*Eur J Cancer, Vol. 27, No. 1, pp. 68–72, 1991.*
*Printed in Great Britain*

*0277–5379/91 $3.00 + 0.00*


# Electrochemotherapy Potentiation of Antitumour Effect of Bleomycin by Local Electric Pulses

**Lluis M. Mir, Stéphane Orlowski, Jean Belehradek Jr and Claude Paoletti**

**In cell culture the cytotoxicity of some anticancer drugs, especially bleomycin, can be greatly enhanced by exposing cells to non-cytotoxic electric pulses. Nude or conventional mice bearing subcutaneous transplanted tumours were treated with intramuscular doses of bleomycin followed by local delivery of electric pulses similar to those used *in vitro*. Tumours were reduced and even eradicated after this electrochemotherapy. Thus the antitumour effects of bleomycin in mice can be considerably potentiated by local electric pulses.**
*Eur J Cancer*, Vol. 27, No. 1, pp. 68–72, 1991.

## INTRODUCTION

WE HAVE compared the *in vitro* cytotoxicity of a panel of anticancer drugs on intact cells and on cells submitted to short, intense, square-wave electric pulses [1]. One of the consequences of such exposure is increased permeability of the cell membrane, which is used to introduce DNA [2–4] and low molecular weight non-permeable molecules [5] into cells. Bleomycin [6] is a cytotoxic agent currently used in some anticancer combinations [7, 8]. The drug causes DNA single and double strand breaks by a catalytic mechanism [9]. However, the diffusion of bleomycin through the plasma membrane is slow and only limited amounts enter the cells [10].

After electric pulse delivery, we observed a substantial reduction (about 700 times) in bleomycin concentration, which reduces cloning efficiency to 50% of controls ($EC_{50}$) [1]. Moreover, after electric field treatment, bleomycin was cytotoxic even at very low external concentrations (starting from $10^{-9}$ mol/l) with a steep dose-response curve [1]. The relative gain in toxicity depends on the survival level considered: we have observed that the external bleomyin concentration required to kill 90% of treated cells (i.e. $EC_{10}$) was reduced 650 000 times after electric field treatment conditions that do not affect cell viability in the absence of the drug. We have now examined the possibility of electric field potentiation of bleomycin *in vivo*.

## MATERIALS AND METHODS

*Cells*

The highly tumorigenic murine LPB cell line is a clonal derivative of TBL.C12, a methylcholanthrene-induced C57B1/6 mouse sarcoma cell line [11]. The B16 melanoma cells, syngeneic to C57B1/6 mice, were obtained by trypsin dissociation of tumours serially passaged *in vivo*. KB cells were derived from cultures of a recently explanted tumour previously initiated in a *nu/nu* mouse with the KB human oral epidermoid carcinoma cell line [12].

*In vivo treatment of cell inocula*

Either 50 000 or 5000 LPB cells (suspended in 100 μl culture medium) were subcutaneously injected into the four legs of pentobarbital anaesthesised (40 mg/kg) 6–8 week-old *nu/nu* mice with Swiss background (from our institute). This unusual inoculation site was chosen because it facilitates the placement of the two flat rectangular electrodes on each side of the cell inoculum. Contact between the electrodes and the skin was ensured by means of electrocardiography paste. Sex and age matched animals were randomly assigned to four experimental groups, each consisting of six mice (i.e. 24 legs inoculated). No statistically significant differences in tumour growth on the anterior and the posterior legs were observed.

Bleomycin (Laboratoires Roger Bellon) was added to the cell suspension just before the injection: cells were injected in the absence (D−) or in the presence (D+) of 0.5 μmol/l bleomycin (which corresponds to a dose of 75 ng locally injected into animals weighing about 25 g). Electric treatments [1], consisting of 8 pulses of 100 μs and 1500 V/cm at 1 Hz were (E+) or were not (E−) administered at the inoculum site immediately after cell inoculation. To assess the tumour latency period, we scored the day on which a nodule of 3 mm in diameter appeared.

