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EFFECT OF ELECTRIC-FIELD INTENSITY ON ELECTROPERMEABILIZATION AND ELECTROSENSITIVITY OF VARIOUS TUMOR-CELL LINES *IN VITRO*

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ABSTRACT

Electropermeabilization (electroporation) is a technique widely used to introduce various membrane-impermeable molecules into cells *in vitro* or *in vivo*. In this study we determined the effect of different electric-field intensities on electropermeabilization and electrosensitivity of a variety of tumor-cell lines *in vitro*. For this purpose we used two assays: propidium iodide uptake for measurement of cell electropermeabilization, and the clonogenic or MTT assay for determination of electrosensitivity. Our results showed that electropermeabilization of almost all cell lines tested occurred at 600 V/cm. In contrast, a marked difference in electrosensitivity existed among these cell lines. Our results could be of great importance for pharmacological and biochemical studies *in vitro*, and for prediction and determination of tumor response *in vivo* to electropermeabilization combined with chemotherapeutic drugs (electrochemotherapy) and gene therapy.

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INTRODUCTION

Electropermeabilization (electroporation) is a technique widely used to introduce different types of exogenous membrane-impermeable molecules into cells *in vitro* or *in vivo* (1). A single electric pulse or a train of electric pulses in the range of a few kV/cm and at a pulse duration of several microseconds causes a reversible permeabilization of the plasma membrane, which forms the basis of this technique (2). Electropermeabilization was first introduced in 1972 by Neumann, who observed changes in the permeability of vesicular membranes that were induced by electrical pulses (3). Electropermeabilization can be either reversible or irreversible, depending on the electrical parameters chosen (1,4,5). Electropermeabilization is now used routinely in many laboratories for electrotransfection, because of its reproducibility and efficiency as compared with other viral and chemical methods. In addition, electropermeabilization is used for electrofusion (2). Recently, this technique was successfully applied for electrochemotherapy, in which it is used as a system for enhancing the delivery of chemotherapeutic drugs into tumors, to increase their antitumor effectiveness (6–13).

Despite a number of studies determining (optimizing) electrical parameters for cell electropermeabilization, little is known about individual responses of different cells to electric pulses (14–16). It has been postulated that optimal electrical parameters for electropermeabilization of cells must be determined empirically for each cell line (15). Although several parameters influence the *in vitro* efficiency of electropermeabilization, such as the nature of the medium in which cells are subjected to electric pulses, pulse duration, and number of pulses, the critical parameter for electropermeabilization is electric field intensity, which must be higher than a critical threshold value (14, 16,17). In the present study we determined the effect of different electric-field intensities on the electropermeabilization and electrosensitivity of a variety of tumor-cell lines. For this purpose we used two assays: propidium iodide uptake for the measurement of electropermeabilization, and the clonogenic or tetrazolium-based colorimetric assay (MTT assay) for determination of electrosensitivity of cells.

MATERIALS AND METHODS

Cell Lines

In the study, 11 different tumor-cell lines and 1 transformed cell line were used. Characteristics of the cell lines are presented in Table 1. MM46T (American Type Culture Collection [ATCC] No. CRL-6423), B16F0 (ATCC No. CRL-6323), B16F1 (ATCC No. CRL-6322), MCF7 (ATCC No. HTB-22), HeLa (generously provided by M. Osmak of the Institute Rudjer Boskovic, Zagreb, Croatia), SA-1 (Jackson Laboratory, Bar Harbor, ME), TBL.C12, TBL.C12 PT, and DC3F cells (generously provided by J. Belehradek Jr. of the Institute Gustave Roussy, Villjuif, France) were grown in Eagle's minimal essential medium (EMEM; Sigma Chemical Co., St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS; Sigma). EAT cells (ATCC No. CCL-77) were grown in NTCT 135 medium (Gibco BRL, Life Technologies, Galthersburg, MD) supplemented with 15% FCS, and IGROV 1 cells (generously provided by J. Bernard of the Institute Gustave Roussy) were grown in RPMI 1640 medium (Sigma) supplemented with 15% FCS. The resistant subclone of IGROV 1 cells, IGROV 1/DDP cells, were grown in RPMI medium supplemented with 15%

