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Electropermeabilization of cells in tissues assessed by the qualitative and quantitative electroloading of bleomycin

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Abstract

Using cells in suspension, electropermeabilization is a technique extensively used to transfect living cells or to introduce a variety of compounds inside the cells. Here we demonstrate the reality of the tissue electropermeabilization using qualitative and quantitative determinations of the electroloading of bleomycin considered as an nonpermeant molecule that serves as an indicator of the permeabilization. In tissues, cell electropermeabilization is achieved for electric field intensities lower than those necessary to permeabilize the same cells in suspension. We also emphasize the importance of the geometry of the electric field lines defined by the electrodes for permeabilizing a whole tissue, for example a tumor.

Key words: Cell electropermeabilization; Tissue electropermeabilization; Electroporation; Bleomycin; Electrochemotherapy; Tumor treatment

1. Introduction

Cell electropermeabilization, also termed cell electroporation, is a technique widely used in molecular and cellular biology laboratories, in order either to transfect bacterial, fungal, plant and animal cells [1–4], or to introduce a variety of compounds inside cells [5]: small metabolites, second messengers, dyes, drugs, oligonucleotides, aminoacyl-tRNAs, peptides, enzymes, antibodies, ... The procedure is usually performed in vitro on dissociated cell suspensions [5,6], and very few reports deal with the use of short, intense electric pulses on tissues [7–15].

We have recently designed a new antitumoral treatment that we have called electrochemotherapy: it combines a current anticancer drug, bleomycin, and local electric pulses delivered at the tumor site. After preclinical studies in mice [16–18], a phase I clinical trial on subcutaneous metastatic nodules has proven electrochemotherapy feasibility in humans [19]. This therapeutical approach was based on our previous observations of the very large increase of bleomycin cytotoxicity on cultured cells after cell electropermeabilization [20,21]. Indeed, bleomycin is almost unable to enter intact nonpermeabilized cells and its potential very high intrinsic cytotoxicity is restricted by a poor plasma membrane crossing [21]. In vivo as well, the observed efficacy of the electrochemotherapy probably implies in situ cell electropermeabilization. Our purpose in the present work was to demonstrate the reality of tissue electropermeabilization using qualitative and quantitative determinations of the electroloading of bleomycin considered as an nonpermeant molecule that serves as an indicator of permeabilization.

2. Materials and methods

2.1. Chemicals

Bleomycin (Laboratoire R. Bellon, Neuilly, France) was dissolved in sterile 0.9% NaCl. Radioactive

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bleomycin was obtained by stable chelation of ^{57}Co (440 Ci/mmol, New England Nuclear) as previously described [22]. ^{57}Co was isotopically diluted 100-fold and mixed to an excess of bleomycin in a 90% molar ratio in order to avoid the presence of unchelated cobalt. Radioactivity was measured using an Automatic Gamma Counting System MR 252 (Kontron). In mice, bleomycin in 100 μl of 0.9% NaCl was injected intravenously (i.v.) into the retroorbitary sinus.

2.2. Electrical treatments

Electrical treatment consisted of eight square-waved, 100-μs long electric pulses delivered at a frequency of 1 Hz by a PS 15 electropulsator (Jouan, France) [16,18]. The intensity of the electric field, indicated in the text for each experiment, was checked by a digital storage oscilloscope VC-6025 (Hitachi) connected to the electropulsator. Electric pulses were delivered through two stainless steel strips 10 mm wide, 0.5 mm thick and either 6 mm apart, for in vivo treatments, or 2 mm apart for ex vivo and in vitro treatments. In vivo, electrode contact with the skin was ensured by means of electrocardiography gel. Ex vivo, tumor fragments were placed between the electrodes, in direct contact with them.

2.3. Cells and tumors in mice

T-DC-3F clone 4 is a clone derived by us from DC-3F cells, a Chinese hamster lung transformed fibroblast cell line [23], that shows the same caryological pattern as the DC-3F stock cell line, similar tumorigenicity and a similar sensitivity to bleomycin (data not shown), and which is able to grow again in vitro after dissociation of an in vivo explant. Cells were maintained in vitro under previously described culture conditions [20]. Determination of the cytotoxicity of the bleomycin upon the electropermeabilized T-DC-3F clone 4 cells was performed as previously reported for other cell strains [20,21,24]. Briefly, $1 \cdot 10^6$ cells were resuspended in 50 μl of S-MEM (Gibco) supplemented with 0.5 mM $CaCl_2$. They were mixed with bleomycin and immediately exposed to the electric pulses at room temperature, and, 5 min later, diluted 10000 times. Survival was determined by cloning efficiency: 500 cells were seeded in triplicate per 60 mm diameter culture dish; colonies were counted after 5 days and results were expressed as percentage with respect to the number of colonies obtained with the appropriate control cells electropermeabilized in the absence of bleomycin. In that last case, absolute cloning efficiency was about 60%.

