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Distribution of Bleomycin in Ethylnitrosourea-induced Gliomas in rats*†

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Abstract—*We used a microbioassay to study the distribution of bleomycin in rat brain tumors induced in newborn Sprague–Dawley rats with 1-ethyl-1-nitrosourea (ENU, 50 mg/kg s.c.). Upon suspected successful tumor induction bleomycin (0.1 g/kg i.v.) was administered, and 2 hr later bleomycin concentrations in major organs and tumor tissues were bioassayed using* Bacillus subtilis *PCI 219 IMC. To determine their histology, the tumors were stained by the immunofluorescence- or immunoperoxidase method using antiserum to astroprotein in addition to the conventional staining methods. There were 11 gliomas each of the brain and spinal cord, 14 schwannomas of the trigeminal nerve and 4 adenomas of the pituitary gland; they developed within 8 (gliomas), 7.3 (schwannomas) and 15 (adenomas) months on average after ENU treatment. The bleomycin concentration and the tumor:plasma concentration ratio were 7.69 ± 2.84 μg/g and 0.13 ± 0.05 (brain gliomas), 7.10 ± 3.15 μg/g and 0.27 ± 0.12 (spinal cord gliomas), 5.40 ± 1.41 μg/g and 0.23 ± 0.05 (schwannomas), 4.83 ± 1.05 μg/g and 0.21 ± 0.08 (adenomas). Normal brain- and spinal cord tissues scarcely contained bleomycin.*

INTRODUCTION

WE PREVIOUSLY reported on the uptake of various carcinostatics by gliomas induced by implanting 20-methylcholanthrene pellets into mouse brains [1–3]. These tumors are infiltrative, histopathologically similar to human gliomas and can be used in uptake studies. Furthermore, they may be more suitable models of human brain tumors than the frequently used transplantable tumors. However, implantation of the pellets may produce changes in the blood-brain barrier; moreover, the induced tumor grows along the route of pellet insertion and often on the brain surface. In addition, the mouse brain is too small to provide a sufficient amount of test material for analysis.

The animal model of brain tumors which circumvents these disadvantages of the mouse model has been developed in rats by percutaneous administration of 1-ethyl-1-nitrosourea (ENU) after initial failures of experiments in which we exposed rats of various strains to derivatives of nitrosourea. Using this rat model we studied the delivery of systemically administered bleomycin [4–8] to this brain tumor.

MATERIALS AND METHODS

Experimental brain tumors

ENU (12.5 mg) was suspended homogeneously in 1 ml of physiological saline, mixed with a thermomixer (Thermonics Co., Tokyo) [9–11], and the resulting suspension (50 mg/kg of ENU) was subcutaneously injected into the abdominal area of newborn Sprague–Dawley (S.D.) rats (Nihon Dohbutsu K. K., Osaka) (Fig. 1).

Rats manifesting generalized weakness and/or neurologic signs were presumed to have brain tumors and used in the experiments.

Administration of bleomycin and preparation of tumor tissue specimens

Rats with presumed brain tumors were injected into the tail vein with 0.1 g/kg of bleomycin

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suspended in physiological saline (20 mg/ml) (Fig. 1); 2 hr later they were exsanguinated by heart puncture and autopsied immediately. The brain and the spinal cord were removed *en bloc* and cut into coronal or transverse sections aseptically under the dissectioning microscope. The induced tumor, which was easily detected by its soft consistency and gray discoloration, was dissected from the normal brain or spinal cord under the microscope. All the tumor tissues collected from the different sections were weighed accurately in total with a digital balance and microbioassayed by the procedure illustrated in Fig. 2. One of the sections containing the tumor was fixed in 10% formaldehyde solution or 95% alcohol and stained with hematoxylin-eosin (HE), phosphotungstic acid-hematoxylin (PTAH) or Laidlow's connective tissue stain (LCT) for histological examination. Immunohistological diagnosis was made by using an antiserum to astroprotein [12]. The bleomycin distribution in the normal brain, spinal cord, trigeminal nerve, liver, lungs, spleen, skin and kidneys was also assayed (wet wt basis) in carcinogen-treated and tumor-free rats.