Table 1. Cell-Line Characteristics

Name	Species	Type
DC3F	Chinese hamster	Fibroblast (lung)
IGROV 1	Human	Carcinoma (ovary)
SA-1	Mouse	Fibrosarcoma
MCF7	Human	Carcinoma (breast)
B16F0	Mouse	Melanoma
TBL.Cl2	Mouse	Sarcoma
TBL.Cl2 PT	Mouse	Subclone of TBL.CL2 cells resistant to CDDP
HeLa	Human	Carcinoma (cervix)
IGROV 1/DDP	Human	Subclone of IGROV 1 cells resistant to CDDP
B16F1	Mouse	Melanoma
MM46T	Mouse	Sarcoma
EAT	Mouse	Carcinoma

FCS and 1 μg/ml cisplatin (Platinol, Bristol Myers Squibb, Wien, Austria). All cells were kept at 37°C in a humidified atmosphere with 5% CO_2, and were routinely subcultured once or twice per week, depending on the growth rate. For the experiments, cells in the exponential growth phase were used. All cells were grown in monolayers.

Determination of Electropermeabilization and Electrosensitivity

Electropermeabilization as well as electrosensitivity were determined as described previously (8). Briefly, electropermeabilization of the plasma membrane was measured by means of propidium iodide uptake, and electrosensitivity was measured by colony-forming assay or MTT assay (18). Cells from the exponential growth phase were prepared and were trypsinized and washed twice at 4°C, first in medium corresponding to that for the particular cells, supplemented with 10% FCS for inactivation of trypsin (Sigma), and then in serum-free medium supplemented with 0.5 mM $CaCl_2$. The cell suspension (2.2×10^7 cells/ml in 67.5 μl) was mixed with 7.5 μl propidium iodide (100 μM; Sigma) for measurement of propidium iodide uptake, or with medium supplemented with 0.5 mM $CaCl_2$ for colony-forming assay or MTT assay. Each of these mixtures (50 μl) was placed between two flat, parallel, stainless-steel electrodes (length = 6 mm, width = 6 mm, interelectrode distance = 2 mm) and subjected to 8 square-wave electric pulses (pulse width = 100 μs, repetition frequency = 1 Hz) of different electric-field intensities, ranging from 100 to 1800 V/cm. After exposure of cells to electric pulses, the cells were incubated for 5 min at room temperature (24°C). To measure the propidium iodide uptake, 25 μl of cell suspension was resuspended in 1 ml of 0.01 M phosphate-buffered saline (PBS) (pH 7.4) and analyzed immediately with a FACSort (Becton Dickinson, Mountain View, CA) flow cytometer. The percentage of stained cells was determined in comparison with control cells that were not subjected to electric pulses.

Electrosensitivity of cells was determined by means of either the colony-forming assay or MTT assay. The electrosensitivity of IGROV 1, IGROV 1/DDP, MCF7, B16F0, B16F1, TBL.Cl2, TBL.Cl2 PT, HeLa, DC3F, and MM46T cells was determined by

means of the colony-forming assay, and that of the other cell lines (SA-1 and EAT) by means of the MTT assay. For the colony-forming assay, cells exposed to electric pulses were diluted and seeded in quadruplicate in 60-mm Petri dishes (Costar, Badhoevedorp, The Netherlands). After 7–14 days, depending on the cell line used, colonies were fixed, stained with Crystal violet (Kemika, Zagreb, Croatia), and counted. Colonies containing fewer than 50 cells were disregarded. The survival of cells treated with electric pulses was presented as the percentage of colonies obtained from the untreated control cells. For the MTT assay, cells were diluted and seeded in 96-well microtiter plate (Costar). After 3 days, MTT dye ([3-(4,5-dimethylthiazol-2-yl)=2,5-diphenyl-formazan bromide]; Sigma) was added and the cells were further incubated for 3 h. Thereafter, the medium was aspirated and the formed formazan crystals were dissolved in 100 μl dimethyl sulfoxide (Sigma). Absorbance of the solutions was measured with a microplate reader (Anthos, Salzburg, Austria) at 570 nm. The survival of cells treated with different electric-field intensities was presented as the percentage of the absorbance obtained with the untreated control cells. Three independent experiments were performed for each cell line.

Electrosensitivity of cells was characterized with the electrosensitivity index (EI), which was calculated by the formula, $EI = 1/(IF_{50} \times 2r)$, where IF_{50} is the electric-field intensity (V/cm) at which cell survival is reduced by 50%, and 2r is the cell diameter (cm) (15).

Determination of Cell Size

To determine cell size, cells were trypsinized and resuspended in a solution composed of PBS, NaCl, KH_2PO_4, EDTA, and bovine serum albumin (BSA) (Sigma) in order to preserve cellular integrity. Smears prepared from these samples were air-dried and stained with Giemsa staining solution (Kemika). The diameter of the cells was obtained by means of a Cyto-Savant cell image analyzer (Oucometrics Technol. Corp. Vancouver, Canada). Because of the fixation by air-drying, the cell diameters were on average 15% larger than they would have been for living cells in suspension as measured with, for example, a light microscope equipped with an ocular scale. However, the relative differences in diameters of different cells were retained.