T-DC-3F clone 4 tumors were regularly obtained in nude mice after the subcutaneous injection of $2 \cdot 10^6$ cells. For in vivo experiments, animals were injected i.v. with 100 μg of bleomycin 3 min before the electric pulses delivery. For ex vivo experiments, 3 min after the i.v. injection of 1 μg of bleomycin, tumors were excised and sliced to obtain several equivalent fragments. Slices from a same tumor were used in order to reduce the heterogeneity between tumors resulting from possible differences in tumor vascularization and in bleomycin diffusion from the point of injection. Slices were placed between and in direct contact with two electrode plates and exposed to electric fields of various intensities. After dissociation of the individual slices through trypsinization, the relative cloning efficiency of the cells was determined from 5000 or 1000 Trypan blue negative cells seeded in triplicate per 60 mm Petri dish. Results were expressed as the percentage of colonies obtained with the appropriate control cells as described in the text. Absolute cloning efficiency of untreated cells from untreated mice was about 10%.

The highly tumorigenic murine LPB cell line is a clonal derivative of TBL.Cl2, a methylcholanthrene-induced C57Bl/6 mouse sarcoma cell line [25]. Subcutaneous tumors of about 6–7 mm in average diameter were regularly obtained 7 to 9 days after the inoculation of $5 \cdot 10^5$ cells in the flanks of age matched female C57Bl/6 mice. The in vivo electric treatment of these tumors was performed as previously described [16,18]. These immunocompetent mice with the syngeneic tumor were used in experiments with 1 μg radiolabelled bleomycin for which mice were confined under safety conditions that were not compatible with the sterile environment necessary to the survival of the immunodeficient nude mice.

2.4. VX2 tumor implantation and treatment in rabbits

White female New Zealand rabbits weighing 2.5–3 kg were used. The VX2 tumor (ATCC designation: CRL 6503) was provided by Dr. Orth (U 190 INSERM, Institut Pasteur, Paris, France) and was maintained by serial passage in carrier rabbits. Hepatic implantation of the VX2 carcinoma was accomplished under general i.v. anesthesia using ketamine hydrochloride (50 mg/kg, Ketamine, Parke Davis) and xylazine 2% (0.1 ml/kg, Rompun, Bayer) through a small subxyphoid incision. $10 \cdot 10^6$ VX2 cells, freshly prepared as described in Ref. [26], were injected with a 27-gauge needle in the median hepatic lobe. 15 days later, rabbits were anesthetized again and the median hepatic lobe was exposed through a subxyphoid incision for the in situ treatment of the transplanted tumor. Bleomycin (0.5 mg/kg body wt.) was given i.v. in about 45 s and 5 min later, eight electric pulses of 100 μs and of 800 V/cm were delivered at a frequency of 1 Hz through two acupuncture needles, inserted in the liver 6 mm apart, at each side of the tumor. At different times after the

treatment of the hepatic tumor, rabbits were killed. Their livers were removed and the regions corresponding to the treated tumors were fixed in Bouin's solution, sliced and hematoxylin-eosin stained.

3. Results

3.1. Determination of the electric field intensity threshold required for the permeabilization of the T-DC-3F cells either isolated or constituting a tumoral tissue

(a) In vitro experiments. T-DC-3F clone 4 cells in suspension were submitted to electric pulses of various intensities in the presence of 30 nM of bleomycin. Up to 800 V/cm, cloning efficiency of the electropulsed cells was equivalent to that of the unpulsed cells (Fig. 1). For electric pulses intensities greater than 1000 V/cm, only 2 to 3% of the cells survived. A clear threshold appeared for an electric pulses intensity around 800 V/cm, with a steep transition to the permeabilized state.

(b) In vivo experiments. Electric pulses were delivered 3 min after the bleomycin i.v. injection and the correlation between antitumor efficacy and the intensity of the electric pulses was tested. Preliminary experiments using nonlimiting electric field intensities (1350 V/cm) and 100 μg of bleomycin showed complete regressions in all the animals (8/8). With lower amounts of bleomycin and the same field intensity, the number of complete regressions was dose-dependent: 4/6 at 40 μg and 0/11 at 10 or 20 μg of bleomycin. Thus, for the following experiments, we chose an amount of bleomycin, 100 μg, which resulted in a nonlimiting concentration of the bleomycin in the tumor interstitial fluid.