Bioassay of tissues by antimicrobial activity

This procedure was as previously reported [1, 2, 6, 7, 13]. Tissue samples were weighed quickly and carefully under aseptic conditions using a Mettler digital balance (model H10Tw). Then they were finely cut with scissors and suspended in a volume of phosphate-buffered saline (PBS, M/15, Sörensen's PBS, pH 7.2) which represented an integral number of times (3–5 times) their wet weight. Each suspension was homogenized with a Potter-Elvehjem homogenizer (Kayagaki Irika K.K., Tokyo) or a microtissue grinder (T.M. Kontes Glass Co., U.S.A.) and centrifuged (3000–5000 rev/min, 15 min). Sterile paper discs (Toyo Roshi, Tokyo; for assay of antibiotics, 8 mm in diameter, thick type) were impregnated with the supernatant and placed on the thin-layer surface of a normal agar plate containing about 10^6 spores/ml of *Bacillus subtilis* PCI 219 IMC (5 ml/plate of nutrient agar:polypeptone, 10 g; special meat extract, 5 g; agar powder, 20 g; distilled water, 1 l.; pH 7.2) to allow the diffusion of bleomycin at 5°C for 2 hr. The plates were then incubated at 37°C for 16 hr and the diameters of the growth-inhibited zones were measured in 2 perpendicular directions ($n = 6$) with a pair of calipers. Standard curves were prepared to calculate the amount of bleomycin contained in the tissue. The mean values (μg/g, ± S.E.M.) for bleomycin concentration were obtained from the data calculated with three plates prepared for one specimen.

RESULTS

There were 43 rats with presumed ENU-induced tumors; they were treated with bleomycin. Of the 43 rats, 31 (72%) developed tumors in the central nervous system (CNS); there were 11 gliomas each of the brain and spinal cord, 14 schwannomas of the trigeminal nerve and 4 adenomas of the pituitary gland. Of the 31 tumor-bearing rats, 9 (29%) manifested tumors of the same, or different, kind at 2 or more sites. Figures 3 and 4 show the gross and histopathological appearance of a glioma in the cerebral hemisphere. The utilization of immunohistological

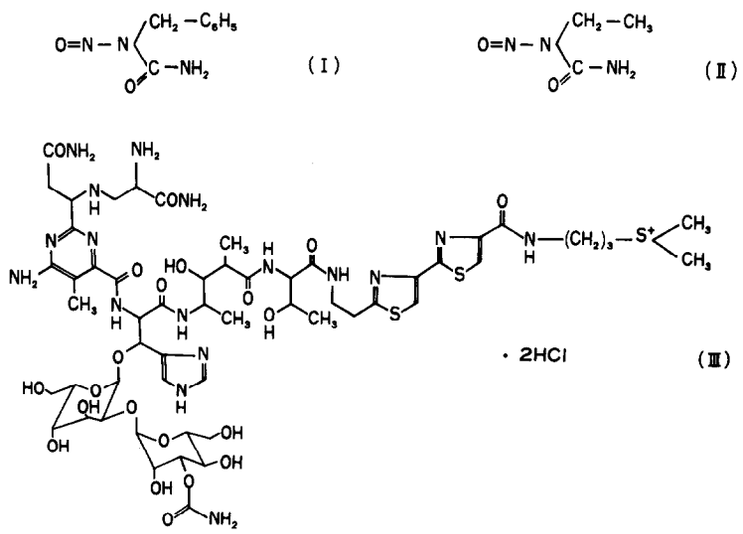

Fig. 1. Chemical structures of the carcinogen (I, benzylnitrosourea, II, ethylnitrosourea) and carcinostatic bleomycin A_2 [III, major component (60–70%) of commercial bleomycin mixture—chemical formula: $C_{55}H_{84}N_{17}O_{21}S_3.2HCl$; mol. wt: 1487.414] [37].