RESULTS

Electropermeabilization and electrosensitivity of various tumor-cell lines were determined with respect to electric-field intensity. Eight electric pulses of 100 μs duration at a frequency of 1 Hz and a field strength of 100–1800 V/cm were used in our experiments. Typical results of propidium iodide uptake by the cells, as visualized with flow cytometry, are presented in Figure 1. The amount of propidium iodide in the cells increased with increasing electric-field intensity. Electropermeabilization and electrosensitivity of cell lines are shown in Figure 2. From the data obtained, it was evident that different cells respond differently to the same treatment with electric pulses. Electropermeabilization in the tested cell lines was achieved at electric-field intensities of 400–600 V/cm. At 800 V/cm, the percentage of permeabilized cells among almost all cells studied reached a maximal value. The percentage of electropermeabilized cells did not reach 100% in any of the cell lines tested, indicating that a small proportion of cells could not be permeabilized. On the other hand, the electrosensitivity of the cell

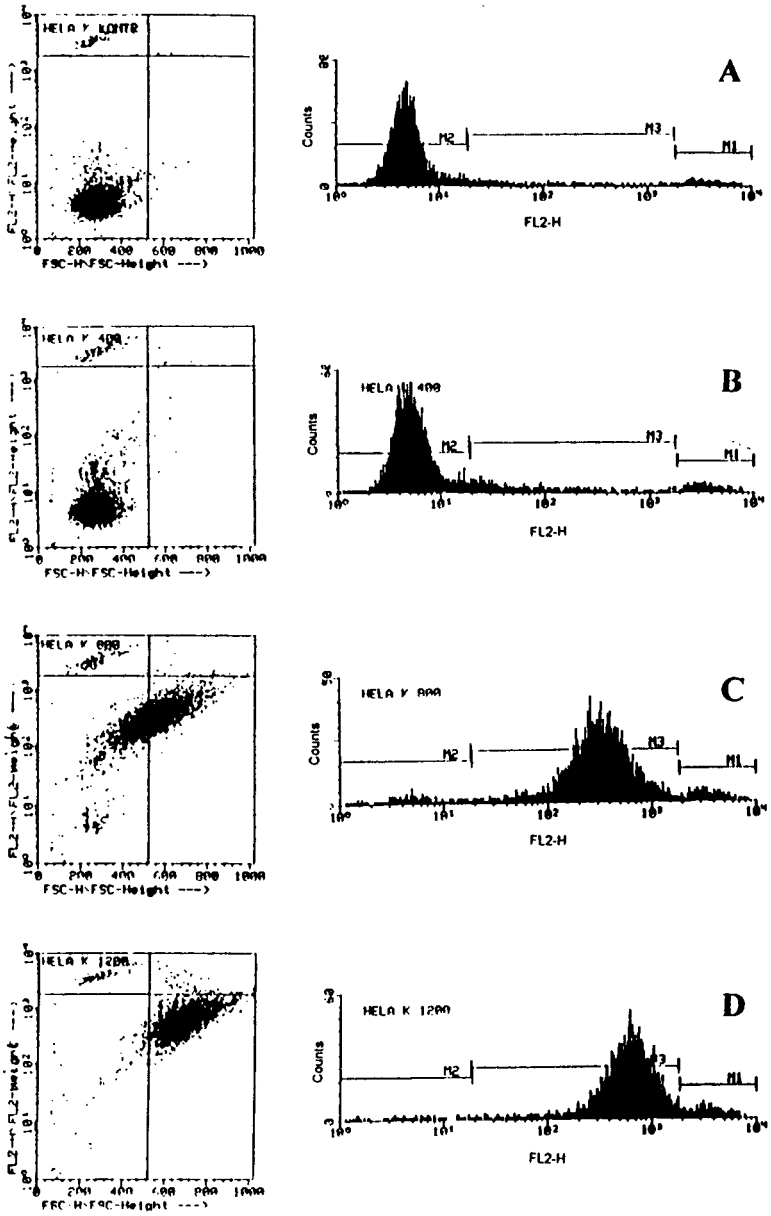

FIGURE 1. Electropermeabilization of HeLa cells visualized through flow cytometry. Cells without (A = control) or exposed to electric pulses (B = 400 V/cm, C = 800 V/cm, D = 1200 V/cm) were incubated with propidium iodide. Red fluorescence (FL-2), representing cell uptake of propidium iodide, was measured. Fluorescence cell sorting (FCS) parameter represents cell magnitude. Cell cluster in upper left quadrant (A = control) represents cells that were dead before exposure to electric pulses.

