Tumor Growth Retardation, Cure, and Induction of Antitumor Immunity in B16 Melanoma-bearing Mice by Low Electric Field-enhanced Chemotherapy[1]

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ABSTRACT

Purpose: **The exposure of cells *in vitro* to trains of low voltage-pulsed electric fields in the range of 20–100 V/cm was previously shown to induce an efficient uptake of macromolecules with molecular weight in the range of M_r 300–2,000,000 via an endocytic-like process. This study examines the antitumor effectiveness of treatment based on similar exposure of solid tumors in mice to low electric fields (LEFs) in the presence of chemotherapeutic agents.**

Experimental Design: **LEF was applied to ~5 mm in diameter (60–70 mm^3) s.c. B16-F10.9 melanoma tumors by percutaneously placed electrodes after intratumoral injection of either *cis*-platinum(II) diamminedichloride, Taxol, 5-fluorouracil, or bleomycin.**

Results: **Significant eradication of primary tumors, prolongation of survival, and complete cure of some of the C57Bl/6 mice from both primary tumors and metastases were achieved using this technique with *cis*-platinum(II) diamminedichloride, bleomycin, and Taxol (13.5, 8, and 26% cure rate, respectively). Mice cured by LEF-enhanced chemotherapy and challenged with a tumorigenic dose of B16-F10.9 cells lived significantly longer than first time inoculated ones, and 23.5% of the challenged mice did not develop tumors at all. Spleen cells from the cured mice that were inoculated together with B16-F10.9 cells inhibited the primary tumor growth in intact mice. Histological analysis of tumor sections of LEF-enhanced chemotherapy-treated mice revealed multiple necrotic areas, apoptosis, and massive infiltrates of T lymphocytes and macrophages. Low voltage electrochemotherapy with Taxol was shown to be more effective than surgical removal of the tumor with Taxol.**

Conclusions: **These findings indicate that LEF-enhanced chemotherapy is an effective treatment of animals bearing metastatic melanoma.**

INTRODUCTION

Successful treatment of solid tumors by chemotherapy depends on the effective penetration of the therapeutic agent into the target cells of the tumor. To achieve this, the blood-borne chemotherapeutic agent enters the tumor vasculature and reaches the cancer cells via distribution through the vascular compartment, transport across the microvascular wall, and transport through the interstitial compartment toward the cancer cells. The final step of this overall process is the penetration of the cytotoxic agents into the cells through the plasma membrane. To overcome the permeability barrier of the cell membrane for nonpermeant drugs, a treatment of solid tumors that combines a cytotoxic drug with locally delivered permeabilizing electric pulses has been previously applied (1). This treatment, known as ECT[3], uses short and intense electric pulses that transiently and reversibly permeabilize the cells through the process of electroporation. Electroporation is generally defined as formation of transient hydrophilic pores by induction of a high transmembrane potential difference (>200 mV) after the exposure of cells to microsecond pulsed electric fields of intensity in the range of 300-3000 V/cm (2). The effectiveness of incorporating agents into cells by electroporation is limited by the short lifetime and the diameter of the electrically induced aqueous pores. ECT-based clinical trials of melanoma, adenocarcinoma, basal cell carcinoma, Kaposi's sarcoma, and squamous cell carcinoma have been reported previously (3, 4).

It was previously demonstrated that exposure of cells to trains of LEFs (in the range of 20–100 V/cm) leads to efficient uptake of macromolecules with molecular weight in the range of M_r 300–2,000,000 into cells (5). The uptake of macromolecules does not proceed through electroporation but through an endocytic-like mechanism. Therefore, this electrically induced endocytosis in combination with intratumoral injection of chemotherapy can be used to effectively incorporate antineoplastic drugs into the cells of the solid tumor as a new modality of cancer therapy.

In this study, we demonstrate that exposure of B16-F10.9

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[3] The abbreviations used are: ECT, electrochemotherapy; LEFCT-EC, low electric field-enhanced cancer chemotherapy; LEF, low electric field; LEF-chemotherapy, LEF in combination with chemotherapy; cisplatin, *cis*-platinum(II) diamminedichloride; RT-PCR, reverse transcription-PCR; IL, interleukin; 5-FU, 5-fluorouracil; MST, mean survival time.

melanoma tumors in mice to low pulsed electric fields combined with local intratumoral injection of chemotherapeutic agents is an effective treatment. We coined this new methodology as LEFCT-EC.

MATERIALS AND METHODS

Animals. C57BL/6 male mice were obtained from the breeding colony of Tel Aviv University (Tel Aviv, Israel). Mice were used at the ages of 8–12 weeks. Animal care and experimentation was carried out in accordance with Tel Aviv University guidelines.

Tumor Cell Line and *in Vivo* Tumor Growth. B16-F10.9, a highly metastatic subclone of the B16-melanoma, was kindly provided by Dr. Lea Eisenbach (Weizmann Institute of Science, Rehovot, Israel). Cells were maintained in supplemented DMEM as described previously (6). Tumor cells (10^5/100 μl of PBS) were injected s.c. on the left side of the back toward the rear end of the mouse.

