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Intrinsic Sensitivity of Tumor Cells to Bleomycin as an Indicator of Tumor Response to Electrochemotherapy

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Electrochemotherapy (ECT) involves the use of locally applied electric pulses to increase delivery of chemotherapeutic drugs into cells in tissues. ECT with bleomycin (BLM) is a very effective local treatment, but different tumors have different response rates to ECT. The aim of our study was to compare the responsiveness of SA-1 and EAT tumors to BLM and ECT *in vitro* and *in vivo*, in order to find possible reasons for the observed difference in response rate. The difference in sensitivity to ECT *in vitro* between the SA-1 and EAT cells was 10-fold and was the same as the difference in sensitivity to chronic BLM exposure, as measured by tetrazolium-based colorimetric (MTT) assay. This difference in sensitivity between SA-1 and EAT to ECT was also reflected in tumor cure rate. A six-times lower dose of BLM was needed to obtain local tumor control in SA-1 than in EAT tumors. Therefore, we suggest that the difference in sensitivity to BLM and ECT predominantly reflects the difference in intrinsic sensitivity of the cells to BLM.

Key words: Electrochemotherapy — Bleomycin — Electroporation — Experimental tumor — Mice

Bleomycin (BLM) is a water-soluble glycopeptidic antibiotic with limited antitumor effectiveness, and is currently used only in combined chemotherapeutic schedules in the treatment of cancer.[1)] The major reason for its limited antitumor effectiveness is the hampered transport of BLM through the plasma membrane. However, once inside the cell, BLM is highly cytotoxic, inducing single and double strand DNA breaks.[2)]

Different approaches have been tested to increase the antitumor effectiveness of BLM,[3–5)] mainly with the aim of facilitating entry of the drug into the cells. Electropermeabilization (electroporation), i.e., a technique for introduction of molecules into cells by exposure of the cells to intense electrical pulses, proved to be very effective. Under specific conditions, electropermeabilization is a reversible process which does not impair cell viability.[6)] Use of electropermeabilization to increase BLM uptake into the cells and consequently to increase the antitumor effectiveness was demonstrated *in vitro*, *in vivo* and also in clinical trials.[7–21)] This treatment was termed electrochemotherapy (ECT) and was also extended to other chemotherapeutic drugs.[22–24)] Its application to cisplatin has proven to be very effective.[23, 24)] Preclinical studies of ECT with BLM were performed on a variety of transplantable and spontaneous tumors in immunocompetent and immunodeficient mice.[9–18)] In all of these studies ECT proved to be an effective antitumor treatment, inducing a prolonged tumor growth delay compared to BLM treatment only. In addition, ECT results in tumor cures. In these preclinical studies a certain degree of variation in responsiveness to ECT was observed among the tumor types treated, as well as in the first clinical trials on basal cell carcinoma, malignant melanoma, head and neck squamous cell carcinoma and breast adenocarcinoma.[7–24)]

The aim of our study was to compare the responsiveness of SA-1 and EAT tumors to BLM and ECT *in vitro* and *in vivo*, in order to confirm the existence of a difference in responsiveness to ECT and to cast light on the possible reasons for it.

MATERIALS AND METHODS

In vitro assay for sensitivity of cells to BLM and ECT

Fibrosarcoma SA-1 cells (Jackson Laboratory, Bar Harbor, ME) and Ehrlich Lettre ascites carcinoma cells (EAT; American Type Culture Collection, Rockville, MD) were used. SA-1 cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 10% FCS and EAT cells in NCTC 135 medium supplemented with 15% FCS. Both SA-1 and EAT cells were routinely subcultured twice per week and were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

Sensitivity of SA-1 and EAT tumor cells to chronic exposure to BLM, as well as sensitivity to combined treatment with BLM and electric pulses (ECT), was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[25)] To determine the sensitivity to chronic exposure of SA-1 and EAT cells to BLM, these cells were plated in 96-well microtiter plates in 100 μl of medium containing BLM in the concentration range from 10^{-8} to 10^{-3} *M*. After 3 days, 100 μg of MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan bromide, Sigma, St. Louis, MO) was added to each well and the cells were further incubated for 4 h. The medium