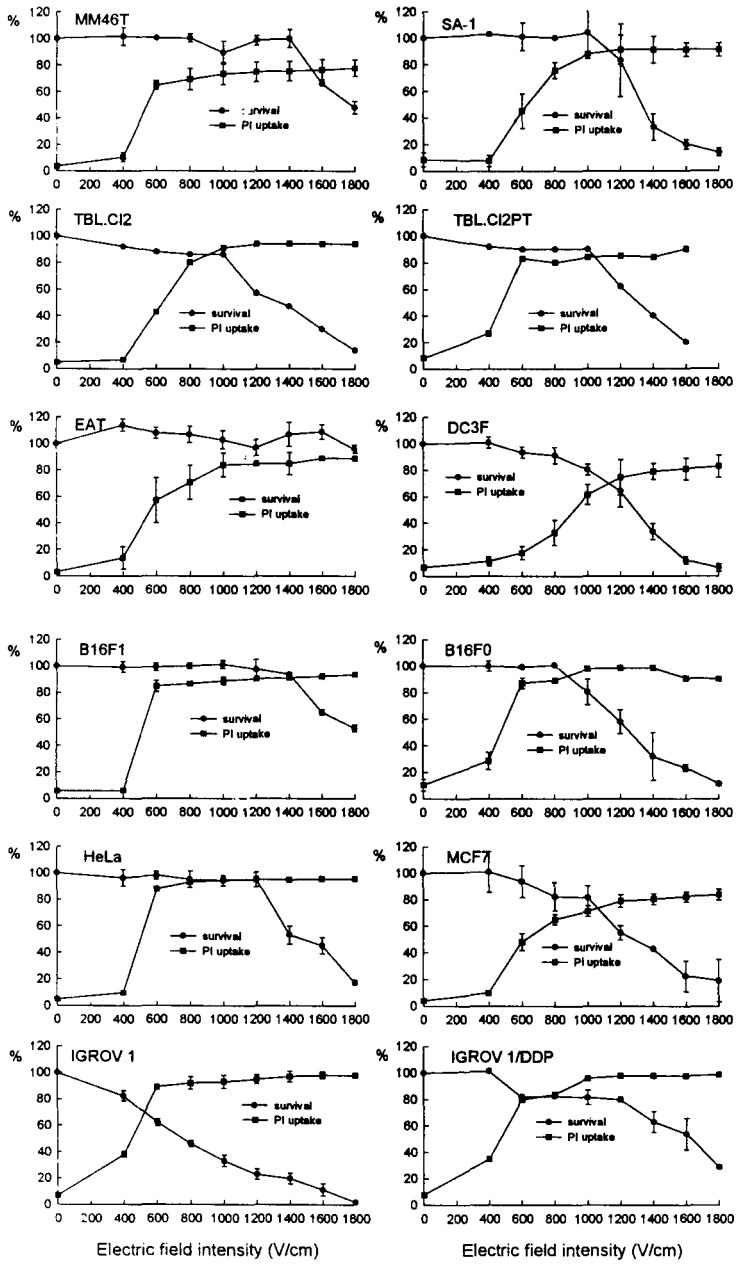

FIGURE 2. Electropermeabilization and electrosensitivity of cell lines. Electropermeabilization was determined by means of propidium iodide (PI) uptake, and electrosensitivity was determined by survival of cells as measured by means of clonogenic or 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Data points represent the mean values of 3 independent experiments with standard error bars.

Table 2. Electrosensitivity of Tumor Cells*

Name	IF_{50}† (V/cm)	24 ($\times 10^{-4}$ cm) AM ± SE‡	EI§ (V–1)
IGROV 1	750	19.8 ± 1.8	0.68
SA-1	1320	12.8 ± 1.7	0.59
DC3F	1300	18.6 ± 0.5	0.41
MCF7	1280	20.6 ± 2.0	0.38
B16F0	1360	19.7 ± 2.5	0.37
TBL.Cl2	1340	20.4 ± 2.0	0.37
TBL.Cl2 PT	1310	21.0 ± 1.5	0.36
HeLa	1450	21.5 ± 2.9	0.32
IGROV 1/DDP	1640	20.3 ± 2.1	0.30
B16F1	1850	18.6 ± 1.3	0.29
MM46T	1780	20.8 ± 1.9	0.27
EAT	ND¶	21.2 ± 6.5	ND¶