LEF Cancer Therapy Protocol. Mice were subjected to a single treatment of LEFCT-EC, once the tumor reached the size of 5 mm in diameter (60–70 mm^3; 11–17 days after tumor cells inoculation). A volume not exceeding 100 μl of the chemotherapeutic agents was injected into the tumor loci. The exposure to electric field was carried out 3–4 min after intratumoral injection of the chemotherapeutic agents. To expose tumors to electric fields, we used stainless steel electrode needles (Karlsbader insect pins No 0; BioQuip Products, Rancho Dominquez, CA), soldered at their brassy ends with thin isolated copper wires. The electrodes were arranged in a triangle with the cathode in the middle and three anodes around it. The distance between the anodes and the cathode was 5 and ~9 mm between the anodes. The tumor was contained within this triangle, and normal tissue was included in the treatment field. The needles penetrated ~7 mm percutaneously into and near the immediate vicinity of the tumor, and they were connected to an electric pulse generator (Grass S48 Stimulator). The electric parameters (previously found as optimal for *in vitro* uptake of molecules) were the same in all of the experiments: field strength, 40 V/cm; repetition frequency, 500 Hz; and pulse width, 180 μs. In most of the experiments, the animals were exposed to the electric stimulus for 12 min. Mice treated with either LEF alone or LEF-chemotherapy were anesthetized before treatment.

Reagents. The following anticancer drugs were used in this study.

Cisplatin (no. P-4394; Sigma, Rehovot, Israel) was dissolved in PBS before injection. Taxol (Mead Johnson Oncology Products, A Bristol-Myers Squibb, Princeton, NJ) was obtained as a sterile nonpyrogenic solution, which contains 6 mg/ml paclitaxel, 527 mg/ml purified Cremophor EL (polyoxyethylated castor oil) and 49.7% (v/v) dehydrated ethanol. Bleomycin (MegaPharm, Hod Hasharon, Israel), and 5-FU (Abic, Netanya, Israel) were dissolved in PBS.

RNA Isolation and RT-PCR. Spleens (three/group) were sterilely ectomized 7 days after the appropriate treatment (~20 days after tumor inoculation). The spleen cells were prepared, counted, and taken for RNA isolation. RNA was extracted using Tri Reagent (no. TR118; Molecular Research Center Incorporated, Cincinnati, OH). Reverse transcription and PCR were performed using reverse transcription system kit (no. A3500; Promega, Madison, WI). RNA extraction and subsequent reverse transcription and PCR were conducted according to the protocols provided by the companies manufacturing the kits. PCR products were run in 1.5% agarose gel. The gels were photographed. Absorbance measuring of the bands of amplified c-DNA was performed using the program "Tina 2.10*g*." The ratios of cytokine absorbance to appropriate actin absorbance were compared, and the value obtained in normal mice splenocytes was considered as 100%.

DNA primers of the cytokines of interest (IL-2, IL-4, IFN-γ) and of β-actin were purchased from BioTechnology General (Rehovot, Israel).

The sequences of DNA primers for PCR were: IL-2 5′ primer-GACACTTGTGCTCCTTGTCA and 3′ primer-TCAATTCTGTGGCCTGCTTG; IL-4 5′ primer-TCGGCATTTTGAACGAGGTC and 3′ primer-GAAAAGCCCGAAAGAGTCTC; IFN-γ 5′ primer-AACGCTACAGACTGCATCT and 3′ primer-TGCTCATTGTAATGCTTGG; and β-actin 5′ primer-ATGGATGACGATATCGCT and 3′ primer-ATGAGGTAGTCTGTCAGGT.

Other reagents used were oleum ricini (Floris, Nesher, Israel), ethanol (BioLab, Jerusalem, Israel), eosin alcoholic with methanol (Pioneer Research Chemicals, Colchester, United Kingdom), Harris's hematoxylin (Pioneer Research Chemicals), buffered formaldehyde solution (no. 5551830; Frutarom, Haifa, Israel), xylenes (no. 5554180; Frutarom), methylsalicylate (Merck, Darmstadt, Germany), isopropanol for RNA isolation (Merck), 1-bromo-3-chloropropan for RNA isolation (no. BP-151; Molecular Research Center Incorporated), agarose (no. V3121; Promega), and tris-borate-EDTA buffer (no. 065841; Amresco, Solon, OH).

Anesthesia. Mice were treated by LEF, LEF-chemotherapy, and surgery after i.p. injection of an anesthetic mixture composed of imalgen (100 mg/kg) and xylazine hydrochloride (6.25 mg/kg). The anesthetic compounds were dissolved in PBS and injected 0.2–0.25 ml/mouse.

Statistical Analysis. The survival time plotting (Kaplan-Meir test), survival comparison between groups (Mantel-Cox test), and tumor volume differences between groups (Kolmogorov-Smirnov test) were carried out using StatSoft Statistica statistical software. The mortality rate 5 days after LEF or LEF-chemotherapy was 10%. These cases of mortality were considered as treatment caused and therefore excluded.

Tumor Volume Determination. Tumor growth was followed by measuring the three mutually orthogonal tumor diameters (Dx, Dy, and Dz). The volume (V) was calculated using the formula ($V = \pi X\ Dx \times Dy \times Dz/6$).