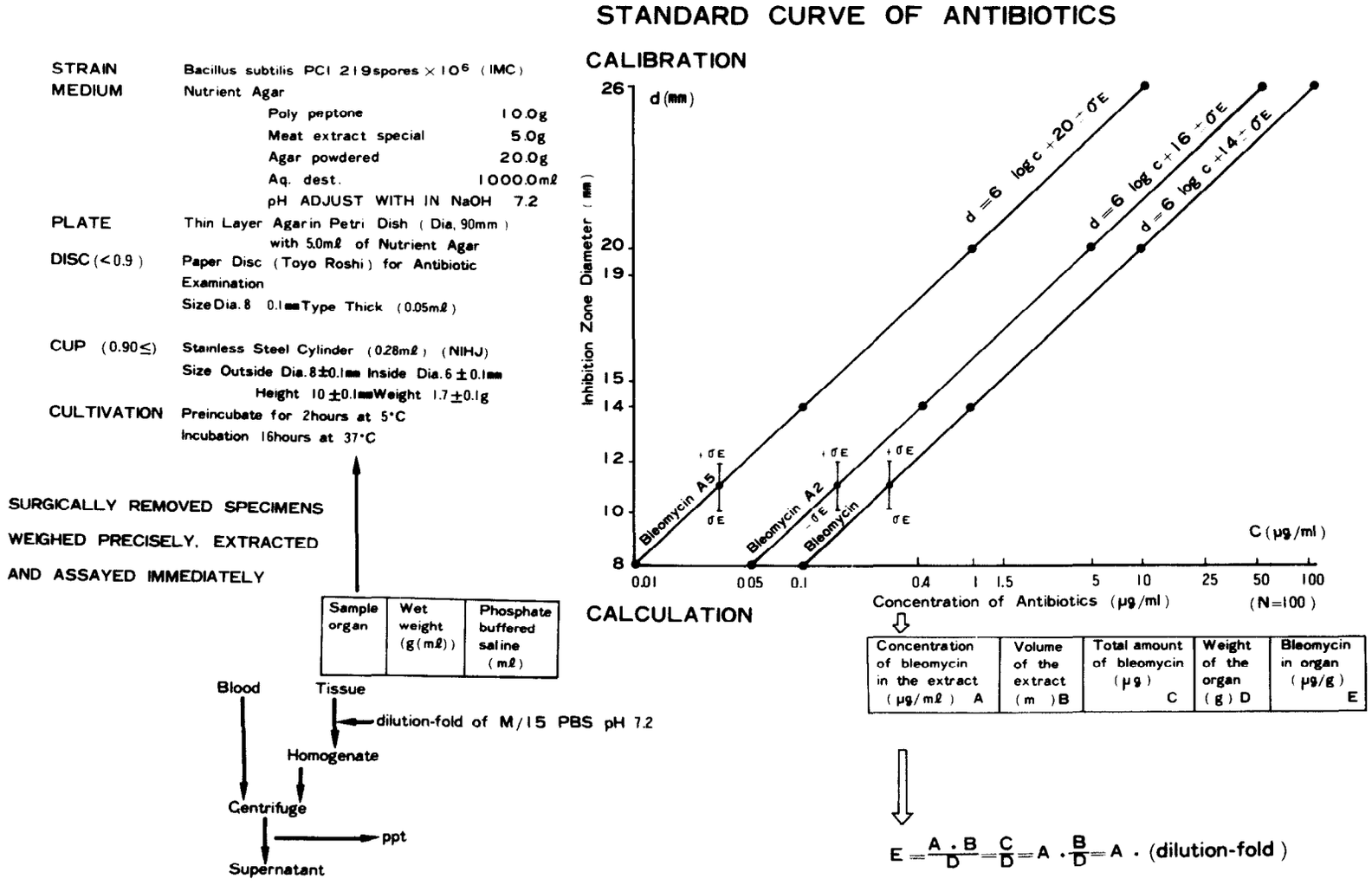

Fig. 2. Procedure of bioassay by agar plate disc (cup) method and standard curves of antibiotics.

staining using antiserum to astroprotein, in addition to the conventional staining methods, made histological diagnosis of the glioma easier. The immunohistological diagnoses of these brain tumors have been reported elsewhere [12]. The interval between ENU injection and the appearance of neurological manifestations and/or generalized weakness was 8.0 ± 0.5 months for gliomas of the brain ($n = 11$), 8.1 ± 0.6 months for gliomas of the spinal cord ($n = 11$), 7.3 ± 1.0 months for schwannomas of the trigeminal nerve ($n = 14$), and 14.9 ± 1.3 months for adenomas of the pituitary gland ($n = 4$) (Fig. 5). Table 1 shows the bleomycin concentration in the gliomas induced in the brain and spinal cord and the tumor:plasma bleomycin concentration ratios.