Two different criteria can be used to evaluate the effects of the electric pulses, i.e., tumor growth arrest and complete tumor regression. If we consider the growth of the tumors occurring rapidly after the treatment (determinations made 2 days after the treatment), at 580 V/cm we already observed a total arrest of the tumor growth (Table 1). However, for lower electric pulse intensities (400 V/cm), no effect was observed, suggesting that a threshold exists for tumor growth stabilization. Only with large electric pulse intensities (1050 V/cm or more), was a decrease in the size of the tumors observed as soon as 2 days after the treatment. Such effects imply that cell death occurred, suggesting that bleomycin is efficient and, consequently, that cells in the tumor are permeabilized.

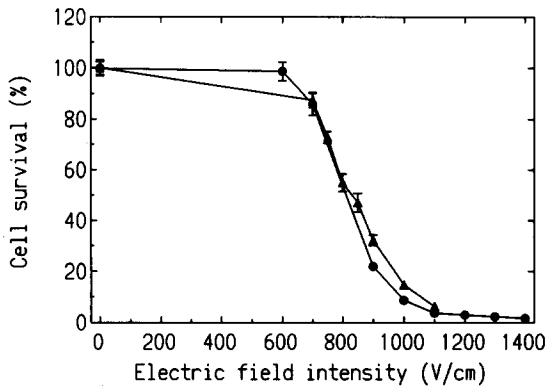

Fig. 1. Relative cloning efficiency of cultured T-DC-3F clone 4 cells exposed in vitro to electric pulses of various electric field intensities in the presence of 30 nM of bleomycin. Each symbol corresponds to a separate experiment.

Table 1
Antitumor effects, i.e., stabilization of tumor growth or complete regressions (CR) achievement, resulting from the in vivo delivery of electric pulses of various electric field intensities to T DC-3F tumors in nude mice injected with 100 μg of bleomycin

Electric pulses intensity	Number of stabilizations	Periods of stabilization (days)	Number of CR	Periods of CR (days)
0 V/cm	0/5	–	0/5	–
400 V/cm	0/5	–	0/5	–
580 V/cm	5/5	2 to 4	0/5	–
740 V/cm	5/5	3 to 15	0/5	–
890 V/cm	5/5	6 to 10	2/5	2 and 17
1050 V/cm	5/5	–	5/5	6 to 10 and 1 cure [a]

[a] Cure is defined as a CR that did not present any recurrency 60 days after the treatment.

If we consider the appearance of complete regressions (CR) a few days later, between 4 and 8 days after the treatments, another threshold appears. Indeed (Table 1), above 890 V/cm all (5/5) the tumors underwent CR, whereas below this value, no CR were observed. At 890 V/cm, 2/5 CR were observed, corresponding to the two smallest tumors in the experimental group.

In fact, considering the whole of the antitumor effects as a function of the intensity of the electric pulses delivered (percentage of CR, duration of CR, percentage of growth arrests, duration of growth arrests) (Table 1), there is a progressive shift from the almost complete absence of effects (400 V/cm) to the occurrence of long lasting CR (1050 V/cm).

(c) Ex vivo experiments. Bleomycin was injected intravenously in nude mice bearing subcutaneous T-DC-3F clone 4 tumors. Three min later, the mice were killed and the tumors were rapidly removed and sliced. Indeed, at this time, the tumors obviously contain the highest, homogeneously distributed extracellular bleomycin concentration after the i.v. injection of the drug, otherwise electrochemotherapy as usually performed [18] would not be efficient. When 1 μg of bleomycin was injected i.v. to mice and no electric pulses delivered to the tumor slices, the absolute

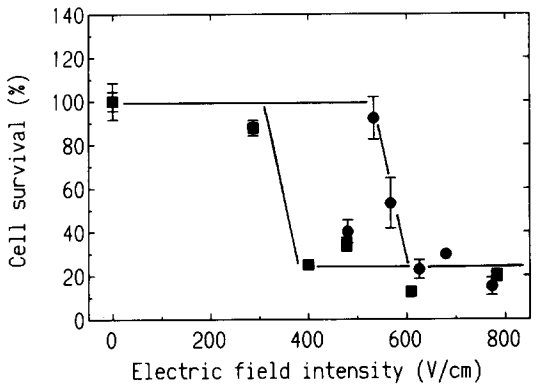

Fig. 2. Relative cloning efficiency of the cells obtained by dissociation of T DC-3F clone 4 tumor slices exposed ex vivo to electric pulses of various electric field intensities. Each closed symbol corresponds to a separate experiment performed using several slices of one single tumor. Other experiments gave similar results, the decrease in the cell cloning efficiency being always observed for electric intensities comprised in the interval defined by the two experiments here reported.

cloning efficiency of the cells from the tumor dissociated was similar to that of cells obtained from control tumors removed from uninjected mice (data not shown). This indicates that without electric pulses the intracellular influx of the drug after the 1 μg i.v. injection was insufficient to induce any cytotoxicity.