Histology. Antibodies used for immunohistochemistry were rat antimouse CD3 (no. MCA1477; Serotec, Raleigh, NC), rat antimouse F4/80 antigen (no. MCA497; Serotec), and the monoclonal antibody F7-26 (no. 804-192-L001; Alexis, San Diego, CA). Tumors with a diameter of 10–12 mm were used for histological examination. The tumors were removed, placed in buffered formaldehyde solution for 24 h, and then transferred to 70% ethanol. The tissues were processed by the standard paraffin technique, and 5-μm slices positioned on slides were stained with H&E for histopathological examination. To detect T lymphocytes, macrophages, and to visualize apoptosis in the

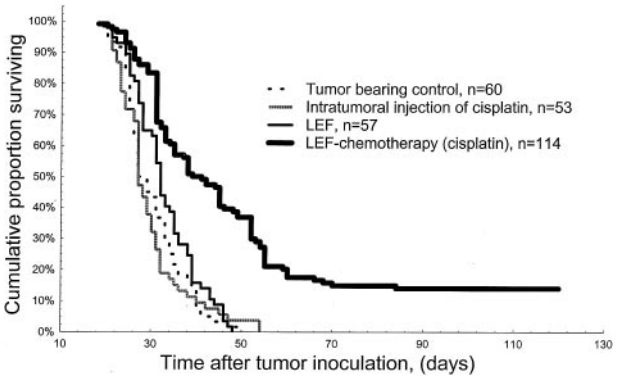

Fig. 1 Survival of C57BL/6 mice, bearing B16-F10.9 melanoma, after LEFCT-EC with intratumoral cisplatin administration. Mice bearing 60–70 mm^3 s.c. melanoma were treated with cisplatin (4 mg/kg) intratumorally and/or with electric stimulation (procedure duration, 12–20 min; field strength, 40 V/cm; repetition frequency, 500 Hz; pulse width, 180 μs). The results are plotted using Kaplan-Meir technique (n, number of animals/group).

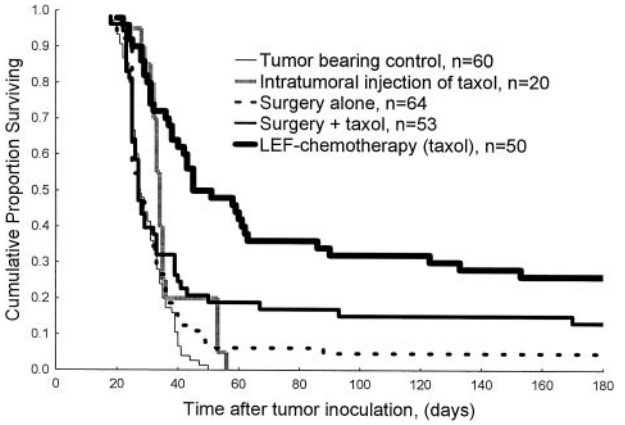

Fig. 2 Survival of C57BL/6 mice, bearing B16-F10.9 melanoma, after LEFCT-EC with intratumoral Taxol administration. Mice bearing 60–70 mm^3 s.c. melanoma were treated either with Taxol (20 mg/kg) alone or Taxol in combination with electric stimulation (procedure duration, 12 min; field strength, 40 V/cm; repetition frequency, 500 Hz; pulse width, 180 μs). In two other groups, the tumors were surgically removed, and in one group, the mice received 20 mg/kg Taxol s.c. near the site of excision. The results are plotted using Kaplan-Meir technique (n, number of animals/group).

tumor sections, we performed immunostaining with anti-CD3, anti-F4/80, and F7–26 antibodies, respectively. Monoclonal antibodies F7-26 were generated against calf thymus single-stranded DNA and selected on the basis of reactivity with mouse apoptotic cells. F7-26 antibodies specifically bind deoxycytidine and require for binding single-stranded DNA of at least 25–30 bases in length. By this way, F7-26 can distinguish between apoptotic and necrotic cells. The immunostaining of paraffin-embedded tumors sections was accomplished according to the protocols provided by the companies manufacturing the antibodies.

The following experimental groups were used: (*a*) nontreated tumor-bearing mice; (*b*) tumor-bearing mice treated with the chemotherapeutic drug intratumorally; (*c*) tumor-bearing mice treated with LEF; and (*d*) tumor-bearing mice treated with chemotherapeutic drug intratumorally and LEF (LEF-chemotherapy).

RESULTS

Effect of LEFCT-EC on the Survival of B16-F10.9-bearing Mice Using Various Chemotherapeutic Agents

In the first phase of this study, the electric pulse treatment was given in combination with various chemotherapeutic agents. Cisplatin, Taxol, bleomycin, and 5-FU were used in this study.

Antitumor Effect of LEFCT-EC with Cisplatin. We tested the duration of the electric stimulation in LEFCT-EC procedure, required for optimal results. Cisplatin (4 mg/kg body weight) was injected intratumorally followed by electrostimulation for 12, 15, or 20 min. There were no significant differences between the groups subjected to the different exposure periods. Yet, the mortality rate within a few days after the treatment was higher in the groups that were exposed to electric fields for 15 or 20 min compared with the ones exposed for only 12 min. Thus, we selected the shortest exposure period for the trials.