*Cell lines are ordered according to their electrosensitivity index, from most to least electrosensitive cells.
†IF50 = electric-field intensity at which cell survival was reduced by 50%.
‡AM ± SE = arithmetic mean ± SEM.
§EI = electrosensitivity index.
¶ND = could not be determined, due to the technical limitations of the electropulsator.

lines tested differed substantially (Table 2). The most sensitive cells were IGROV 1 cells and the most resistant cells were EAT carcinoma cells. For the latter cells, IF_{50} could not be determined because of technical limitations of the electropulsator. Since the threshold for electropermeabilization was almost the same for all cell lines tested, the optimal range of electric-field intensities at which the cells were permeabilized and remained viable was dependent only upon the electrosensitivity of the cells. There were also differences in electrosensitivity between subclones of B16 melanoma cells, with subclone B16F0 more sensitive than subclone B16F1. The electrosensitivity of parental and resistant TBL.Cl2 cells was the same, in contrast to the electric-field intensity at which the maximal number of cells were permeabilized. The electric-field intensity at which this value was achieved was 600 V/cm for cells resistant to cisplatin, and 1000 V/cm for parental cells. The parental and resistant subclones of IGROV 1 cells showed the opposite effect: the threshold value for electropermeabilization was the same for both, while parental cells were more electrosensitive than the resistant ones, and the slope of the survival curve of parental IGROV 1 cells was not as steep as that of resistant ones.

DISCUSSION

In this study we determined the effects of different electric-field intensities on the electropermeabilization and electrosensitivity of various tumor cell lines *in vitro*. We found that the threshold value of electric-field intensity for electropermeabilization

was almost the same for all cell lines tested, and was between 400 and 600 V/cm. However, different types of tumor cells exhibited different electrosensitivities.

Since the threshold value for electropermeabilization was almost the same for all the cell lines tested, the electrosensitivity of cells is the parameter that was responsible for the observed differences in the response of cells to electric pulses. The electrosensitivity of cells is therefore also responsible for determination of the optimal range of electric-field intensities at which the plasma membrane is permeabilized but cells remain viable. According to the Laplace equation, which predicts that the voltage at the surface of a cell is directly proportional to the cell diameter, smaller cells should have a greater threshold value for electropermeabilization (2). However, our results with propidium iodide uptake did not support this relationship, since all cells had a threshold value between 400 and 600 V/cm. Therefore, the differences in electrosensitivity of cells could not be attributed to the threshold value for electropermeabilization. Furthermore, the EI of cells showed that some of the smallest cells (SA-1 sarcoma) were very sensitive and one of the largest types of cells, EAT cells, were insensitive to treatment with electric pulses. O'Hare *et al.* have also shown marked differences in responses of several types of mammalian cells to electropermeabilization (15). Our results are in accordance with their study, and further support the statement that the effects of electric pulses on cells must be determined empirically, since they could not be predicted on the basis of currently known parameters. In contrast to the study by O'Hare *et al.*, we did not find that electrosensitivity was dependent on tissue type. They observed that lymphoid cells were the most electrosensitive, followed by epithelial and fibroblastic cells, while endothelial cells were the least electrosensitive. In our study, cells of the same tumor type were either very sensitive (SA-1 sarcoma, IGROV 1 carcinoma) or insensitive (MM46T sarcoma, IGROV 1/DDP carcinoma). The important observation is also that a majority of cells exhibited an EI index between 0.41 and 0.27 V^{-1}. However, the most sensitive cells—IGROV 1 carcinoma and SA-1 fibrosarcoma cells—had almost twice the IE index of other cells. The reason for this observation could not be attributed either to the cell size or to tissue type, indicating that other cell properties also influence the electrosensitivity of cells.

In conclusion, our data on the effects of electric-field intensities on electropermeabilization and electrosensitivity of various tumor-cell lines could be useful for biochemical studies of cells, such as studies of intracellular physiology involving second messengers, cellular structure, and biochemical pathways, in which cells must remain functional. In addition, our data also form the basis for studies of intracellular mechanisms of action of cytotoxic molecules, for investigation of the role of the plasma membrane in the effects of cytotoxic drugs (resistance studies), and for *in vitro* the screening of drugs with potential use in electrochemotherapy, as well as for prediction and determination of tumor response to electrochemotherapy and gene therapy *in vivo*.

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