a needle biopsy obtained from that tumor.