The LEF-chemotherapy treatments with intratumoral application of 4 mg/kg cisplatin, resulted in a significant increase in the survival of the LEF-chemotherapy treated mice, as compared with the other treatment and control groups (Fig. 1). The MST (MST ± SE) of LEF-chemotherapy-treated mice (150 days after tumor inoculation) was 51.1 ± 2.8 days, whereas nontreated tumor-bearing animals had a mean survival of 30.3 ± 0.9 days. Chemotherapy alone and electrostimulation alone yielded survival time of 29.5 ± 1.1 and 32.8 ± 1.0 days, respectively. Moreover, in the LEF-chemotherapy group, 13.5% of the animals were disease free 150 days after tumor inoculation. A Mantel-Cox statistical analysis showed a significant improvement ($P < 0.000004$) in the survival of LEF-chemotherapy-treated mice, compared with all other groups. Neither cisplatin nor electrostimulation alone had any significant effect.

Antitumor Effect of LEFCT-EC with Taxol. Similar experiments were performed with paclitaxel as a chemotherapeutic agent (20 mg/kg intratumorally). The results presented in Fig. 2 show that chemotherapy alone had no effect on the survival rate. The cure rate in the LEF-chemotherapy with Taxol group reached 26% 180 days after tumor inoculation ($P = 0.00021$, LEF-chemotherapy *versus* Taxol). The mean survival of LEF-chemotherapy-treated mice was 83.5 ± 9.0 days, and chemotherapy alone 36.7 ± 2.0 days. It was noticed that at the site of LEF-chemotherapy treatment tumor destruction occurred, and necrotic tissue was formed. In mice where the primary tumor disappeared (49%), a healing process was evident, with regrowth of the hair at that site (Fig. 3).

Because paclitaxel was dissolved in castor oil and ethanol, we tested the effect of LEF stimulation in a control group that was also injected intratumorally with 100 μl of a mixture consisting of oleum ricini + ethanol (1:1 v/v) before the electrostimulation. This mixture was selected to imitate the commercial solvent of paclitaxel, which consists of Cremophor EL (polyoxyethylated castor oil) and dehydrated ethanol in a 1:1

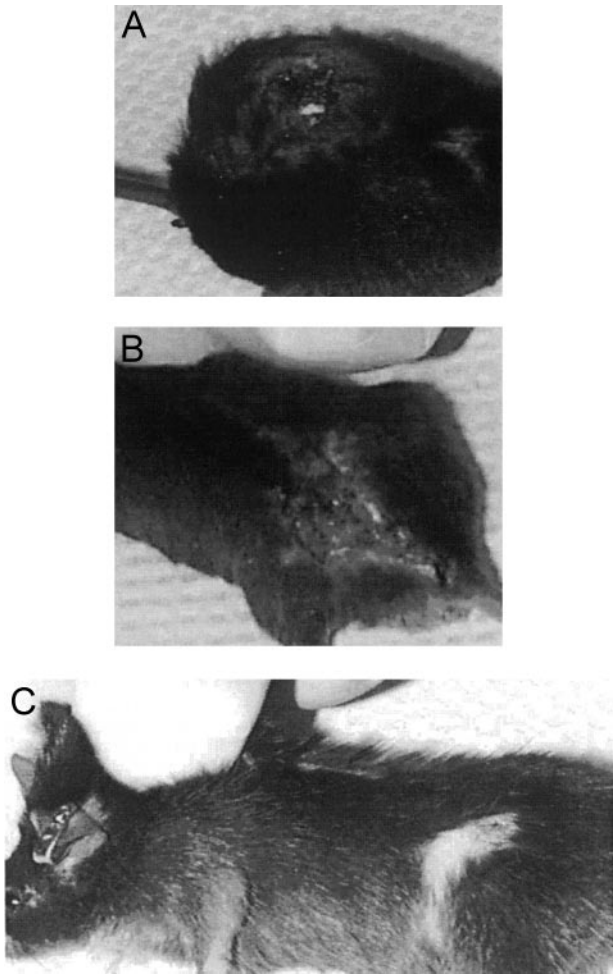

Fig. 3 C57BL/6 mice, bearing B16-F10.9 melanoma, after LEFCT-EC with intratumoral Taxol administration. Mice bearing 5 mm in diameter (60–70 mm^3) s.c. melanoma were treated with Taxol (20 mg/kg) in combination with LEF stimulation (procedure duration, 12 min; field strength, 40 V/cm; repetition frequency, 500 Hz; pulse width, 180 μs). *A*, nontreated tumor-bearing mouse; *B*, LEFCT-EC-treated mouse 2 weeks after treatment; and *C*, LEFCT-EC-treated mouse 2 months after treatment.

ratio. In this control group, the MST was 59.1 ± 6.8 days, and 13.5% of the mice were tumor free 150 days after tumor inoculation.