The mean bleomycin concentration in each type of tumor was calculated and compared to normal tissue values (Table 2). Bleomycin was delivered to all gliomas of the brain and spinal cord but not to normal brain tissue, and only trace amounts were demonstrated in normal spinal cord. Schwannomas of the trigeminal nerve contained approximately twice the bleomycin concentration of the corresponding normal tissue; the bleomycin level in pituitary adenomas was significantly higher than in normal pituitary gland tissue. To determine the significance of the differences between tumor- and corresponding normal tissues we applied the *t*-test of Gehan [14]. Table 2 also lists the tissue:plasma bleomycin concentration ratios. The bleomycin distribution pattern in rat liver, lung, spleen and kidney was similar to that we previously reported for mice [1].

DISCUSSION

Bleomycin is a complex of water-soluble, lipid-insoluble glycopeptides with a molecular weight of about 1500. We subcutaneously (s.c.) injected normal rats with bleomycin and found that its delivery to the brain and spinal cord is prevented by the blood–brain barrier. On the other hand, considerably high concentrations of this antibiotic were detected in the present ENU-induced tumors, suggesting that the blood–brain barrier in the tumor had undergone some changes and that under this condition bleomycin can exert an antitumor effect against CNS tumors. However, before drawing conclusions regarding the uptake of bleomycin by primary malignant gliomas, questions related to its molecular size, inactivation, arrival at the cancer site and elimination rate need to be answered.

Studies on the pathology and treatment of brain tumors have been aided by the establishment of experimental brain tumor models [15–18]. The brain tumors induced in rats orally or intravenously administered with various alkylnitrosoureas [9–11, 19, 20] exhibited growth-, invasion- and histopathological features strongly reminiscent of human brain tumors [21–24].

As the induction of these tumors does not require surgical invasion of the brain for transplantation- or implantation purposes, they closely resemble spontaneously developing human brain tumors. Therefore, they represent a pathologic model more suitable for the observation of the blood–brain barrier and of the distribution of carcinostatics.

On the other hand, this experimental model presents some disadvantages in that the rate and required period of tumor induction are not uniform. To our knowledge, no previous study has been reported on the uptake of carcinostatics by these tumors. Therefore we injected newborn rats s.c. with ENU and then administered

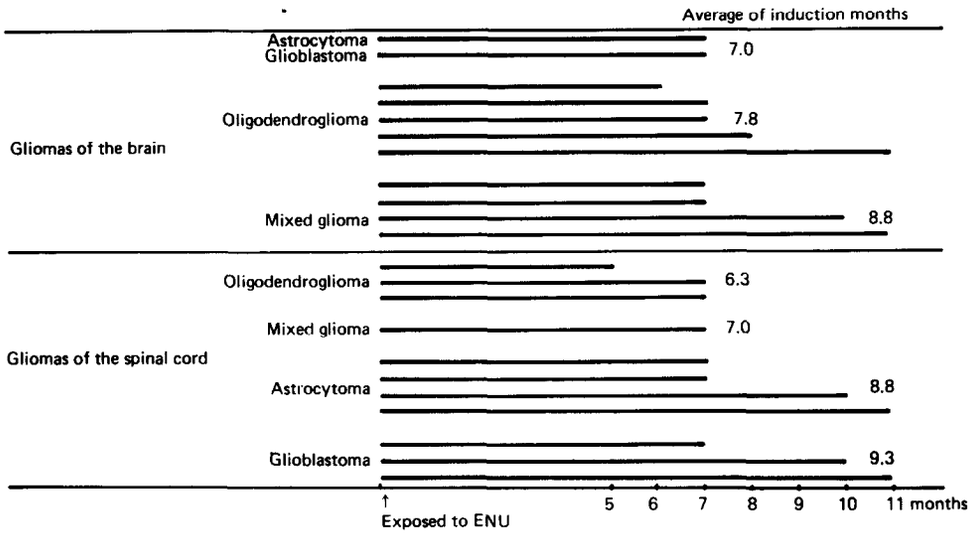

Fig. 5. Intervals between ENU injection and the appearance of neurological manifestations and/or general weakness indicative of successful tumor induction.