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was then removed and the formed formazan crystals were dissolved in 100 μl of dimethyl sulfoxide (Sigma). The plates were shaken for 99 s and the absorbance of the resulting solution was measured at 540 nm using an Anthos microplate reader (Anthos, Austria). Survival of cells treated with different BLM concentrations is presented as percentage absorbance with respect to that of the control, i.e. untreated cells. These experiments were repeated 3 times.

The sensitivity of SA-1 and EAT cells to ECT was determined as described earlier.[23] Briefly, exponentially growing cells were trypsinized, washed three times and resuspended in a medium supplemented with 0.5 m*M* $CaCl_2$ at a population density 2.2×10^7 cells/ml. Ninety microliters of the cell suspension was mixed with 10 μl of different BLM concentrations ranging from 10^{-8} to 10^{-3} *M*. Half of this mixture was placed between two stainless steel electrodes 2 mm apart and subjected to 8 square wave electric pulses, with a pulse length of 100 μs, amplitude of 200 V and a repetition frequency of 1 Hz. Both SA-1 and EAT cells exposed to 200 V were 90% permeabilized as measured by the propidium iodide uptake method, and the survival was not changed (survival after electric pulses was 104±7% for SA-1 cells and 103±12% for EAT cells).[26] Electric pulses were generated by a Jouan GHT 1287 electropulsator (Jouan, Saint Herblain, France). After the pulsing procedure, the cells were incubated for 5 min at room temperature, diluted in BLM-free medium and plated in 96-well microtiter plates (1000 cell/well, 6 wells per BLM concentration). The second half of the cell suspension was treated in the same way as the first half, except for the electric pulse treatment. After 3 days, MTT assay was performed. The survival curve of cells treated with ECT was normalized to that of the electric pulse treatment alone group. The experiment was repeated 3 times. From the survival curves, IC_{30} values (BLM concentration required to reduce cell survival by 30%) were determined. The difference in sensitivity was calculated at the IC_{30} level. Since the drug was present in the medium surrounding the cells only for 5 min (ECT protocol, Fig. 2), it was not possible to calculate the IC_{50} value for EAT cells treated with BLM. Thus, calculation of the IC_{30} values enabled us to compare different treatments.

Mice and tumors Inbred A/J (Rudjer Boskovič, Zagreb, Croatia) and CBA mice (Institute for Pathology, University of Ljubljana, Ljubljana, Slovenia), weighing 20–25 g, were used. Mice were kept in a conventional animal colony at 24°C and in a natural day/night light cycle. Tumor cells for induction of subcutaneous tumors were obtained from the ascitic form of the tumors in mice, serially transplanted every 7 days. Tumors were induced in the right flank of the mice by inoculation of 5×10^5 viable (determined by trypan dye exclusion test) SA-1 cells in A/J mice and 5×10^6 viable EAT cells in CBA mice. Treatment started 6–8 days post-inoculation when the tumors reached approximately 45 mm^3 in volume. Mice subjected to the specific experimental protocol were randomly divided into experimental groups, consisting of 6–10 mice.

Electrochemotherapy *in vivo* BLM (Bleomycinum, Mack, Germany) was dissolved in phosphate-buffered saline at concentrations ranging from 2–2000 μg/ml. BLM was injected intravenously as a bolus into the lateral tail vein of the pre-heated mice at doses of 1, 10, 50, 100, 250 and 1000 μg per animal (approximately 0.05–50 mg/kg). BLM solution was prepared fresh for daily injections.