When electric pulses were applied to tumor slices in the absence of bleomycin in the tumor, almost no loss of cell viability was observed, at least for intensities up to 1200 V/cm (data not shown). On the contrary, in the presence of bleomycin, a clear decrease in the relative cell cloning efficiency was observed between 300 and 400 V/cm, or between 500 and 600 V/cm, depending on the individual tumor considered (Fig. 2). For greater field intensities, a plateau of only 20% of cell survival was observed.

3.2. In vivo electroloading of bleomycin into electropermeabilized tumors

1 μg of radiolabelled cobalt(57)-bleomycin (^{57}Co-bleomycin) was injected to 12 age-matched C57Bl/6 mice bearing a LPB tumor on their left flank. Six of these mice were treated as by usual electrochemotherapy, i.e., their tumors received eight transcutaneous electric pulses of 100 μs and of 1300 V/cm delivered at the frequency of 1 Hz, 3 min after the ^{57}Co-bleomycin i.v. injection. The six control mice did not receive any electric pulse. From each mouse, a blood sample

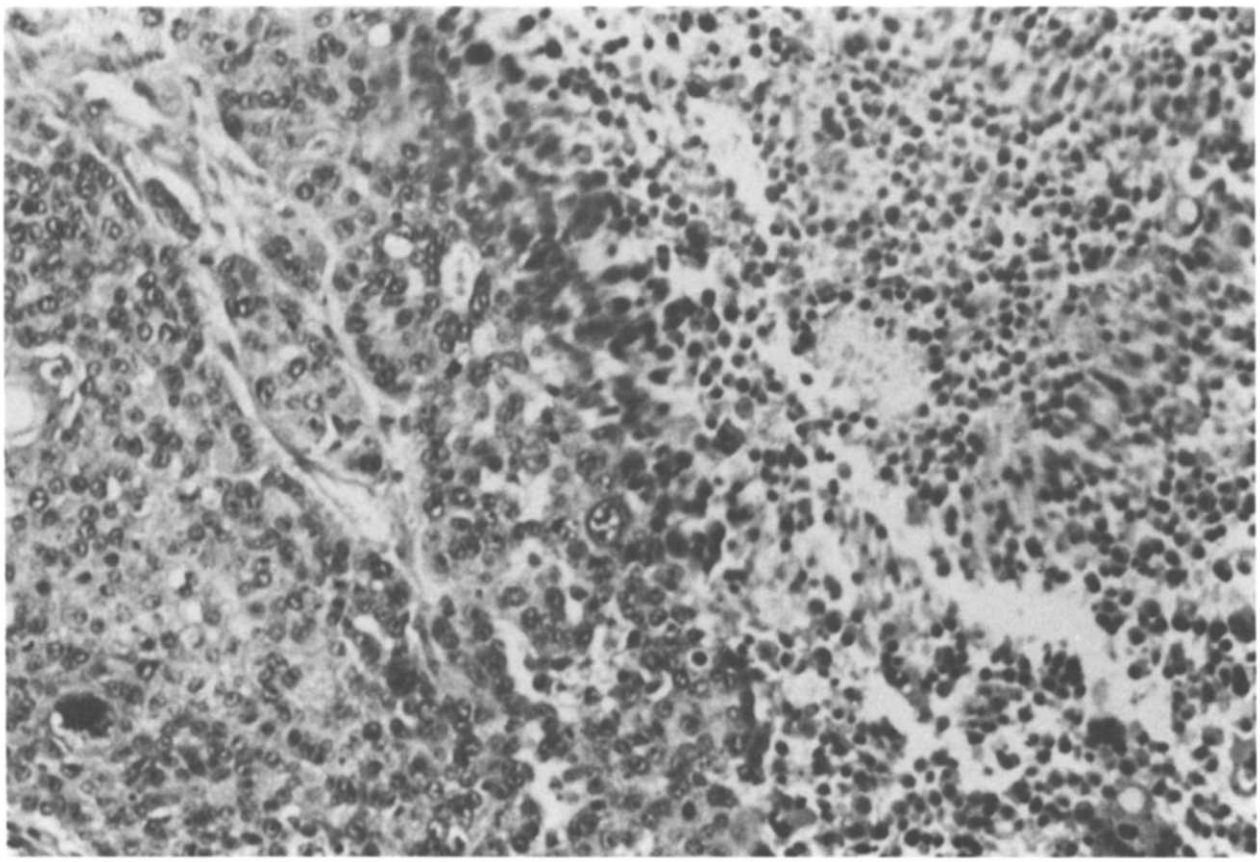

Fig. 3. Microphotography showing a section of a liver treated with electric pulses of 800 V/cm delivered through two needles. The necrosed area (right) is separated from the healthy part of the tumor (left) by a straight limit.