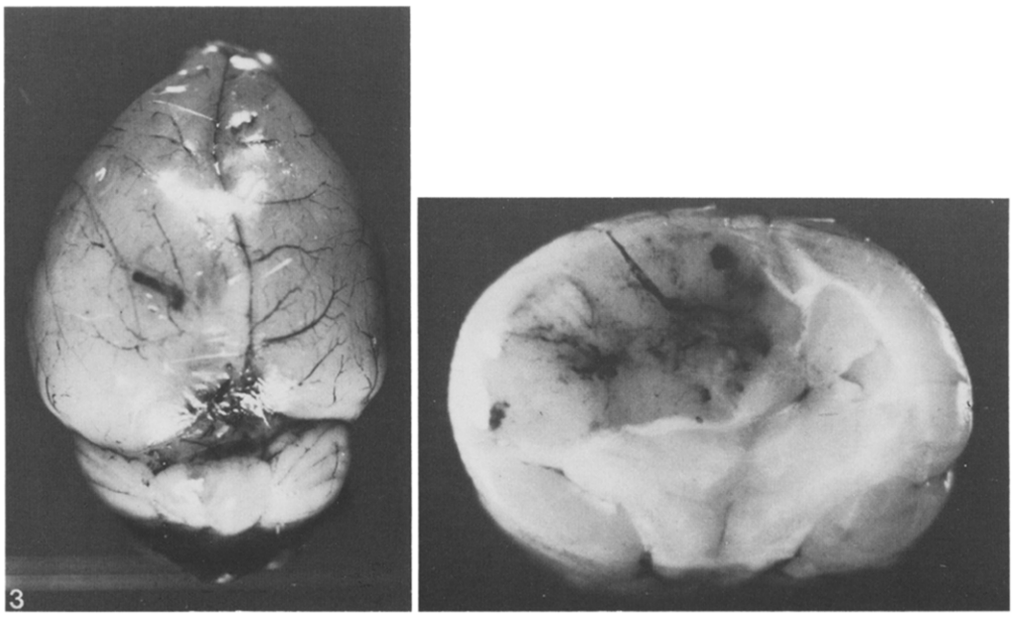

Fig. 3. ENU-induced glioma in the rat brain. Left: the brain, showing a tumor in the left hemisphere; right: coronal brain section; note the large infiltrative tumor attended by hemorrhage.

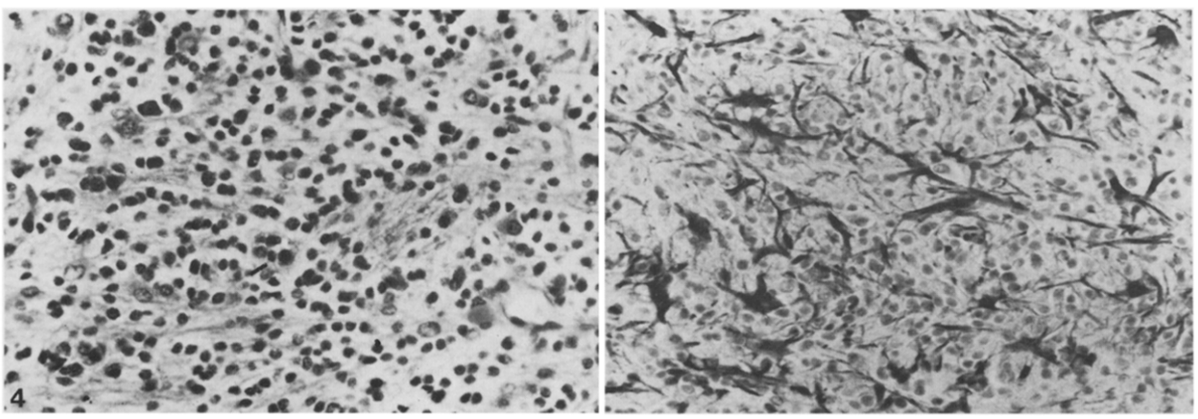

Fig. 4. Micrographs of the tumor presented in Fig. 3. Left: H&E stain, × 400; right: immunoperoxidase preparation, × 400, exhibiting 2 types of cells in the tumor; one type is stained from the cell body to the tips of the cell processes. This method led to the histological diagnosis of mixed glioma comprising astrocytoma and oligodendroglioma.