*Tumour treatment*

To produce solid tumours, either 150 000 LPB cells or $2 \times 10^6$ KB cells were injected subcutaneously into flanks of nude mice, and either 150 000 LPB cells or $10^6$ B16 cells into the flanks of C57B1/6 mice. The tumours of about 7 mm in average diameter (the maximum size which could be encompassed by our electrodes with a distance of 6.6 mm between them and 10 mm long) were obtained 7–9 days later. Animals bearing tumours were then randomised into four experimental groups designated D+E+, D+E−, D−E+ and D−E−, each comprising usually 10 mice ($n$=6 for experiments with KB cells).

The animals of the D+E+ and D+E− groups received two intramuscular injections of 250 μg bleomycin into both thighs (i.e. total dose 500 μg, which corresponds to roughly one-tenth of the $LD_{50}$ [13]). 30 min after bleomycin injection, the amount of time necessary to allow bleomycin to reach the tumour [13], the D+E+ animals received electric pulses (1500 V/cm, 100 μs, 1 Hz, 8 pulses) delivered with electrodes placed on the both sides of the protruding tumour; again contact was ensured by electrocardiography paste. The D−E+ group received the same electric pulses without a bleomycin injection. The D−E− group received neither bleomycin nor electric pulses.

The tumour's longest diameter ($a$) and the next longest diameter ($b$) perpendicular to $a$ were measured with a caliper at

Correspondence to L.M. Mir.
The authors are at the Laboratoire de Biochimie-Enzymologie, PR II (URA 158 CNRS, U 140 INSERM), Institut Gustave Roussy, 39 rue Camille Desmoulins, F-94805 Villejuif Cedex, France.
Revised 4 Jun. 1990; accepted 31 Oct. 1990.

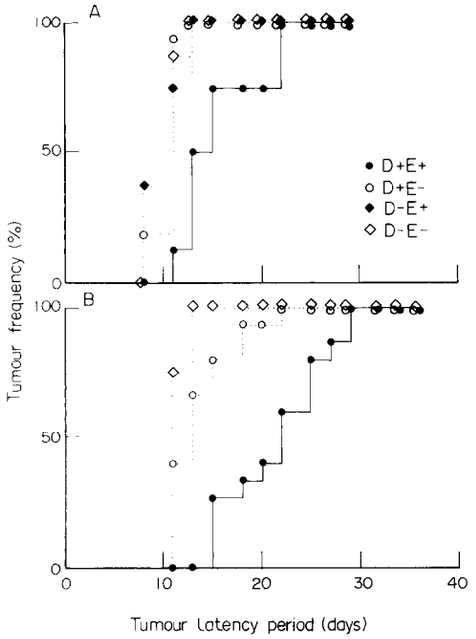


**Fig. 1. Increase of latency period before tumour appearance after electrochemotherapy on inocula of (A) 50 000 and (B) 5000 LPB cells in legs of nude mice.**

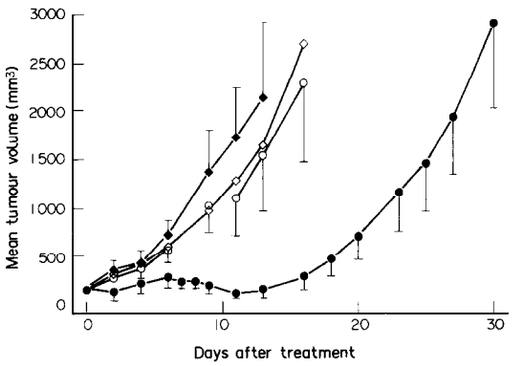


**Fig. 2. Effects of bleomycin and electric pulses on LPB sarcomas in flanks of nude mice. ● = D+E+; ○ D+E−; ◆ = D−E+ and ◇ = D−E−.**

regular times. The tumour volume was calculated by $V = ab^2\pi/6$, derived from the formula previously developed by Auerbach *et al.* [14]. The mean (S.D.) tumour volume were calculated.