Electric pulses were delivered percutaneously by two flat parallel stainless-steel electrodes (two stainless-steel strips, length 35 mm, width 7 mm with rounded corners) separated by 8 mm, which were placed at the opposed margins of the tumor. Good contact between the electrodes and the skin was assured by the use of a conductive gel. Eight square-wave electric pulses of 1040 V amplitude, 100 μs pulse width and 1 Hz repetition frequency were generated by an electropulsator (Jouan GHT 1287; Jouan). Electric pulse treatment lasting 8 s was performed without anesthesia. The ECT protocol consisted of intravenous bolus injection of BLM (1, 10, 50, 100, 250 or 1000 μg/animal) followed 3 min later by electric pulse application on the tumor.

Treatment evaluation and statistical analysis Tumor growth was followed by measuring three mutually orthogonal tumor diameters (e_1, e_2, e_3) with a vernier calliper on each consecutive day. Tumor volumes were estimated according to the formula $\pi\cdot e_1\cdot e_2\cdot e_3/6$. The arithmetic mean (AM) and standard error of the mean (SE) of the tumor volumes were calculated for each experimental group.

Doubling times of tumors (DT) were determined for each individual tumor. Tumor growth delay was calculated for each individual tumor by subtracting DT of each tumor from mean DT of the control group and averaged for each experimental group. In order to compare the antitumor effectiveness of the treatments between SA-1 and EAT tumors, which have different growth rates, specific tumor growth delay was calculated by dividing the growth delay of experimental groups with DT of the control group.[27] Specific tumor growth delay is the normalized tumor growth delay, and thus more closely reflects the amount of cell killing after the treatment, providing a more accurate comparison of the antitumor effectiveness of ECT on SA-1 and EAT tumors.[27]

The response to treatment was scored as complete response when the tumor was no longer palpable. Mice that were in complete response 100 days after the treatment were considered as cured. Tumor control probability was calculated using Logit analysis. The difference in sensitivity to ECT between SA-1 and EAT tumors was

calculated at TCD_{50} value (tumor control dose 50 is the BLM dose in ECT treatment that, on average, would be expected to achieve tumor control in half of the animals).[28)]

RESULTS

Cell sensitivity to BLM and ECT *in vitro* Cell survival after chronic exposure to BLM was determined by MTT assay. To determine the sensitivity of SA-1 and EAT cells to BLM, cells were incubated in the presence of the drug for 72 h. Cell survival was determined by means of MTT assay. BLM was cytotoxic to both cell lines at concentrations above 10^{-6} *M* (Fig. 1). SA-1 cells were more sensitive to BLM than EAT cells; the IC_{30} value for SA-1 cells was 6×10^{-6} *M* BLM, whereas that for EAT cells was 6×10^{-5} *M* BLM. The difference in sensitivity to BLM calculated at the IC_{30} value was 10-fold and was similar at all levels of cell survival.

To determine the sensitivity of SA-1 and EAT cells to ECT, cells were incubated in the presence of BLM during exposure of the cells to electric pulses and 5 min thereafter. Exposure of cells to electric pulses greatly potentiated BLM cytotoxicity towards both cell lines tested, though by itself, it did not affect cell survival (Fig. 2). For SA-1 cells treated with ECT, the IC_{30} value was 2×10^{-9} *M* BLM, which is 6×10^{4} times lower than the IC_{30} value for cells treated with BLM only ($IC_{30}=1.2\times10^{-4}$). ECT was less effective on EAT cells; the IC_{30} for EAT cells treated with ECT was 2×10^{-8} *M*, which is 2.5×10^{4} times lower than the IC_{30} value for cells treated with BLM only ($IC_{30}=5\times10^{-4}$ *M*). The difference in sensitivity to ECT between SA-1 and EAT cells was 10-fold and was the same as the difference in sensitivity of the cells to chronic BLM exposure. Furthermore, in both cell lines ECT (5 min exposure of cells to BLM) was 3×10^{3} times more cytotoxic than chronic treatment of the cells with BLM.

Antitumor effectiveness of ECT *in vivo* The antitumor effectiveness of ECT with respect to BLM dose was tested on subcutaneous SA-1 and EAT tumors in mice. ECT was performed by exposure of tumors to electrical pulses 3 min after intravenous injection of BLM.

