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Electrochemotherapy with bleomycin in SA-1 tumor-bearing mice—natural resistance and immune responsiveness

Gregor Serša, Vladimir Kotnik,[1] Maja Čemažar, Damijan Miklavčič[2] and Antonija Kotnik[3]
Department of Tumor Biology, Institute of Oncology, Zaloška 2, 1000 Ljubljana, Slovenia.
Tel: (+386) 61 323 063 extn 2933; Fax: (+386) 61 131 41 80. [1]Institute of Microbiology and Immunology, Medical Faculty, University of Ljubljana, Zaloška 4, 1105 Ljubljana, Slovenia. [2]Faculty of Electrical Engineering, University of Ljubljana, Tržaška 25, 1000 Ljubljana, Slovenia. [3]Virology Department, Institute of Public Health of the Republic of Slovenia, Bohoričeva 15, 1000 Ljubljana, Slovenia

Electrochemotherapy is an antitumor treatment that utilizes locally delivered electric pulses to increase the effectiveness of chemotherapeutic drugs in cells and tissues. Electric pulses permeabilize tumor cells to allow non-permeant drugs such as bleomycin to enter the cells. Although preclinical data indicate that immune responsiveness of the organism is important for obtaining cures of the tumors after electrochemotherapy with bleomycin, it is not known how electrochemotherapy affects the immune system of the organism. The aim of the study was to determine the effects of electrochemotherapy with bleomycin on natural resistance and immune responsiveness. Natural resistance was evaluated by phagocytic and intracellular killing activity (oxidative burst) in monocytes and polymorphonuclear granulocytes from venous blood, and immune responsiveness by blast transformation of spleen mononuclear cells to mitogens. The percentage of monocytes in venous blood able to elicit oxidative burst was significantly increased 7 days after the electrochemotherapy and returned to normal values after 14 days. In addition, increased blast transformation of spleen mononuclear cells by stimulation with concanavalin A (T lymphocytes activity) was found 14 days after electrochemotherapy treatment. The results of our study demonstrate that electrochemotherapy with bleomycin affects the immune system of the organism.

Key words: Bleomycin, electrochemotherapy, fibrosarcoma, immune responsiveness.

Introduction

Electrochemotherapy is an antitumor treatment that utilizes electric pulses to increase drug delivery into cells and tissues.[1] Short intense electric pulses transiently and reversibly increase plasma membrane permeability without impairing cell viability.[2–6] The increased plasma membrane permeability enables non-permeant drugs to diffuse into the cells to reach their intracellular targets and thus exert their cytotoxicity. For example, *in vitro* cytotoxicity of bleomycin, netropsin, methotrexate, actinomycin D and cisplatin is potentiated several fold by exposing cells to electric pulses.[7–10]

In preclinical studies the feasibility of electrochemotherapy *in vivo* was demonstrated on a variety of tumors in different strains of mice. The treatment is performed by application of electric pulses to the tumor by percutaneously placed electrodes several minutes after systemic or local drug administration. Electric pulses as single treatment do not exert an antitumor effect and do not induce side effects. Electrochemotherapy with the chemotherapeutic drugs bleomycin or cisplatin proved to be effective in local tumor control, inducing partial and complete responses of the tumors.[10–21] Electrochemotherapy with bleomycin requires a low amount of bleomycin which is ineffective without electric pulses and therefore does not induce side effects. Preclinical data on electrochemotherapy with bleomycin were confirmed in the first clinical trials on cutaneous and s.c. tumor nodules of squamous cell carcinoma, basal cell carcinoma and malignant melanoma.[22–25]

Studies on electrochemotherapy with bleomycin that were performed on the tumors induced in immunocompetent and immunodeficient mice demonstrated that immune responsiveness of the organism is involved in obtaining cures.[14] In addition, it was demonstrated that a higher cure rate of the tumors was obtained by stimulation of T-dependent immune responsiveness after electrochemotherapy with bleomycin.[15] Furthermore, since cell electropermeabilization is a universal phenomenon occuring in all types of living cells,[6] and bleomycin generates highly cytotoxic DNA double-strand breaks when introduced into the cells,[8] all histological types of tumors should respond to electrochemo-

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Correspondence to G Serša

therapy at approximately the same level. However, different levels of responsiveness of different tumors in mice treated with the same electrochemotherapy protocol were demonstrated. This different level of tumor responsiveness to electro-chemotherapy might be also due to different levels of tumor immunogenicity.[20]

Although preclinical data indicate that immune responsiveness is involved in obtaining cures of the tumors after electrochemotherapy with bleomycin, it is not known how electrochemotherapy affects the immune system of the organism. The aim of the study was to determine the effects of electrochemotherapy with bleomycin on natural resistance and immune responsiveness.