Comparison of Surgery and LEFCT-EC. Next, LEFCT-EC efficacy in comparison to conventional surgery and chemotherapy treatments was evaluated. C57BL/6 male mice (age of 8–12 weeks) were injected with 10^5 B16-F10.9 cells s.c. Once the tumor reached the size of 5 mm in diameter (11–17 days after inoculation), it was surgically removed. Some of the mice received a single dose of Taxol (20 mg/kg) s.c. in close proximity to the site of the operation 1 day after tumor resection. The results presented in Fig. 2 indicate that <5% of mice were cured by surgery alone, and the use of Taxol in addition to surgery increased the cure rate to 13%. MST ± SE of surgery and surgery plus Taxol groups was 37 ± 4 and 55 ± 7 days, respectively.

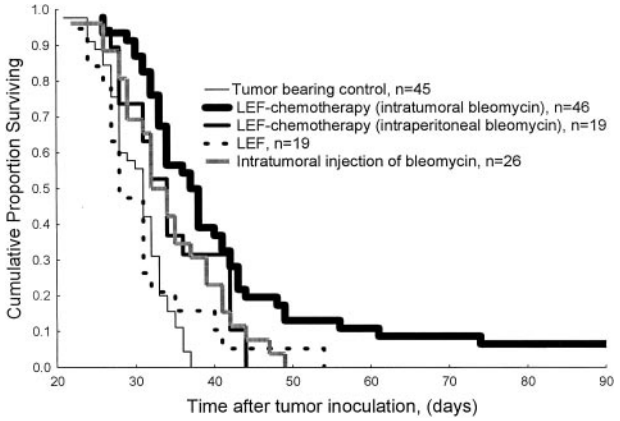

Fig. 4 Effect of the different methods of bleomycin administration on the survival of C57BL/6 mice, bearing B16-F10.9 melanoma, after LEFCT-EC. Mice bearing 60–70-mm^3 s.c. melanoma were treated with bleomycin (8 units/kg) intratumorally or i.p. and/or with electric stimulation (procedure duration, 12 min; field strength, 40 V/cm; repetition frequency, 500 Hz; pulse width, 180 μs). The results were plotted using Kaplan-Meir technique (n, number of animals/group).

It is evident that LEF-chemotherapy treatment resulted in a significantly better effect in all tested parameters (complete cure rate of 26% and MST of 84 ± 8 days), when compared with both surgical groups (LEF-chemotherapy *versus* surgery plus Taxol, $P = 0.0013$).

Histological Examination of Tumors. To characterize some of the factors involved in the efficient primary tumor destruction after LEF or LEF with Taxol, tumors were ectomized at different times after treatment. Tumors were prepared and stained by H&E, as well as reacted with anti-CD3, anti-F4/80, and F7-26 antibodies. Swollen cells with condensed nuclei were observed 3–4 h after LEF and LEF-chemotherapy. Most noticeable necrotic lesions with infiltrate were seen 48–72 h after LEF-chemotherapy. Massive infiltration of T lymphocytes and macrophages, as compared with the untreated tumors, was observed 48–72 h after the treatment (data not shown). Three to 4 h after LEF or LEF-chemotherapy, infrequent apoptotic regions were detected by immunostaining with F7-26 antibody. Two to 3 days after LEF-chemotherapy or LEF alone, apoptotic cells became rare or absent in some of the sections. In the untreated tumors, apoptotic cells were not found (data not shown).

Antitumor Effect of LEFCT-EC with Bleomycin. A dose of 8 units/kg body weight of bleomycin was used for these experiments. The survival of mice treated with the different protocols is shown in Fig. 4. Nontreated mice had a MST of 30.2 ± 0.6 days, and those treated with electric field alone possessed MST of 31 ± 1.7 days. Mice, which were treated with bleomycin alone, had a MST of 34.3 ± 1.3 days, whereas LEF-chemotherapy, with intratumoral injection of bleomycin, resulted in a notable prolongation of the MST to 47 ± 5 days (180 days after tumor inoculation). Furthermore, in the LEF-chemotherapy group, 8.2% of the animals were alive and free of a visible tumor 180 days after tumor inoculation. Mantel-Cox analysis revealed a statistically significant difference between survival of mice in the LEF-chemotherapy group compared with

Table 1 Effect of treatment on tumor growth

Treatment[b]	Volume (mean ± SE, mm^3)[a]			
	Cisplatin	Bleomycin	5-FU	Taxol
LEF-chemotherapy	83 ± 57	573 ± 138	143 ± 52	71 ± 25
Chemotherapy	1684 ± 204	2456 ± 220	460 ± 54	927 ± 177
LEF	914 ± 323	1792 ± 250	588 ± 143	126 ± 34[c]
Untreated tumor-bearing animal	4972 ± 358	5823 ± 604	4802 ± 549	5078 ± 535

[a] Tumor size was measured 7 days after treatment (18–24 days after tumor inoculation) and 21 days after tumor inoculation in the nontreated tumor-bearing mice. The results are expressed as mean ± SE of measurements taken from 25–60 mice/group.
[b] B16-F10.9 melanoma-bearing mice were treated by LEF-chemotherapy with cisplatin (4 mg/kg), bleomycin (8 units/kg), 5-FU (150 mg/kg), and Taxol (20 mg/kg) by chemotherapy alone or LEF alone or not treated.
[c] In this group, a mixture of oleum ricini with ethanol was injected intratumorally.