The antitumor effectiveness of ECT was BLM dose-dependent (Fig. 3) in both tumor models. SA-1 tumors

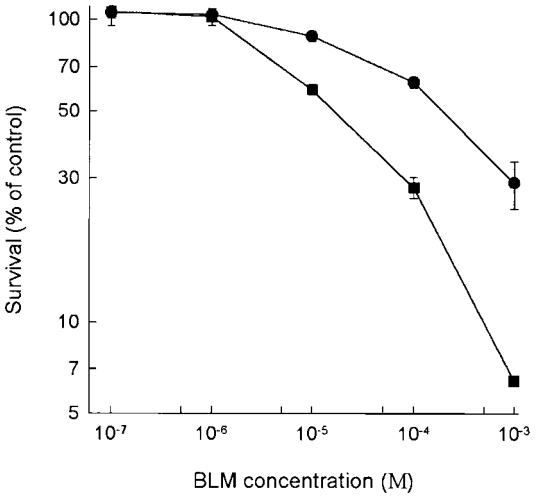

Fig. 1. Survival of SA-1 and EAT cells after chronic exposure (72 h) to various BLM concentrations. Cell survival was determined by means of MTT assay. Data are AM±SE from quadruple plates. ● EAT, ■ SA-1.

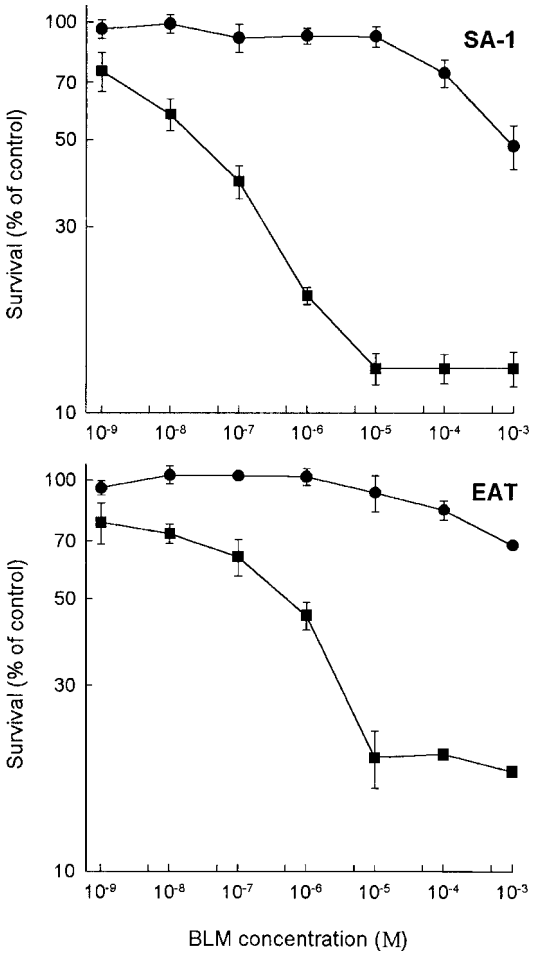

Fig. 2. Survival of SA-1 and EAT cells treated with ECT and BLM. The survival curve of ECT-treated cells was normalized with respect to electric pulse treatment (surviving fraction of SA-1 cell exposed to electric pulses alone was 1.04±0.07 and that of EAT cells was 1.03±0.12). Cell survival was determined by means of MTT assay. Data are AM±SE from quadruple plates. ● BLM, ■ ECT.