Materials and methods

Animals and tumor model

In the experiments, an inbred strain of A/J mice of both sexes was used, purchased from Rudjer Bošković Institute (Zagreb, Croatia). They were maintained at constant room temperature (24°C) with a natural day/night light cycle in a conventional animal colony. Before the experiments, the mice were subjected to an adaptation period of at least 10 days. Mice in good condition, without fungal or other infections and 10–12 weeks of age, were included in experiments.

In the study, fibrosarcoma SA-1 tumor (Jackson Laboratory, Bar Harbour, ME) syngeneic to A/J mice was used. Tumor cells were obtained from the ascitic form of the tumors in mice, serially transplanted every 7 days. Solid s.c. tumors, located dorsolaterally, were initiated by an injection of 5×10^5 SA-1 cells in 0.1 ml 0.9% NaCl solution. The viability of the cells was over 95% as determined by the Trypan blue dye exclusion test. Six days after transplantation when the tumors reached approximately 40 mm^3 in volume, mice were randomly divided into experimental groups and subjected to a specific experimental protocol on day 0.

Electrochemotherapy protocol

Bleomycin (Mack, Jllertissen, Germany) was injected i.v. in a bolus into the lateral tail vein of the mice. The dose used was 5 mg/kg (approximately 100 μg per mouse) and was well tolerated by the mice. Electric pulses were delivered by two flat, parallel stainless steel electrodes 8 mm apart (two stainless steel strips: length 35 mm, width 7 mm with rounded corners) and placed percutaneously at the opposite margins of the tumor. Good contact between the electrodes and the skin was assured by means of a conductive gel. Eight square-wave pulses of 1040 V amplitude, with a pulse width of 100 μs and repetition frequency of 1 Hz, were generated by an electropulsator Jouan GHT 1287 (Jouan, St-Herblain, France). In the electrochemotherapy protocol, mice were treated with electric pulses 3 min after bleomycin injection. Treatments were performed without anesthesia and were well tolerated by the mice.

Tumor growth was followed by measuring three mutually orthogonal tumor diameters (e_1, e_2 and e_3) with a caliper on each consecutive day. Tumor volumes were calculated by the formula $V = \Pi \times e_1 \times e_2 \times e_3/6$. From these measurements, the arithmetic mean and standard error of the mean (SE) were calculated for each experimental group. The doubling time was determined for each individual tumor and the tumor growth delay (GD) from the mean doubling time of experimental groups.[10] The response to electrochemotherapy was scored according to WHO guidelines as: (i) progressive disease if tumors increased in size, (ii) no change if tumors reduced in size by less than 50%, (iii) partial response if the size of the tumor was reduced by more than 50% and (iv) complete response if they became unpalpable. The growth curves of electrochemotherapy treated tumors were presented for tumors that regrew separately from those that were in complete response up to 120 days after the treatment.

Determination of natural resistance

Natural resistance was evaluated by phagocytic activity and ability to elicit oxidative burst by production of toxic oxygen radicals of the monocytes and polymorphonuclear granulocytes. Cells were obtained from the venous blood, drawn from the retro-orbital sinus of the mice.

Phagocytic activity was tested with a Phagotest (Becton Dickinson-Orpegen, Heidelberg, Germany). Heparinized blood was mixed with FITC-labeled *Escherichia coli* cells in plastic test tubes. Negative control tubes were incubated at 0°C and testing tubes at 37°C for 10 min. Then phagocytosis was stopped and quenching performed. Erythrocytes were lysed, and monocytes and polymorphonuclear granulocytes washed. Monocytes or polymorphonuclear granulocytes were gated out on a FACSort

flow cytometer (Becton Dickinson, Mountain View, CA) and the percentage of FITC *E. coli* positive cells was calculated.