Table 2
Radioactivity retained by different tissues, not directly exposed to the electric pulses, and by the tumors, exposed (EP) or not exposed (NEP) to the electric pulses

	NEP [a]	EP [b]	Ratio [c]
A. Average values (±S.D.)			
Initial blood [d] (cpm/μl)	67±9	79±10	1.2 n.s.
Tumor [e] (cpm/mg)	15±2	61±20	4.1 $P<10^{-3}$
B. Normalized average values (±S.D.) [f]			
Blood	0.13±0.02	0.11±0.02	0.8 n.s.
Brain	0.05±0.01	0.05±0.01	1.0 n.s.
Thigh muscle	0.04±0.01	0.05±0.02	1.3 n.s.
Abdomen muscle	0.06±0.02	0.11±0.07	1.8 n.s.
Skin	0.11±0.02	0.12±0.06	1.1 n.s.
Spleen	0.12±0.03	0.18±0.09	1.5 n.s.
Lungs	0.29±0.02	0.30±0.04	1.0 n.s.
Kidney	0.60±0.08	0.71±0.21	1.2 n.s.
Liver	0.72±0.18	0.80±0.13	1.1 n.s.
Tumor	0.22±0.03	0.79±0.28	3.6 $P<10^{-3}$

[a] NEP: mice whose tumor was not exposed to the electric pulses; values are the means of independent determinations in six mice.
[b] EP: mice whose tumor was exposed to the electric pulses; values are the means of independent determinations in six mice.
[c] The Student's *t*-test was used for statistical analysis (n.s., non significant).
[d] Radioactivity present in the blood 4.5 min after the i.v. injection of the bleomycin.
[e] Radioactivity present in the tumors 3 days after the treatment.
[f] Normalization was performed according to individual initial blood levels which average values are given in part A of the table. Values, in cpm/mg of tissue 3 days after the treatment, divided by the cpm/μl of blood, are the means of independent determinations in six mice.

was taken 4.5 min after the ^{57}Co-bleomycin injection in order to determine the individual ^{57}Co-bleomycin blood level at that time. On an average, the value found was 40 nM (Table 2). Since ^{57}Co-bleomycin as well as the electric pulses per se are non cytotoxic [22,27], tumor development was similar in both groups, treated with electric pulses and untreated groups, of mice. Three days later, after the renal and fecal elimination of most of the ^{57}Co-bleomycin injected, the mice were killed and various organs, including tumors, were sampled. We then measured the total radioactivity of each sample and its weight, and the radioactivity per mg was normalized with respect to the individual initial (at 4.5 min) blood level of ^{57}Co-bleomycin. For any of the organs except the tumors, no significant differences in radioactivity retention were detected as a consequence of the electric pulses delivery to the tumors (Table 2). On the contrary, the tumors treated by the electric pulses retained 3.6–4-times more ^{57}Co-bleomycin than those that did not receive the electric pulses (Table 2).

3.3. Histology of a partially treated tumor tissue

9 days after the treatment by bleomycin and electric pulses (800 V/cm) of VX2 tumors transplanted in the liver of rabbits, the histological analysis of slices of these tumors showed a definite straight line separating the necrosed from the healthy parts (Fig. 3). At this time, the necrosed area was heavily infiltrated by macrophages (Fig. 3).

4. Discussion

Our previous work showing clear antitumor efficacy of the electrochemotherapy has suggested that local cell electropermeabilization occurs after the in vivo delivery of adequate electric pulses to tumors [16,17,19]. We therefore undertook experiments designed to unambiguously demonstrate the achievement of electropermeabilization of cells organized in tissue structures.

In recent in vitro experiments, we measured bleomycin electroloading in electropermeabilized DC-3F cells in suspension [21] using radiolabelled ^{57}Co-bleomycin of very high specific activity [22]. Bleomycin and Co-bleomycin are nonpermeant molecules (we previously defined a molecule as 'nonpermeant' with respect to the plasma membrane if it is unable to flow freely through the plasma membrane and is devoid of any membrane transport system [24]). On intact, nonpermeabilized cells, bleomycin associates with cell membrane binding sites [28] and is also present in the endocytic compartment [29]. On electropermeabilized cells, there is another component of bleomycin association: the electroloaded bleomycin, which is the bleomycin that can directly reach the cytosol through the electropores and then remain entrapped there [21]. This study provided us with the theoretical basis necessary to measure the bleomycin electroloading in cells of a tissue exposed to the electric pulses in vivo.