Objective responses were scored as: (i) partial regression (PR), (ii) complete regression (CR) and (iii) cure according to WHO guidelines. The therapeutic result was termed cure if CR at a particular site was maintained without recurrence at least 120 days after treatment.

## RESULTS

*Effect on cell inocula*

We chose a bleomycin concentration that *in vitro* was unable to kill more than 10% of cells not exposed to electric fields but which was at least 5 fold the concentration necessary to kill *in vitro* 100% of LPB cells thus exposed (data not shown). No macroscopic signs of necrosis were observed on the skin under the electrodes. Locomotion was not impeded in the treated animals. The only secondary effect observed was a potentiation of the pentobarbital-induced anaesthesia.

Three independent experiments were done with inocula of 50 000 LPB cells in the legs of nude mice and yielded similar results. One typical experiment is shown in Fig. 1A: the median number of days before the appearance of tumours was 14.3 for the mice which received both cells suspended in bleomycin and electric pulse delivery (D+E+) and 10.5 (D−E−), 10.3 (D−E+) or 10.5 (D+E−) for the control groups. The tumour latency periods for the three control groups were not mutually significantly different (Duncan and Tukey tests on harmonic means), whereas the delay in tumour growth in the D+E+ group was significant ($P < 0.01$). Since the number of LPB injected cells (50 000) was above the minimal cell number necessary to initiate a tumour, we did additional experiments with as few as 5000 LPB cells per inoculum. The effect of bleomycin combined with electrical pulses on the delay in tumour growth was greater (Fig. 1B): the median number of days before the appearance of tumours was 21.7 for the D+E+ group compared with 10.6 (D−E−) and 12.6 (D+E−). The delay in tumour growth observed in the D+E+ group compared with that of the control groups was again significant ($P < 0.01$). These data suggest that only a small fraction of cells survived electrochemotherapy. Thus, *in vivo*, electrical pulses delivered via the skin potentiated bleomycin's cytotoxicity to fresh inocula of suspended cells.

*Tumour treatment in nude mice*

An arrest in LPB tumour growth was observed in all mice receiving electrochemotherapy ($n=14$), whereas bleomycin alone ($n=10$ animals) or electric pulses alone ($n=10$) had no effect on tumour growth compared with the control D−E− group ($n=10$ animals) (Fig. 2). The regression of tumour volume in the D+E+mice was partly masked by local oedema: the consistency of the volumes measured was more flaccid than that of the untreated tumours. Transient superficial skin scabs were observed under the electrodes after electrochemotherapy. They progressively disappeared within 2–3 weeks. However, necrosis of the tumour tissue was found on histological sections as early as 24 h after treatment (data not shown). Nevertheless, in nude

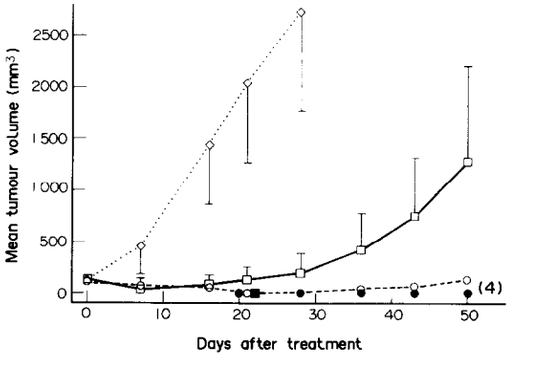


**Fig. 3. Effects of bleomycin and electric pulses on KB carcinomas in flanks of nude mice. ○ = 500 and □ = 50 μg bleomycin. For controls only the D+E− group has been drawn (◇). S.D. not drawn in right part of dashed curve as only 1 mouse showed recurrence. ■ = 1 transient CR, mouse treated with 50 μg bleomycin (this value was not included in calculation of mean tumour volume); ● = CRs, D+E+ mice treated with 500 μg bleomycin. No. in brackets refers to 4 CRs still at day 50 among 6 treated mice.**