Table 2 Effect of LEFCT-EC on eradication of the primary tumor[a]

	Percentage tumor-free animals		
	Cisplatin	Bleomycin	Taxol
Animals that died	16	12	23
Animals that survived	13.5[b]	8[c]	26[c]
Total	29.5	20	49

[a] B16-F10.9 melanoma-bearing mice were treated by LEF-chemotherapy with cisplatin (4 mg/kg), bleomycin (8 units/kg), or Taxol (20 mg/kg). The results were calculated from groups of 25–60 animals/group.
[b] Determined 150 days after tumor inoculation.
[c] Determined 180 days after tumor inoculation.

control ($P = 0.00001$) and LEF ($P = 0.00011$) groups, and significantly different from the chemotherapy-treated one ($P = 0.021$).

We also tested the significance of the mode of drug injection on the efficacy of LEFCT-EC. Mice with 5-mm diameter tumors received bleomycin (8 units/kg) either intratumorally or i.p. The results presented in Fig. 4 reveal a longer MST of the LEF-chemotherapy group that received the chemotherapy intratumorally (47 ± 5 days), as compared with the LEF-chemotherapy treated by i.p. injection (35 ± 1.4 days).

Antitumor Effect of LEFCT-EC with 5-FU. LEFCT-EC with 5-FU (150 mg/kg) was not beneficial in term of life expectancy prolongation of treated mice. The only positive effect achieved was the inhibition of primary tumor growth (Table 1).

Effect of LEFCT-EC on Elimination and Size Reduction of the Primary Tumors

To assess the effect of LEFCT-EC on tumor development, we also recorded the rate of destruction of the primary tumor and the average size of these tumors. The primary tumor was eliminated in some of the mice treated by LEFCT-EC with either cisplatin (29%), Taxol (49%), or bleomycin (20%; Table 2). A certain proportion of the mice cured of their primary tumors died >50 days after tumor implantation. These mice that died without recurrence of the tumor at the primary site developed lung metastases as revealed by autopsy (16, 23, and 12% for cisplatin, Taxol, and bleomycin, respectively). Mice treated by LEFCT-EC in which the primary tumor disappeared and did not die from a metastatic disease at least 150 days after tumor inoculation were considered completely cured (13, 26, and 8% for cisplatin, Taxol, and bleomycin, respectively; Table 2). In comparison, treatment by surgery and Taxol cured 20% of the animals from their primary tumor, and only 13% were completely cured. All of the animals treated with LEF or chemotherapy alone died with a primary tumor that was either not destroyed or regrew at the site of treatment.

Tumor volume was recorded seven days after treatment (18–24 days after tumor inoculation; Table 1), and mice with untreated tumors were measured on the 21st day. In each group tumor volume value was obtained as an average of 25–60 tumors. In all LEF-chemotherapy treatments, tumors were much smaller than in control groups (LEF-chemotherapy *versus* LEF, $P < 0.01$ for cisplatin, 5-FU, and bleomycin groups, and $P < 0.05$ for Taxol group). The most notable effect on the primary tumor was achieved by LEFCT-EC with Taxol. The solvent of Taxol (mixture of oleum ricini with ethanol) in combination with LEF stimulation had also a very strong effect on the primary tumors and reduced tumor size by 40-fold in comparison with the untreated tumors.

The Development of an Antitumoral Reaction after LEFCT-EC

It was of interest to determine whether mice, cured by LEF-enhanced chemotherapy, were rendered resistant to a tumorigenic dose of B16-F10.9 melanoma cells. For this purpose, mice that were cured by LEFCT-EC and survived for 120–180 days after initial tumor inoculation were injected with 2×10^5 B16-F10.9 cells s.c. The results presented in Fig. 5 show that cured mice exhibited a significantly prolonged survival as compared with first-time inoculated normal mice. It can be also observed that mice cured by LEFCT-EC with Taxol were rendered significantly more resistant to the challenge, as compared with the animals cured by LEFCT-EC with cisplatin ($P < 0.035$). MST ± SE of first-time inoculated normal mice was 31.3 ± 0.8, whereas mice cured by LEFCT-EC with either cisplatin or Taxol and subsequently challenged with tumor cells survived for longer time periods (48.7 ± 6.1 and 73.2 ± 11.0 days, respectively). Moreover, ~23.5% (4 of 17) of the mice, cured earlier by LEFCT-EC with Taxol, rejected the second tumor inoculation.