Table 1. *Bleomycin concentration in gliomas of the brain and spinal cord* 2 hr *after i.v. administration of* 0.1 mg/g *body weight*

	Rat No.	Histology	Bleomycin concentration in tumor, μg/g*	Tumor:plasma concentration ratio
Brain	E1078	Astrocytoma	0.63 ± 0.03	0.03
	E0678	Glioblastoma	21.21 ± 2.12	—
	E5777	Oligodendroglioma	16.83 ± 2.18	0.36
	E0578	Oligodendroglioma	1.21 ± 0.04	0.11
	E0778	Oligodendroglioma	24.20 ± 1.69	—
	E1178	Oligodendroglioma	0.46 ± 0.02	0.03
	E2278	Oligodendroglioma	17.49 ± 1.92	0.48
	E3577	Mixed glioma	0.76 ± 0.06	0.01
	E3677	Mixed glioma	0.50 ± 0.05	0.02
	E3877	Mixed glioma	0.04 ± 0.01	0.06
	E0478	Mixed glioma	1.24 ± 0.14	0.07
Spinal cord	E3477	Glioblastoma	1.23 ± 0.13	0.18
	E3777	Glioblastoma	2.33 ± 0.27	0.07
	E2378	Glioblastoma	0.95 ± 0.09	0.07
	E3577	Astrocytoma	31.50 ± 2.52	0.41
	E0178	Astrocytoma	3.18 ± 0.41	1.35
	E0878	Astrocytoma	26.55 ± 2.38	—
	E1078	Astrocytoma	3.57 ± 0.49	0.19
	E4077	Oligodendroglioma	3.60 ± 0.54	0.10
	E0478	Oligodendroglioma	0.58 ± 0.03	0.03
	E1078	Oligodendroglioma	3.57 ± 0.57	0.19
	E0578	Mixed glioma	0.99 ± 0.06	0.09

*Mean ± S.E.M. ($n = 6$).

Table 2. *Bleomycin concentration in ENU-induced rat CNS tumors and by normal tissue* 2 hr *after i.v. administration of* 0.1 mg/g *body weight*

Tissues	No. of tumors	Bleomycin concentration in tissue, μg/g*	Tumor:plasma concentration ratio*
Tumor tissue			
Glioma of the brain	11	7.69 ± 2.84**	0.13 ± 0.05**
Glioma of the spinal cord	11	7.10 ± 3.15***	0.27 ± 0.12***
Schwannoma of the trigeminal nerve	14	5.40 ± 1.41	0.23 ± 0.05
Pituitary adenoma	4	4.83 ± 1.05***	0.21 ± 0.08
Normal tissue			
Brain	23	0.00	0.00
Spinal cord	13	0.16 ± 0.08	0.01 ± 0.01
Trigeminal nerve	9	2.01 ± 0.47	0.09 ± 0.02
Pituitary gland	3	0.20 ± 0.16	0.01 ± 0.01
Liver	40	0.61 ± 0.46	0.04 ± 0.01
Lung	40	7.20 ± 1.07	0.34 ± 0.05
Spleen	14	0.64 ± 0.56	0.04 ± 0.01
Skin	40	11.30 ± 1.79	0.45 ± 0.06
Kidney	40	37.01 ± 6.46	1.43 ± 0.19

*Mean ± S.E.M.

Significantly different from normal tissue: ** $P < 0.001$; *** $P < 0.05$.

bleomycin to test the delivery of this carcinostatic agent to the tumor and non-tumor tissue. We chose bleomycin because it is incorporated in bioactive form by the tissue and can therefore be readily microbioassayed [1, 2, 6, 13, 25–27]. Furthermore, our present experimental design made it possible to compare our uptake results with those obtained for 20-methylcholanthrene-induced- and human gliomas [25, 26, 28–31].

Chemical assay, radioassays, bioassays and immunoassays are available for the microassay of body fluids and tissues for antibiotics, but they all have advantages and disadvantages. There are only limited kinds of antibiotics assayable by the chemical methods, and such methods involve risks of determining even the *in vivo* metabolites which are no longer biologically active. The methods using radioisotopes are sensitive enough to detect antibiotics administered in animal experiments at such low levels as below their usual clinical doses, while these methods likewise involve risks of determining both the antibiotics metabolised *in vivo* and the radioactive substances which no longer retain biological activities such as antimicrobial activity due to their binding to protein. Moreover, they require labeled compounds which are stable and have high specific activity to be synthesized as tracers, and also call for special experimental apparatus and facilities. Immunoassay is an excellent method for monitoring the blood levels of aminoglycoside antibiotics, which permits the relatively rapid microassay for such antibiotics. However, this method is also accompanied by problems of high activity-specific antisera and very limited kinds of assayable antibiotics.