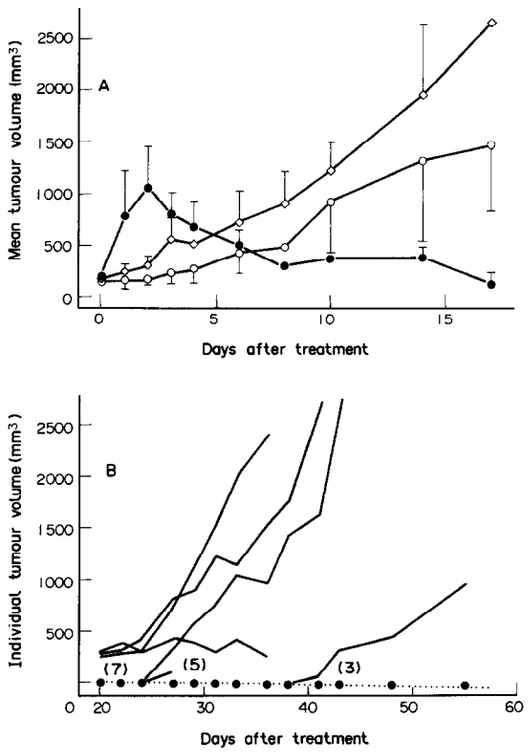


**Fig. 4. Effects of bleomycin and electric pulses on LPB sarcomas in flanks of C57Bl/6 mice. (A) Mean tumour volumes until day 17. D−E+ group was omitted in this experiment; when it was done, it never differed significantly from the other two controls, namely D+E− and D−E− (see also Figs 2 and 5). ● = D+E+, ○ = D+E− and ◇ = D−E− mice. (B) Individual course of D+E+ tumours from day 20 onwards (10 survivors out of 16 treated mice) 7 mice had CR (●). Lines show individual recurrences. No. in brackets refers to CRs achieved.**

mice, LPB tumours were never completely eradicated: after a transient interruption in growth (and a probable PR masked by oedema) of 12–15 days, tumour progression resumed with growth slopes similar to those of control tumours.

For the KB carcinomas treated under the same conditions, CRs were observed in all the D+E+ treated mice; only 1 early recurrence was detected later (Fig. 3). For animals with a longer CR, tumours did not reappear 50 days after the treatment. KB carcinomas were also treated with only 50 μg bleomycin and the same electrical field. Tumour growth was arrested in all cases and 1 transient CR was detected at day 22 (Fig. 3).

*Tumour treatment in C57Bl/6 mice*

In immunologically reactive C57Bl/6 mice bearing LPB sarcomas, a more severe oedema than that found in nude mice was always detected at the tumour site after electrochemotherapy with 500 μg bleomycin. This resulted in a rapid increase in the mass perceivable, which, nevertheless, began to regress 3–4 days later (Fig. 4A). Oedema was never detected in controls. In mice with the LPB tumour in the right flank (Fig. 4A), after combined treatment, 6 out of 16 D+E+ mice died after 4–10 days without, however, any further development of tumour. Necropsy and histological examination revealed necrosis in the external part of the liver, just beneath the treated tumour. 20 days after electrochemotherapy, sarcomas disappeared completely in 7 of the remaining 10 mice (Fig. 4B). Of these 7, 2 developed an early recurrence (at day 23) and 1 a late recurrence at day 41 (Fig. 4B); 1 animal in CR was killed at day 45 for necropsy and histological examination which confirmed the absence of tumour tissue; 1 animal died without any apparent tumour; and 2 mice were cured (absence of tumour 250 days after the treatment).

Similar results were obtained in C57Bl/6 mice bearing LPB tumours at the left flank, except that only 3 of 21 animals died a few days after treatment, again without any further tumour development. Among the 18 survivors, we observed 4 CRs. 2 were still free of their initial tumour 210 days after the combined treatment (data not shown).