Ability to elicit oxidative burst by production of toxic oxygen radicals of monocytes and polymorphonuclear granulocytes in venous blood was determined with a Burst test (Becton Dickinson-Orpegen). Heparinized blood was mixed with opsonized *E. coli* cells in plastic test tubes and incubated for 10 min at 37°C. Then, dihydrorhodamine-123 (DHR-123) was added and cells were incubated for another 10 min at 37°C. Erythrocytes were lysed and monocytes and polymorphonuclear granulocytes washed. Monocytes or polymorphonuclear granulocytes were gated out on the flow cytometer and the percentage of rhodamine-123 (R-123) positive cells was calculated. Two measured parameters were evaluated in analysis, i.e. percentage of cells able to elicit oxidative burst and the main peak of this activity.

Determination of immune responsiveness

Immune responsiveness was evaluated by blast transformation tests performed on mononuclear cells isolated from spleens. Spleens were aseptically removed from the mice and a single cell suspension prepared by squeezing the spleens between two sintered glass plates and washing the cells with Hank's balanced salt solution. Erythrocytes were lysed by incubating the cell suspension for 3 min in buffered 0.83% NH_4Cl at room temperature and mononuclear cells collected from suspension with centrifugation. Mononuclear cells were counted in a hemocytometer and prepared as 1×10^6 cells/ml suspension in RPMI 1640 medium (Sigma, St Louis, MO) supplemented with 10% heat inactivated fetal calf serum (Sigma), penicillin, streptomycin and 5×10^{-5} M mercaptoethanol (Sigma). These cells were then used in the blast transformation test. Tests were performed in 96-well flat-bottom microtiter plates (Costar, Baldhoevedorp, The Netherlands). The mononuclear cell suspension (100 μl) was pipetted to different concentrations of concanavalin A (Con A) (0.94–15 μg/ml, Sigma), lipopolysaccharide (LPS) (12.5–200 μg/ml; Sigma) and pokeweed mitogen (PWM) (1.7–26.7 μg/ml; BioChrom, Berlin, Germany). After 72 h of incubation at 37°C, 5% CO_2 and 95% humid atmosphere, cultures were tritiated with 50 μl (18 kBq) [^{3}H]thymidine (NEN, Les Ulis, France) for 4 h. Cells were collected on glass fiber filters and radioactivity was measured in a liquid scintillation counter (Pharmacia, Upssala, Sweden).

Statistical analysis

Differences between median values of experimental groups were evaluated with the Mann–Whitney *U*-test for comparison of the two groups, and the Kruskal–Wallis ANOVA and median test for comparison of more than two groups. A level of $p < 0.05$ was taken as indicative of significant differences.

Results

Antitumor effectiveness of electrochemotherapy

Antitumor activity of electrochemotherapy with bleomycin was tested on s.c. SA-1 tumors in mice. Treatment with bleomycin and electric pulses as single treatments induced moderate antitumor effect (0.2 ± 0.1 and 1.3 ± 0.2 days GD, respectively). However, combined treatment with electric pulses 3 min after i.v. injection of bleomycin proved to be very effective; 52% of the tumors were in complete response for 120 days and were therefore termed cured. The remaining 10 tumors regrew with GD 34.0 ± 3.7 days after approximately 10 days in partial response (Figure 1).