Table I. Antitumor Effectiveness of Electric Pulses and BLM Treatments on SA-1 and EAT Tumors *in vivo*

Exp. group	Tumor type					
	SA-1			EAT		
	n	DT[a] (days) (AM±SE)[c]	SGD[b]	*n*	DT (days) (AM±SE)	SGD
Control	45	1.8±0.05		42	4.6±0.3	
Electric pulses	7	3.1±0.2	0.7	7	6.8±1.0	0.5
BLM						
1 μg	20	1.8±0.1	0.0	—[d]	—[d]	—[d]
10 μg	19	1.9±0.1	0.1	28	5.6±0.4	0.2
50 μg	20	2.0±0.1	0.1	13	4.4±0.3	0.0
100 μg	20	1.9±0.1	0.1	29	6.2±0.3	0.3
250 μg	10	2.4±0.2	0.3	19	6.1±0.5	0.3
500 μg	—[d]	—[d]	—[d]	7	8.0±0.6	0.7
750 μg	—[d]	—[d]	—[d]	6	6.9±0.7	0.5
1000 μg	—[d]	—[d]	—[d]	7	7.7±0.4	0.7

a) Tumor doubling time was calculated from the tumor volumes.
b) Specific tumor growth delay was calculated from the tumor doubling times.
c) Arithmetic mean±standard error of the mean.
d) Not tested.

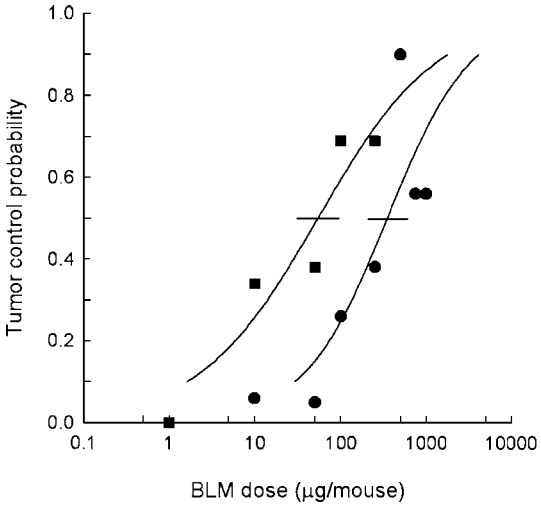

Fig. 3. Dose-response curve of SA-1 and EAT tumors treated by ECT with different BLM doses, evaluated in terms of tumor control probability. Mice bearing subcutaneous tumors were treated with BLM and 3 min later tumors were exposed to electric pulses. The TCD_{50} value is the BLM dose that, on average, would be expected to achieve control of half of the treated tumors. ● EAT, ■ SA-1.

were more sensitive to ECT than EAT tumors; local tumor control in SA-1 tumors was obtained at lower BLM doses than in EAT tumors. The TCD_{50} value for SA-1 tumors was 55 μg BLM (95% confidence interval: 33–92 μg BLM) and that for EAT tumors was 346 μg BLM (95% confidence interval: 200–600 μg BLM). The difference in sensitivity to ECT between SA-1 and EAT tumors was 6-fold and was comparable to the difference in sensitivity of cells to BLM and ECT obtained in *in vitro* experiments.

Treatment of SA-1 and EAT tumors with either BLM or electric pulses as a single treatment had only a minor effect on tumor growth. Specific tumor growth delays of these control treatments ranged from 0.0 for the lowest BLM dose to 0.7 for experimental groups treated with higher BLM doses (Table I).

DISCUSSION

We found that SA-1 tumor cells were more sensitive to BLM than EAT cells, after chronic exposure, as well as at 5 min incubation. The difference in sensitivity was also apparent *in vitro* ECT-treated cells, as well as *in vivo* in ECT-treated tumors. Therefore the difference in sensitivity of SA-1 and EAT tumors might be due to the difference in intrinsic sensitivity of these two cell lines to BLM.