We extended these experiments by applying the same combined treatment to C57Bl/6 mice bearing transplantable syngeneic B16 melanoma in their left flank. The growth of this tumour was extremely rapid for the three control groups whereas an excellent response to electrochemotherapy was obtained (Fig. 5). After a transient increase in volume at the tumour site, oedemas and tumours regressed. Overall, 4 mice had CR. 17 days after the treatment, tumour was no longer detectable in 3 out of 8 surviving animals (the D+E+ group initially contained 11 mice). In 1 case tumour had not reappeared 200 days later.

We did an additional control experiment to compare these results with the most active treatment with bleomycin alone—a daily intramuscular injection of 1 mg for 5 consecutive days in C57Bl/6 mice bearing LPB sarcoma. This dose is not far from the lethal dose and had a considerable toxic effect: weight loss, hirsutism and death a few days later. A deceleration was noted, however, in tumour growth rate, which was more likely related

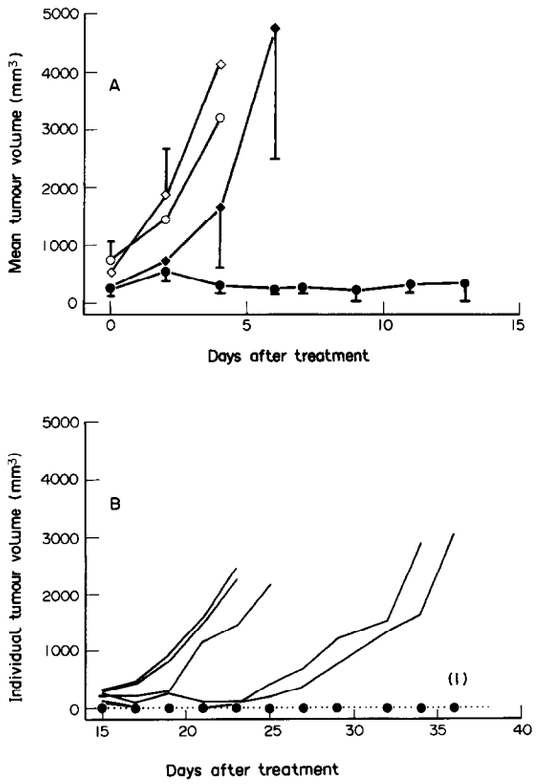


**Fig. 5. Effects of bleomycin and electric pulses on B16 melanomas in flanks of C57Bl/6 mice. (A) Mean tumour volumes until day 13. ● = D+E+, ○ = D+E−, ◆ = D−E+ and ◇ = D−E− mice. (B) Individual course of D+E+ tumours from day 15 onwards (8 survivors out of 11 treated mice). At day 17, 3 mice had CR (●). 1 late recurrence (at day 23) is shown in right part of figure.**

*Table 1. Effects of single combined treatment of LPB sarcomas as function of intensity of electric pulses* (500 μg *bleomycin*)