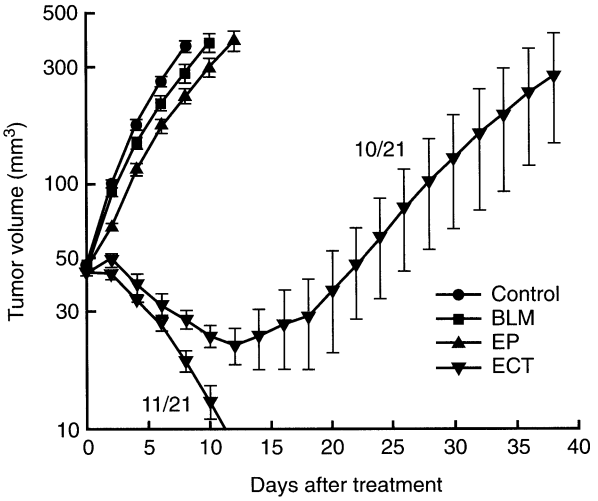

Figure 1. Antitumor effectiveness of electrochemotherapy (ECT) with bleomycin (BLM) on s.c SA-1 tumors. Mice were treated with 100 μg bleomycin i.v. and/or eight electric pulses (EP) 3 min after treatment with BLM. Tumor growth curves represent the arithmetic mean ± SE of the tumor volumes measured every second day. The growth curves of ECT-treated tumors are presented for tumors that regrew separately from those that were in CR up to 120 days after the treatment.

Natural resistance and immune responsiveness

Natural resistance and immune responsiveness of the mice without tumors, tumor-bearing mice which were not treated, and mice treated with electric pulses, bleomycin and electrochemotherapy were evaluated 7 days after the treatment. Besides, in the electrochemotherapy group the natural resistance and immune responsiveness was also evaluated 14 days after treatment. These time points were selected according to the antitumor effectiveness of the electrochemotherapy observed in the previous experiment; 7 days after the treatment tumors were progressively diminishing, while 14 days after the treatment antitumor effectiveness was the most pronounced and approximately 50% of the animals were expected to be cured.

Natural resistance was evaluated by determining the phagocytic activity and ability to elicit oxidative burst by production of toxic oxygen radicals of monocytes and polymorphonuclear granulocytes after electrochemotherapy (Figure 2). Phagocytic activity of monocytes and polymorphonuclear granulocytes did not change in tumor-bearing mice compared to mice without tumors. Also, the treatment of tumor-bearing mice with electric pulses, bleomycin and electrochemotherapy did not affect the phagocytic activity of monocytes and polymorphonuclear granulocytes. However, the percentage of monocytes able to elicit oxidative burst by production of toxic oxygen radicals measured by the burst test was increased 7 days after the electrochemotherapy treatment and returned to normal values 14 days after the electrochemotherapy. In contrast, no significant increase was observed in polymorphonuclear granulocytes. The intensity of microbicidal activity of these monocytes and polymorphonuclear granulocytes, measured by peak channel intensity, was not significantly changed.

Immune responsiveness was evaluated by blast transformation tests of spleen mononuclear cells after electrochemotherapy (Figure 3). Blast transformation of T lymphocytes after Con A stimulation of spleen cells was not changed in tumor-bearing mice compared to mice without tumors. T lymphocyte activity was not affected either after bleomycin, electric pulses and electrochemotherapy treatment on day 7. In contrast, T lymphocyte activity was increased 14 days after electrochemotherapy treat-