Our *in vitro* experiments demonstrated that SA-1 cells are 10 times more sensitive to BLM, as well as to ECT, than EAT cells. This difference in sensitivity to BLM could be due to differences in the mechanisms responsible for the resistance of tumor cells to BLM. Several different mechanisms of tumor resistance to BLM have been proposed: decreased drug accumulation inside the cell, increased DNA repair, and metabolic inactivation of BLM due to the BLM hydrolase.[29] Since BLM is a hydrophilic molecule, it does not freely diffuse into the cells.[2] One of the proposed transport mechanisms for BLM internalization is endocytosis.[30] In addition, a membrane protein of

approximately 250 kDa that specifically recognizes and binds BLM was discovered. This protein is involved in BLM internalization and thus in its cytotoxicity.[31] It was also demonstrated that the quantity of this membrane protein correlates with BLM cytotoxicity.[31] However, this mechanism internalized only a small quantity of BLM into the cells. In our study we bypassed the plasma membrane restriction of BLM internalization by employing electropermeabilization. By electropermeabilization using specific parameters and a defined extracellular concentration of BLM, the same quantity of BLM molecules should be introduced into different types of cells.[7] In addition, if intrinsic cytotoxicity is defined as the cytotoxicity of the drug present inside the cell[2] then our *in vitro* data demonstrate that the observed difference in sensitivity of cells to ECT may be due to the difference in intrinsic sensitivity of cells to BLM. Taking into account the three different mechanisms of resistance to BLM, we can speculate that either EAT cells have more BLM hydrolase than SA-1 cells or that they are capable of repairing more of the DNA damage produced by BLM.

Comparison of our data on *in vitro* BLM cytotoxicity and the data available in the literature indicates that EAT cells are among the most BLM-insensitive cells.[32, 33] For EAT cells chronically treated with BLM the IC_{50} value was 2×10^{-4} *M* BLM, while for other cells such as HeLa, A-253, KB and Hepd the IC_{50} values range from 10^{-6} to 10^{-9} *M* BLM.[32, 33] From this point of view, the obtained results on potentiation of BLM cytotoxicity by exposure of cells to electric pulses is promising since they demonstrate that ECT is very effective in the case of BLM-insensitive tumor cells. The IC_{30} value of ECT-treated cells was 3000 times lower than that of cells chronically treated with BLM. The potentiation was the same in both SA-1 and EAT cells.

Also, ECT *in vivo* was more effective on SA-1 tumors than on EAT tumors. A six-times lower BLM dose was sufficient for effective tumor control of SA-1 as compared with EAT tumors, in terms of TCD_{50}. The difference in sensitivity to ECT *in vivo* was comparable to the difference in sensitivity to ECT *in vitro* (6- and 10-fold difference, respectively). From the biological point of view this difference in the sensitivity of the cells *in vitro* and the tumors *in vivo* is similar. Therefore, the results suggest that the difference in intrinsic sensitivity of tumor cells to BLM is also the reason for the observed difference in responsiveness of tumors to ECT *in vivo*. Since BLM, once inside the cell, is a very potent cytotoxic drug, tumors regardless of the histological type respond very well to ECT. In preclinical studies 36–100% of complete responses were obtained with mouse B16 melanoma, SA-1 and LPB sarcoma, spontaneous mammary carcinoma and human KB epidermal carcinoma tumors treated with ECT.[8, 9, 13, 14, 16] In clinical trials, 50–100% of complete responses were obtained on basal cell carcinoma, malignant melanoma, head and neck squamous cell carcinoma and breast adenocarcinoma tumors treated with ECT.[18–21] Thus, in the clinic, ECT offers an approach to treat accessible cutaneous and subcutaneous tumor lesions of different histological types. Specifically, ECT is convenient for treatment of patients who are not eligible for other therapies due to their physical status or in cases where other therapies have failed. As demonstrated in clinical trials, ECT can be performed on an outpatient basis and can be safely repeated several times.[18–21]

In conclusion, our study indicates that the different responsiveness of tumors to ECT might be due to different intrinsic sensitivity of cells to BLM. Since BLM is a very potent cytotoxic drug once inside the cell, a great potentiation of BLM cytotoxicity is obtained even in BLM-insensitive tumors. This can be of clinical importance in the treatment of tumors that are less sensitive to BLM and where BLM is currently not a treatment of choice due to its low antitumor effectiveness.

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