| | Day | | | |
|---|---|---|---|---|
| V/cm | 0 | 11 | 27 | 120 |
| 1500 | | | | |
| Mean (S.D.) | 69 (39) | 76 (33) | 810 (550) | |
| Tumours | 12 | 3 | 7 | 0 |
| CR | — | 8 (66%) | 4 (33%) | 3 (25%) |
| 1400 | | | | |
| Mean (S.D.) | 72 (52) | 23 (14) | 520 (360) | |
| Tumours | 13 | 2 | 8 | 0 |
| CR | — | 11 (85%) | 4 (31%) | 4 (31%) |
| 1300 | | | | |
| Mean (S.D.) | 55 (31) | 47 (41) | 1100 (700) | |
| Tumours | 13 | 6 | 4 | 0 |
| CR | — | 7 (54%) | 8 (61%) | 7 (54%) |
| 1200 | | | | |
| Mean (S.D.) | 50 (24) | 20 (19) | 820 (900) | |
| Tumours | 13 | 3 | 7 | 0 |
| CR | — | 9 (69%) | 4 (31%) | 4 (31%) |
| 1100 | | | | |
| Mean (S.D.) | 46 (24) | 68 (32) | 1100 (1500) | |
| Tumours | 13 | 3 | 10 | 0 |
| CR | — | 10 (77%) | 3 (23%) | 3 (23%) |
| 1000 | | | | |
| Mean (S.D.) | 43 (14) | 33 (53) | 1700 (1800) | |
| Tumours | 7 | 4 | 6 | 0 |
| CR | — | 3 (43%) | 1 (14%) | 0 |
| 900 | | | | |
| Mean (S.D.) | 70 (42) | 85 (59) | 1500 (1200) | |
| Tumours | 6 | 4 | 5 | 0 |
| CR | — | 2 (33%) | 1 (17%) | 0 |
| 0 | | | | |
| Mean (S.D.) | 32 (32) | 430 (300) | 4300 (2400) | |
| Tumours | 8 | 8 | 8 | 0 |
| CR | — | 0 | 0 | 0 |

to general bleomycin toxicity than to a specific antitumour effect. No tumour growth arrest was observed.

*Variations of electrochemotherapy modalities*

Table 1 shows the biological and therapeutic efficiency of the combined treatment according to the electric field intensity applied. From 1500 V/cm (our initial conditions) down to 1200 V/cm, electric fields remained as effective on LPB tumours (at the left flank), both after short and long intervals following combined treatment. Most of the animals which achieved CR at day 27 were cured. Overall, 35% of the treated mice (18 out of 51) were cured after a combined treatment, whatever the electric field used between 1500 and 1200 V/cm. At lower voltages, fewer CRs were observed, more recurrences occurred (1 as late as day 65 and another on day 98) and fewer (1100 V/cm) or no cures (below 1100 V/cm) were obtained. Thus, with 500 μg bleomycin and 8 pulses, the minimal efficient field intensity required was 1100–1200 V/cm.

Less easy to quantify was the constant observation about local cutaneous and subcutaneous reactions. The more the electric field was reduced, the more oedema and scabs were attenuated or disappeared rapidly. Similarly, no death was observed after the combined treatment when 1400 V/cm or less intense electric fields were applied (in Table 1, only 1 early death was observed, at 1500 V/cm).

*Table 2. Effects of electrochemotherapy on LPB sarcomas as function of number of pulses and of pulse intensity* (500 μg *bleomycin*)

| | 1500 V/cm | | | 1200 V/cm | | |
|---|---|---|---|---|---|---|
| | Day 0 | Day 7 | Day 120 | Day 0 | Day 7 | Day 120 |
| 8 pulses | | | | | | |
| Tumours | 33 | 7 | 0 | 21 | 6 | 0 |
| CR | — | 24 (73%) | 5 (15%) | | 12 (57%) | 6 (29%) |
| 3 pulses | | | | | | |
| Tumours | 20 | 0 | 0 | 30 | 13 | 0 |
| CR | — | 20 (100%) | 3* (30%) | | 17 (57%) | 2† (20%) |

Early deaths occurred with 8 pulses (2 and 3 mice treated with 1500 and 1200 V/cm, respectively).
*Only one cage of 10 mice out of two and †only one cage out of three were followed up to day 120.

Short-term experiments on C57Bl/6 mice bearing a LPB tumour in the left flank revealed that, both at 1500 and at 1200 V/cm, combined treatment with 3 pulses instead of 8 also induced substantial regressions (Table 2). Local reactions, however, were different. After 3 pulses at 1200 V/cm, oedema was almost absent and 3 days after the treatment, already 5 CRs were observed among 30 treated tumours. At 1500 V/cm and 3 pulses and at 1200 V/cm and 8 pulses, oedema was still found but regressed more rapidly than after 8 pulses delivered at 1500 V/cm. Consequently, CR, unmasked by local inflammatory reaction, could be detected earlier. Moreover, no deaths were observed when only 3 pulses were delivered, even at 1500 V/cm. Long-term follow-up showed no major differences in the number of pulses used in the combined treatment (Table 2).