With this aim, we injected tumor-bearing mice with 1 μg of radioactive, non cytotoxic ^{57}Co-bleomycin with or without the delivery of the electric pulses at the tumor site 3 min later. These tumors were obtained by the injection of the tumor cells used in our previous studies [16–18,30] during which the best conditions for the electrochemotherapy of such tumors were determined; consequently, the electric parameters used here in this in vivo study were the same as those used for electrochemotherapy. The major problem with our approach was to distinguish between three drug localizations: (a) the radioactivity in the vascular compartment and the interstitial fluids of the tumor; (b) the radioactivity associated with the extracellular matrix of the cells, tightly bound but not yet internalized; and (c) the radioactivity electroloaded into the cells, effectively internalized into the cell cytosol. To reduce the fraction (a) as much as possible, we decided to wait, after the treatments, for the time necessary for the physiological excretion of most of the bleomycin from the body, i.e., the time necessary for the in vivo wash of the

tissular fluids. Therefore, we determined in preliminary assays a time for the sampling of the tissues, namely 3 days, as being the best compromise between the growth of the tumors and the decrease of the extracellular radioactivity still remaining. Under these conditions, comparison between electric pulses-exposed mice and unexposed mice should give us directly the relationship between the fraction (b) and the fraction (c).

In this experiment, we chose a subcutaneous tumor to determine its electroloading because (i) this localization is easily accessible to transcutaneous electric pulses; (ii) it seemed very easy to distinguish between the volume subjected to the electric pulses, i.e., the entire tumor, and the surrounding areas not subjected to the electric pulses; (iii) unlike any organ, a tumor is not vital for mice survival during the 3 days following the treatment, even if we knew that ^{57}Co-bleomycin and electric pulses are not toxic. Our results (Table 2) clearly indicate that an increase of retention of ^{57}Co-bleomycin in the pulsed tumors can be achieved in vivo using our experimental electric conditions. This increase of radioactivity retention is likely due to the electroloading of ^{57}Co-bleomycin in cells of the tissue subjected to the electric pulses in vivo. This strongly suggests that electropermeabilization was really achieved in these cells.

This amount of electroloaded ^{57}Co-bleomycin can be compared to the increase of ^{57}Co-bleomycin association with cells in suspension after their in vitro electropermeabilization [21]: for ^{57}Co-bleomycin external concentrations in the range of 40 nM, the electropermeabilized cells retained 3–4-times more radioactivity than the nonpermeabilized cells (Fig. 4 in Ref. [21]). Moreover, in this reference, we reported the direct relationship between bleomycin uptake and bleomycin cytotoxicity found after the electropermeabilization of the cells in suspension. Hence, our results showing the in situ electropermeabilization of the tissues exposed to the electric pulses provide us with a convincing explanation of the increase of the antitumoral effects of bleomycin after the delivery of electric pulses to subcutaneous tumors.

We considered it of interest to further investigate electropermeabilization of tissue by another more detailed approach. Indeed, one can imagine a large number of uses of tissue electropermeabilization both in basic biological research and in pharmacological applications, contrasting with the small number of so far published reports. Isolated islets of Langerhans [9,10], intact skeletal muscle fibers [11], intact rice tissues [14] and chicken retinal explants [12] have been submitted to electric pulses that could be able to permeabilize these tissues. Living animals have also been exposed to electropermeabilizing electric pulses, either for the treatment of tumors by electrochemotherapy [16–19,30]

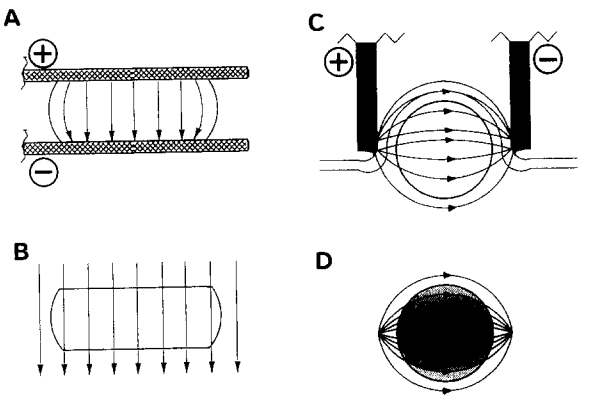

Fig. 4. Schematic drawings of the experimental situation during ex vivo (panel A) and in vivo (panel C) experiments and of the modelization that allows to explain the clear threshold found during the ex vivo treatments (panel B) and the progressive antitumor effects reported during the in vivo manipulations (panel D). Experimental procedures, mimicked in panels A and B, are described in Materials and methods. In the model reported in panel D, the decreasing intensity of the shadowing corresponds to the electropermeabilization obtained by applying either 580, 740, 890 or 1050 V/cm between the electrodes.

or for the in vivo electrotransfection of skin cells after the subcutaneous injection of DNA [15].