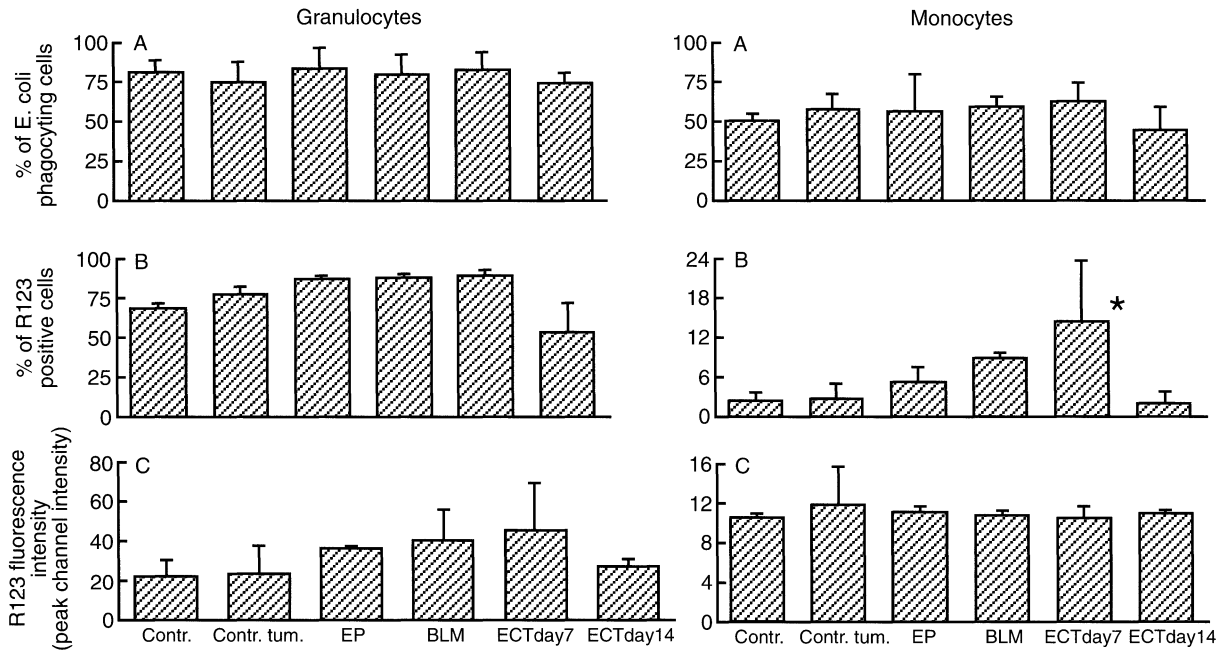

Figure 2. Activity of monocytes and polymorphonuclear granulocytes was determined in venous blood of electrochemotherapy (ECT)-treated mice on day 7 and 14 after treatment. All the pertinent controls were measured on day 7 after treatment. (A) Phagocytic activity of the cells was measured by determining the percentage of FITC *E. coli* positive cells (Phagotest; Becton Dickinson-Orpegen). (B) Ability to elicit oxidative burst by production of toxic oxygen radicals was measured by the percentage of R-123 positive cells (Burst test; Becton Dickinson-Orpegen). (C) The vigorousity of the oxidative burst in the cells was measured by determining the peak channel intensity of R-123 fluorescence. Results are expressed as medians, bars are 75th percentiles, from quadruplicates. $^{*}p < 0.05$.

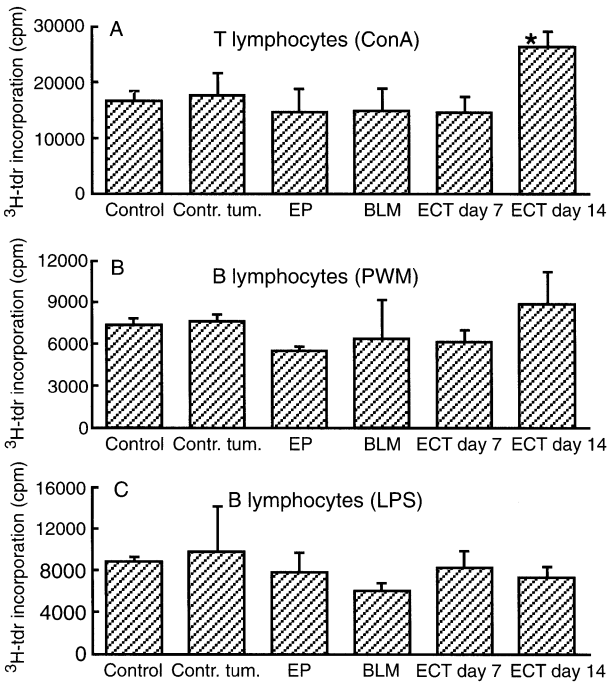

Figure 3. Activity of lymphocytes was evaluated by blast transformation tests of spleen mononuclear cells on day 7 and 14 after electrochemotherapy (ECT). All the pertinent controls were measured on day 7 after treatment. Incorporation of [^{3}H]thymidine was measured after: (A) blast transformation of T lymphocytes after Con A stimulation, and (B and C) blast transformation of B lymphocytes after PWM and LPS stimulation. Results are expressed as medians, bars are 75th percentiles, from quadruplicates. $^*p < 0.05$.

ment. Blast transformation of B lymphocytes after PWM and LPS stimulation of spleen mononuclear cells was not changed in tumor-bearing mice compared to mice without tumors, and also after bleomycin, electric pulses and electrochemotherapy treatment.