The possible development of an immune-mediated antitumoral activity, after LEFCT-EC, was also tested by the Winn assay (7). Mice (six/group) received injections of a

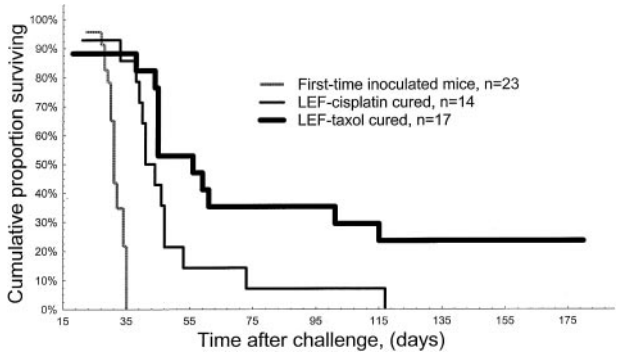

Fig. 5 Resistance of mice, previously bearing B16-F10.9 melanoma and cured by LEFCT-EC with cisplatin or Taxol, to subsequent inoculation of B16-F10.9 melanoma cells. Mice cured by LEF-chemotherapy that survived for 150–180 days after initial tumor inoculation were challenged with 2×10^5 B16-F10.9 cells s.c. The results were plotted using Kaplan-Meir technique (n, number of animals/group).

mixture of B16-F10.9 cells and splenocytes from either LEF-chemotherapy-treated mice or normal mice. The mice for Winn assay were taken 2 months after tumor inoculation. The cells were injected in a proportion of 1:100 (10^5 B16-F10.9 cells $+10^7$ spleen cells) s.c. The tumor volume, 14 days after inoculation, in mice that received spleen cells from LEF-chemotherapy-cured mice was significantly lower than in the control group (286 $\pm$ 125 *versus* 847 $\pm$ 164 mm^3, $P = 0.023$). Although the median survival times were 30 and 25 days in LEF-chemotherapy-cured and control groups, respectively, they were not statistically different.

Cytokines m-RNA Expression by Spleen Cells after LEF Impact

To characterize possible systemic immunological reactions after LEF action, we examined m-RNA expression of major cytokines (IL-2, IL-4, IFN-γ) by spleen cells of treated animals. This was performed using semiquantitative RT-PCR technique. There were five experimental groups: (*a*) normal untreated mice group; (*b*) tumor-bearing mice group; (*c*) intratumoral 20 mg/kg Taxol group; (*d*) LEF group; and (*e*) LEF-chemotherapy with intratumoral 20 mg/kg Taxol group. The results of three separate experiments are summarized in Table 3. In tumor-bearing mice, elevated IL-2 mRNA expression was evident, as compared with normal mice. After LEF treatment, cytokine mRNA expression increased, but LEF-Taxol treatment resulted in a lower cytokine mRNA expression than after LEF only, probably attributable to the suppressive effect of Taxol. However, because of high fluctuations in the results, the differences were not statistically significant.

DISCUSSION

In view of previous findings that indicated increased uptake of molecules by cells exposed to LEFs (5), we tested the efficacy of LEFs in augmenting the capacity of chemotherapy to destroy tumors *in vivo*. Implementation of LEF or intratumoral chemotherapy, separately on B16-F10.9 melanoma tumors, only slightly extended the survival of C57BL/6 mice. However, combining LEF and chemotherapy (LEFCT-EC) not only destroyed the primary tumors but resulted in complete cure of 13.5% of the mice with cisplatin, 8% with bleomycin, and 26% with Taxol (animals alive and free of primary tumors and metastases 150–180 days after tumor inoculation).

Intratumoral injection of bleomycin showed better results with LEFCT-EC than i.p. one. Evidently, a higher concentration of the drug in the tumor tissue caused higher entry into the tumor cells, additionally augmented by the implementation of LEF pulses. However, a combination of LEFCT-EC with 5-FU was not beneficial, presumably because of the lipophilic character of 5-FU, which easily permeate into cells. Thus, its concentration in the cells cannot be significantly enhanced by the LEF. Evidently, the pattern of response to LEFCT-EC depends on the chemotherapeutic agents and route of administration.

Cured mice developed resistance to a subsequent challenge with a tumorigenic dose of B16-F10.9 melanoma cells. The mice cured by LEFCT-EC, which did not develop tumor within 6 months after the first tumor cell challenge, were rendered sensitive to a second B16 melanoma challenge after treatment with cyclosporin A, which causes immunosuppression by T cells' function elimination (data not shown). Spleen cells from LEFCT-EC-cured mice slowed down tumor growth when injected together with B16-F10.9 cells. B16-F10.9 is known as a moderately immunogenic and highly metastatic clone of B16 melanoma. When the primary tumor reaches a diameter of 5 mm, micrometastases are already present in the lungs of these mice (6). Therefore, the existence of long-time survivors and antitumoral resistance of the cured mice indicate that LEFCT-EC induces immune-mediated antitumoral activity that destroys residual disease cells, both at the primary tumor site and in metastatic foci.