As indicator of the achievement of cell electropermeabilization, we used the large difference in bleomycin cytotoxicity on electropermeabilized versus nonpermeabilized cells [20,21]. Indeed, at least in vitro, with adequate external bleomycin concentrations (between 30 and 500 nM), all the electropermeabilized cells are killed whereas virtually none among the nonpermeabilized cells is affected by these drug concentrations [20,21]. In the case of a tissue, this experimental approach was valid only if bleomycin was homogeneously distributed in the interstitial fluid bathing the cells of the tissue. Moreover, it was necessary to use an accurate means to measure the cytotoxicity induced by the combined treatment.

For these reasons, we selected a clone of the DC-3F cell culture line, namely the T-DC-3F clone 4 cells, able to regularly form tumors in nude mice and able to readily grow again in vitro after the dissociation of the tumor into cells. So it was possible to in vivo distribute the bleomycin by the blood flow in the whole interstitial fluid of the tumor, and then, after the electric pulses delivery to the tissue ex vivo, to measure bleomycin cytotoxicity by the relative cloning efficiency of the cells obtained after a careful dissociation of the tissue sample.

We first determined the plot of the in vitro permeabilization of the T-DC-3F clone 4 cells in suspension as a function of the electric field intensity, using bleomycin as a probe for testing the electropermeabilization. The plot of the cell survival as a function of the electric pulse intensity (Fig. 1) shows a clear

threshold for a value of about 900 V/cm, suggesting that less intense electric pulses are unable to permeabilize the T-DC-3F clone 4 in vitro. This value is close to that obtained previously using the same electrical device and a fluorescent dye, Lucifer yellow, to test the permeabilization threshold of the parental DC-3F cells [24].

The results reported in Fig. 2 show a steep reduction in cell cloning efficiency when tissues supplied in vivo with bleomycin are removed and directly exposed to electric pulses of more than 300 to 500 V/cm, clearly evidencing the existence of a threshold. The drop in the cell viability can be attributed to the uptake of bleomycin by the electropermeabilized cells, since in the absence of bleomycin almost no reduction in cloning efficiency was detected for intensities as high as 1200 V/cm. However, this ex vivo threshold is observed for an electric field intensity lower than in the case of the same cells treated in suspension in vitro. Two hypothesis can account for this observation: (i) the fact that cells in a tissue are more close each to other than in suspension implies that the conductivity of the whole tissue (through the conductive extra-cellular fluids that are present in the very reduced extra-cellular space left between the glycocalix of the cells in contact) is lower than that of the conducting medium in which isolated cells are suspended, leading to a lower permeabilizing threshold [31]; (ii) the fact that adjoining cells in a tissue can establish gap junctions between them, thus creating an electrical continuum between these cells, allows to consider the tissue as a set of independent clusters of cells that behave as separated insulated units with an apparent average diameter greater than that of the isolated cells [32].

It is worth recalling here that, at least for the cells in suspension, electropermeabilization results from the induction of a transmembrane potential ΔV_i by the external electric field E and that the value of ΔV_i at the point defined by the polar angle Θ with respect to the direction of E is given by:

$$\Delta V_i = (3/2) r \cdot E \cdot \cos \Theta \quad [33]$$

(assuming the simplifying hypotheses that cells are spherical, of radius r, membrane thickness very small and membrane conductivity negligible). In the articles published until today, only one, by Maurel et al., reports a comparison of the electropermeabilization of the same cells in two different situations, namely in suspension and in monolayer [7]. When these authors checked the permeabilization of attached epithelium-forming embryonic amphibian cells by electric fields applied longitudinally, two transitions were observed. The first one was detected for an electric field intensity lower than that necessary to permeabilize the isolated cells in suspension: it was attributed to the permeabilization of the attached cells spread at the periphery of the epithelium that have an apparent diameter larger than that of the cells in suspension. The second transition was detected for an electric field intensity close to the value that permeabilizes the isolated cells: it was attributed to the permeabilization of the more compacted cells located in the core of the epithelium. However, returning to our results on the ex-vivo permeabilization of tridimensional tissues, the shape of the individual cells in the tissue does not seem to be the main factor that determines the electric field intensities necessary to permeabilize the cells. Indeed, (i) the majority of the cells in the tissue are compacted, not spread, in close vicinity each with other and nevertheless, their permeabilization threshold was lower than that of the cells in suspension, and (ii), as far as it may be experimentally detectable, using electric pulses of higher electric field intensity up to 1200 V/cm, we could not detect a second transition among the cells that remained nonpermeabilized at 600 V/cm.