Discussion

The results of the study show that electrochemotherapy with bleomycin increases a monocyte's ability to elicit oxidative burst by production of toxic oxygen radicals and T lymphocyte's activity in SA-1 tumor-bearing mice.

From the previous studies it is known that immune responsiveness of the organism is important for obtaining cures after electrochemotherapy. All preclinical studies on electrochemotherapy with bleomycin demonstrated that a higher percentage of tumors were cured in immunocompetent mice with transplantable and spontaneous tumors than in immunodeficient nude mice.[11,14,15] For example, 30% of mice were cured after electrochemotherapy with bleomycin on the LPB mouse sarcoma tumors induced in immunocompetent C57Bl/6 mice, whereas only some partial responses were obtained in immunodeficient *nu/nu* mice with a Swiss background.[14] These results demonstrated that the host's immune responsiveness is important in the elimination of the tumor cells that remained viable after electrochemotherapy treatment and could eventually regrow into the tumors.

Electropermeabilization may be performed in all types of living cells.[2] In addition, bleomycin is a potent cytotoxic drug, which exerts its activity already in minor quantities, once inside the cells.[8] Therefore, it is expected that all histological types of tumors should be sensitive to electrochemotherapy with bleomycin. This was demonstrated in many experimental as well as clinical situations. However, in the study performed on three different tumor types in mice with the same treatment protocol, some degree of variability in the responsiveness of the tumors was observed.[20] In that study, it was demonstrated that a very high percentage of cures was achieved on fibrosarcoma SA-1 tumors (62%), whereas there was a much lower percentage on B-16 melanoma (5%) and Ehrlich ascites tumors (19%). For this reason, in this study we selected the SA-1 tumor for evaluation of electrochemotherapy induced changes in the immune system. This difference in tumor responsiveness might be due to many factors; however, one possibility could be that the response is correlated with the immunogenicity of the tumors. Therefore, it must be taken into account that not all tumors growing in immunocompetent hosts are equally responsive to electrochemotherapy with bleomycin and that a higher percentage of cures can be achieved by stimulation of the host's immune response in immunogenic tumors.

In our experiments we found that T lymphocyte activity was increased 14 days after electrochemotherapy, demonstrated by blast transformation of mononuclear spleen cells by Con A. These results are in accordance with the previously published observations that curability of tumors after electrochemotherapy is dependent on T lymphocytes.[15,26] First, in mice treated with OKT_3 monoclonal antibodies which induce a transient immunosuppression by causing the depletion of mature T lymphocytes, only 20% of tumors were cured after electrochemotherapy, whereas in immunocompetent mice 60% or more of tumors were cured.[15] Second, stimulation of the immune responsiveness of the organism, either

by i.p. IL-2 injection or by peritumorally injected IL-2-secreting cells, increased the percentage of tumors cured with electrochemotherapy with bleomycin.[15,26] In addition, adjuvant treatment with IL-2 secreting cells induced 50% cure of untreated contralaterally established tumors of the same origin.[26] Furthermore, $CD4^+$ and $CD8^+$ T lymphocytes were observed in the tumors treated with electrochemotherapy and IL-2.[26]

To our knowledge, all the studies on electrochemotherapy with bleomycin have only evaluated T lymphocyte-mediated immune responsiveness. However, in this study we demonstrated that monocytes also are involved in the defense of the organism treated with electrochemotherapy at least on day 7. This was demonstrated by the ability of monocytes to elicit oxidative burst, an important phase in antimicrobial activity. The percentage of monocytes able to elicit oxidative burst by production of toxic oxygen radicals returns to normal values 14 days after electrochemotherapy. This increased activity of monocytes is important, since they are involved in non-specific tumor cell destruction and in stimulation of other components of the immune system of the organism.

Conclusions

The results of our study demonstrate that electrochemotherapy with bleomycin increases a monocyte's ability to elicit oxidative burst by production of toxic oxygen radicals and T lymphocyte activity in SA-1 tumor-bearing mice. This information may be used in planning adjuvant immunotherapy to electrochemotherapy, separately for stimulation of natural resistance and for immune responsiveness.

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