Broughton *et al.* [25, 26] and Elson *et al.* [32] studied radioimmunoassays as a combination of the 2 methods; Lloyd *et al.* [33] and Galvan *et al.* [34] investigated into PM-2 bacteriophage DNA (bleomycin-specific fragmentation) assay; and Twentyman [35] studied the clonogenic assay of bleomycin cytotoxicity. However, there still remain questions to be answered before these methods are brought into practical use.

On the other hand, microbioassay is a comparative assay method which determines directly the antimicrobial activity of the target antibiotic using the inhibition of cell division as an index, and the test organisms selected for this purpose are practically susceptible enough to antibiotics with high selective toxicity [13, 36]. Needless to say, the loss in the potency of such antibiotics due to their *in vivo* metabolism and their binding to protein needs to be considered, while it is most advantageous in that it is capable of determining only the biologically active form of antibiotics in terms of overall antimicrobial activity in which such effects have been considered [1, 2]. In other words, it offers the following 4 advantages: (i) with a bleomycin recovery rate of not less than 80%, it is a very accurate method; (ii) it is of high precision, with standard deviation of the measured values being within ± 20% of the mean value, and the same operators have been attaining results with high reproducibility under the same experimental conditions for more than 10 yr [1, 2, 6, 7]; (iii) in assays by the thin-layer nutrient agar plate disc method, *Bacillus subtilis* PCI 219 IMC strain has proved very specific for the antimicrobial activity of bleomycin; and (iv) it has proved very sensitive, with the minimum assayable concentration being 0.01 μg/ml (theoretical value). Because of these advantages the microbioassay has been a very reliable, important assay method for screening not only bleomycin but also many other new drugs [1, 2, 6, 7, 27, 28, 31].

We found that bleomycin injected i.v. into rats was incorporated selectively and at high concentrations by tumor tissue, as is the case in human gliomas [28]. When the results of this study were compared with the distribution of bleomycin in human brain tumors [28] it was obvious that the bleomycin administered i.v. was incorporated by the induced tumor tissues selectively at high concentrations, as with human gliomas, compared with the normal portion of the brain. The ratios of these concentrations to the concentration in the plasma also proved very close to the human brain tumor–plasma concentration ratios. From the findings in this study, it is obvious that little or no bleomycin is distributed in active form into the normal brain tissue or CSF.

Evidence to date indicates that the blood–brain barrier and blood–CSF barrier may limit the entrance of bleomycin into brain tissue, or CSF from blood to a certain extent, though even if bleomycin is incorporated into the brain tissue or CSF it is probably metabolized rapidly into an inactive form. However, if its antitumor activity is related to its antibacterial activity, the feelings attained in the present study may be useful for the treatment of brain tumors with bleomycin as a cancer chemotherapy.

To our knowledge, no studies have been reported on the delivery of carcinostatics by spinal cord tumors. Our results show that in rats the bleomycin incorporation by gliomas of the spinal cord was similar to that of brain gliomas. Furthermore, the tumor:plasma ratio for bleomycin in rats was similar to that reported in brain tumor patients [31]. The bleomycin distribution in rat pituitary adenomas also approximated that of human pituitary adenoma [31].

Our data show that the bleomycin delivery to

the 14 schwannomas of the rat trigeminal nerve was about twice that of normal rat trigeminal nerve tissue (Table 2). In only 1 of 3 schwannomas of the human trigeminal nerve was bleomycin delivery observed [28]; however, due to the scarcity of reported schwannoma patients treated with bleomycin, valid comparisons cannot be made at this point.

Based on our results, we suggest that ENU-induced rat brain tumors represent a viable model for the study of the distribution of carcinostatic agents. We are at present investigating the relationship between bleomycin delivery and the tumor type and histology, with special reference to the concentration in central- and marginal portions of the tumors.

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