C57Bl/6 mice bearing LPB tumour in the left flank received three weekly combined treatments at 1500 V/cm with only 3 pulses and only 50 μg bleomycin per treatment to reduce the cutaneous and subcutaneous reactions as well as the global drug toxicity (Table 3). A single treatment at this reduced dose of bleomycin was as efficient as a single treatment with ten times more (compare Tables 1 and 3). When treatment with the low dose was repeated three times, efficacy was enhanced (Table 3). Indeed, the repeated combined treatments resulted in 50% cures and no signs of cumulative drug toxicity.

*Table 3. Effects of single or three-times repeated electrochemotherapy* (1500 *V/cm*) *on LPB sarcomas with* 50 μ*m bleomycin*

| | Day | | | | |
|---|---|---|---|---|---|
| | 0 | 7 | 20 | 35 | 120 |
| Once | | | | | |
| Mean (S.D.) | 84 (42) | 4 | 180 (200) | 1900 (1100) | |
| Tumours | 10 | 1 | 6 | 7 | 0 |
| CR | — | 9 (90%) | 3 (30%) | 3 (30%) | 3 (30%) |
| Thrice | | | | | |
| Mean (S.D.) | 56 (50) | | | 420 (580) | |
| Tumours | 10 | 0 | 0 | 4 | 0 |
| CR | — | 10 (100%) | 10 (100%) | 6 (60%) | 5 (50%) |

## DISCUSSION

Electrochemotherapy, i.e. the combined treatment associating bleomycin and local electric pulses, has a considerable antitumour effect. In conventional C57B1/6 mice, we observed 100% at least PRs in murine LPB and B16 syngeneic tumours, and under the most efficient conditions up to 50% cures. Human KB tumours in nude mice also regressed considerably after electrochemotherapy. As suggested by our previous *in vitro* data [1], the increased permeability of the tumour cells after electric field application should, at least partly, account for the potentiation observed *in vivo*.

Only one attempt to combine bleomycin and electric pulses *in vivo* has been reported. Mohri and Okino [15] obtained PRs of AH-109A hepatocellular carcinoma in Donryu rats after the combined administration of bleomycin and one exponentially decaying intense (5000 V/cm, 2 ms) pulse. These conditions produced oedema even in the absence of drug and caused necrosis of the surrounding skin when used in combination with bleomycin.

In more appropriate conditions as in our present work, electrochemotherapy is appealing because of the extent to which bleomycin's antitumour activity was potentiated. Preliminary experiments with BLM doses as low as 0.5 μg achieved deceleration of tumour growth similar to that observed with the non-potentiated high doses of bleomycin (1 mg per day for 5 days). Thus, the *in vivo* potentiation obtained by the electric treatment was about 10 000 fold.

Our results suggest that a substantial fraction of the tumour cells was killed by the combined treatment. However, all the cells were not probably killed. Indeed, we observed a great difference in response to the same treatment of the same LPB tumour between immunodeficient nude mice and immunologically reactive C57B1/6 mice. Thus, the host's immune response could be instrumental in the elimination of tumour cells after massive cell lysis due to electrochemotherapy.

Whatever the mechanism of potentiation, the local presence of the electric field is essential. First, a minimum intensity of the electric field of at least 1100–1200 V/cm was required for drug potentiation. Secondly, we repeatedly observed that all tumour areas that were not exactly between the electrodes escaped treatment and continued to grow with a slope identical to that of control animals which received bleomycin alone. These findings suggest that most of the treatment failures in C57B1/6 mice can be explained by the fact that the corresponding tumours slightly exceeded the maximum area covered by the electrodes.