In other respects, cell survival plateau level obtained during ex vivo tissue treatments, when tissues were treated at electric field intensities greater than the threshold value, is higher than that observed in vitro, with cells in suspension. The fact that 20–30% of the cells of the tissue survived after the combined exposure to bleomycin and high intensity electric pulses could result from the lack of homogeneity either of the electric field distribution inside the tissue or/and of the bleomycin availability around the cells. Indeed, killing of the mouse and tumor excision were performed at the time when, on the basis of our previous work on electrochemotherapy, tumors are supposed to contain the highest, homogeneously distributed extracellular bleomycin concentration after the i.v. injection of the drug. However, for in vivo experiments we were obliged to use low amounts of bleomycin in order to avoid cytotoxicity of the drug on the nonpermeabilized cells at the time of the enzymatic dissociation of the tumors, whereas in vitro the concentration of bleomycin used was largely in excess due to the considerable difference in cytotoxicity of bleomycin upon electropermeabilized versus intact cells. Alternatively, one cannot exclude that the apparent insensitivity of the cells in the tissue would be the consequence of a factor intrinsically linked to the tissue structure: this result could be similar to the observation that even in the case of dissociated cells exposed in suspension to bleomycin and to optimized electric pulses, a small percentage of cells is not permeabilized and survives the treatment [20,21,24]. Nevertheless, what is important is the existence of a marked threshold and a steep transition which are unambiguous arguments in favor of the electropermeabilization of the cells constituting the tumor tissue.

Finally, we decided to compare the ex vivo tissue electropermeabilization characteristics with the in vivo

exposure of the same tumors to electric pulses of various electric field intensities. As already shown [34], the difference in the temperature of the tissues at the time of the electric pulses delivery (37°C in vivo versus room temperature ex vivo) should not influence the permeabilization threshold. We were also aware of the fact that ex vivo the tissues were exposed to an almost homogeneous electric field generated by two parallel plates, whereas in vivo the geometry was quite different (Fig. 4). For the in vivo experiments, mice were injected intravenously with a large amount of bleomycin (100 μg) to have an excess of the drug, as in the in vitro experiments, taking into account that we were not limited by the trypsinization step of the ex vivo tissue dissociation. Even under these conditions in which every permeabilized cell should die, and contrary to what happens in the ex vivo experiments (T-DC-3F tumor tissue is assuredly permeabilized ex vivo for electric pulse intensities greater than 550 V/cm), antitumor effects were progressively improved with increasing intensities of the electric field over a large range, from 580 to 1050 V/cm. Indeed, effects increased from only a short slow down of the tumor growth at an intensity corresponding to an efficient permeabilization in ex vivo experiments, to a long lasting complete regression of the tumors (Table 1). Almost no cures (1 amongst 5 mice treated at 1050 V/cm) were obtained in these experiments because electrochemotherapy, that is the combination of bleomycin and electric pulses, was performed in immunodeficient nude mice. Nevertheless, this experimental system allowed us to better identify the direct effects of the electric pulses in the absence of any potentiation of the effects of the electrochemotherapy by the hosts' immune system response. All these effects, however, are indicative of a bleomycin-dependent cell killing that can be attributed to tissue electropermeabilization. The progressiveness of the effects could be due to a progressive 'recruitment' of tumor cells that are actually permeabilized: indeed, the higher is the electric field intensity delivered between the electrodes, the greater is the volume of tissue submitted to permeabilizing electric field lines having an intensity greater than that of the ex vivo-determined permeabilizing threshold (Fig. 4).

The choice of a transplanted hepatic tumor in a rabbit allowed us to experimentally approach at best to the theoretical situation depicted in Fig. 4D. Indeed, using two acupuncture needles implanted in the liver located around the tumor as electrodes, histological sections made perpendicular to the direction of needles should correspond to this situation. Then we searched for the existence of a definite straight limit between the necrosed, i.e., the permeabilized, and the healthy, i.e., the nonpermeabilized, parts of a tumor exposed to electric pulses of intermediate intensity (800 V/cm) by histological analysis. In the case of tumors removed nine days after the electrochemotherapy, such a straight limit was clearly observed (Fig. 3). This observation reinforces our demonstration of tissue electropermeabilization as well as our interpretation of the progressive antitumoral effects observed in vivo on mice.

To our knowledge, the results reported in this paper constitute the first direct demonstration of tissue electropermeabilization in vivo as well as ex vivo monitored by both the qualitative biological consequences (the cell cytotoxicity) and the actual quantitative determination of the electroloading of a molecule almost nonpermeant under normal conditions. In the future, tissue electropermeabilization could become a current technique for in situ studies of the cell physiology and metabolism. At the present time, the demonstration of the tissue electropermeabilization achievement reported in this article confirms that the electrochemotherapy efficacy is based on the same mechanisms as those already fully analysed in vitro on cells in suspension, i.e., that electric pulses transiently permeabilize tumor cells and provide access of the bleomycin to the cytosol and thus to its intracellular target, the DNA.

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