Another indication for immunostimulation induced by LEF treatments was the finding that destruction of the tumors by LEF treatment resulted in an increase in the expression of m-RNA of IL-2, IL-4, and IFN-γ by spleen cells of treated animals 1 week after treatment. Cytokine m-RNA expression in the LEF-treated group was always higher then in normal mice and nontreated

Table 3 Effect of treatment on cytokine m-RNA expression by spleen cells[a]

	Normal	TB	Taxol	LEF	LEF-chemotherapy
IL-2	100 ± 57	165 ± 99	99 ± 52	390 ± 254	204 ± 109
IL-4	100 ± 37	101 ± 47	67 ± 24	152 ± 32	74 ± 33
IFN-γ	100 ± 48	89 ± 32	26 ± 19	131 ± 7	47 ± 21

[a] RT-PCR for mRNA of the indicated cytokines was performed in splenocytes derived from normal mice (normal), untreated tumor-bearing mice (TB), TB mice treated with Taxol (Taxol), TB mice treated with LEF (LEF), and TB mice treated by LEF with Taxol (LEF-chemotherapy). The values (mean ± SE) are ratios of cytokine absorbance to the appropriate actin absorbance from three separate experiments. The value of normal mice splenocytes was taken as 100%.

tumor-bearing mice. In mice treated by LEF-chemotherapy (LEFECT-EC), cytokine mRNA expression was higher than in mice treated by Taxol only but lower than in LEF-treated mice. The lower levels of cytokines in the combination of LEF and Taxol are probably because of the immunosuppressive action of Taxol.

We suggest that the LEF-mediated destruction of the tumor triggered a particular excited state, probably by common innate and adaptive immune reactions to the damaged tumor tissue. This notion is supported by the findings of Feng *et al.* (8) who reported that stressed apoptotic tumor cells are better than nonstressed ones in the induction of antitumor immune responses.

The involvement of immunological reactions in LEFCT-EC-induced antitumoral mechanism was additionally supported by histological examinations. Histological tumor specimens revealed that LEFCT-EC-treated tumors underwent massive necrosis after a transient step of apoptotic cell death, and massive infiltration of T cells and macrophages was evident inside the tumor. It was also observed that the destruction of the tumors by LEF treatment was augmented when combined with oleum ricini (the control for the Taxol solvent, Cremophor EL), which exhibited immunostimulatory properties (13.5% cure; Refs. 9, 10). LEF alone had only a marginal effect. This fact indicates that tumor destruction by electric pulses, with or without chemotherapy, may be greatly improved by appropriate immunoadjuvants.

We pointed out previously the potential of immunostimulation when combined with chemotherapy in the treatment of metastatic tumors (6). In this earlier study, we showed that immunostimulation after surgery and chemotherapy can cure mice with metastatic B16 melanoma. A major advantage of LEFCT-EC demonstrated in this article, besides the direct destruction of the primary tumor, is the ability to trigger immune antitumoral responses, and in that respect, it shows a big improvement relative to surgery combined with chemotherapy. LEFCT antitumor immunization capability involves probably mechanisms such as a more efficient exposure of tumor-associated antigens in the destroyed tumor mass and improved accessibility of immune cells to tumor antigens or others. Nevertheless, LEFCT might benefit from additional immunostimulation, and we currently are testing this approach.

In view of the use of ECT as an anticancer treatment, we compared the effects of LEFCT-EC and ECT on B16 melanoma in mice. Mir *et al.* (11) used ECT with belomycin and reported elimination of the primary tumor in 3 of 11 animals, with 1 survivor for 200 days. Sersa *et al.* (12) treated small B16 melanoma tumors (40 mm^3 in volume) with ECT and bleomycin and cured 1 mouse of 20. Sersa *et al.* (12) treated B16 melanoma-bearing mice (40 mm^3) with ECT and cisplatin and reported a median survival time of 41 days (chemotherapy), 44 days (electroporation), and 59 days (ECT), with 14% cure by ECT after 100 days. Jaroszski *et al.* (13) treated B16 melanoma (0.5–0.75 cm in diameter) with ECT and bleomycin using stainless steel plates, with no long-term survivors after a single treatment. Heller *et al.* (14) treated 6–8 mm (in diameter) B16 tumors with ECT, using steel plates and belomycin, with no long-term survivors. High-voltage ECT by itself was not reported to be immunostimulatory. Only when combined with plasmids coding for granulocyte macrophage colony-stimulating factor or IL-2 it was reported to induce long-term antitumor immune response and resistance to a tumor challenge in up to 25% of the mice cured from B16 melanoma (16).

Another electrostimulation-based anticancer treatment was suggested by Nordenstrom (17). This method, termed electrochemical treatment, uses a constant voltage of <10 V direct current for up to 60 min. Treatment of lung cancer patients by this method resulted in short-term cure of 26% of the patients (18).

The effect of electric field on living tissue is multifactorial and complex. Tumor destruction may be a combination of direct cell damage and antivascular effects. We assume that exposure of tumors to low-pulsed electric field in the presence of chemotherapeutic agents in the extracellular compartment may increase the incorporation of the agents into the cytosol. This is expected to increase tumor cell destruction and may render the tumoral mass more antigenic and more accessible to the host's immune response. Activation of inflammatory and immune responses might facilitate the recognition and elimination of tumor cells at primary as well as metastatic tumor sites. High efficacy of LEFCT-EC was also confirmed by the comparison to the traditional surgery and chemotherapy treatment. LEFCT-EC was significantly better than surgery with Taxol in this experimental model. Thus, LEFCT-EC may prove as a safe treatment modality to be used in clinical cancer applied therapy, especially for profoundly localized tumors and for cases where organ or tissue preservation is of major concern.

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