The toxicity observed during the experiments on C57B1/6 mice can be partly explained by the dose of bleomycin (500 μg, i.e. one-tenth of the $LD_{50}$) and by the fact that, due to the slenderness of the mice, the so-called local treatment was in fact a regional treatment. Indeed, necropsy revealed that the external portion of the liver (just beneath the tumour) was also necrosed when treated tumours were located at the right flank. This observation probably explains the difference in toxicity observed during the treatment of tumours located either at the right or at the left flanks. Nevertheless, this toxicity is not a problem since in bigger animals or in man local treatment will have a genuine local action without secondary regional effects and a lower dose (50 μg instead of 500 μg) proved to be efficient and did not cause death (Table 3).

Thus, *in vivo*, the local potentiation of bleomycin by electric pulses results in substantial antitumour activity even with the administration of small amounts of drug which limits side-effects. Electric pulses could also be used with other poorly permeable molecules, such as oligonucleotides directed against oncogene expression products. These molecules have specific targets and can discriminate between transformed and normal cells; however, oligonucleotides do not enter intact cells easily. Nevertheless, their uptake is increased *in vitro* after exposure of cells to electric fields [16].


1. Orlowski S, Belehradek J Jr, Paoletti C, Mir LM. Transient electropermeabilization of cells in culture. Increase of the cytotoxicity of anticancer drugs. *Biochem Pharmacol* 1988, **37**, 4727–4733.
2. Neuman E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lyoma cells by electroporation in high electric field. *EMBO J* 1982, **1**, 841–845.
3. Zerbib D, Amalric F, Teissié J. Electric field mediated transformation: isolation and characterization of a $TK^+$ subclone. *Biochem Biophys Res Commun* 1985, **129**, 611–618.
4. Presse F, Quillet A, Mir LM, Marchiol-Fournigault C, Feunteun J, Fradelizi D. An improved electrotransfection method using square shaped electric pulses. *Biochem Biophys Res Commun* 1988, **151**, 982–990.
5. Mir LM, Banoun H, Paoletti C. Introduction of definite amounts of nonpermeant molecules into living cells after electropermeabilization. *Exp Cell Res* 1988, **175**, 15–25.
6. Umezawa H, Maeda K, Yamauchi T, Toshoiku O. New antibiotics: bleomycin A and B. *J Antibiot* 1966, **19**, 200–206.
7. Sikic BI. Bleomycin. In: Droz JP, Cvitkovic E, Armand JP, Khoury S, eds. *Handbook of Chemotherapy in Clinical Oncology*. Paris, F.I.I.S., 1988, 143–144.
8. Sikic BI, Rosencweig M, Carter SK, eds. *Bleomycin Chemotherapy*. New York, Academic Press, 1985.
9. Povirk LF. Bleomycin. In: Neidle S, Waring MJ, eds. *Molecular Aspects of Anti-cancer Drug Action*. Weinheim, Verlag Chemie, 1983, 157–181.
10. Roy SN, Horwitz SB. Characterization of the association of radiolabeled bleomycin $A_2$ with HeLa cells. *Cancer Res* 1984, **44**, 1541–1546.
11. Belehradek J Jr, Barski G, Thonier M. Evolution of cell mediated antitumor immunity in mice bearing a syngeneic chemically induced tumour. Influence of tumor growth, surgical removal and treatment with irradiated tumor cells. *Int J Cancer* 1972, **9**, 461–469.
12. Eagle H. Propagation in a fluid medium of a human epidermoid carcinoma, strain KB. *Proc Soc Exp Biol Med* 1955, **89**, 362–364.
13. Crooke ST, Bradner WT. Bleomycin, a review. *J Med* 1976, **7**, 333–428.
14. Auerbach R, Morrissey LW, Sidky YA. Regional differences in the incidence and growth of mouse tumors following intradermal or subcutaneous inoculation. *Cancer Res* 1978, **38**, 1739–1744.
15. Okino M, Mohri H. Effects of a high voltage electrical impulse and an anticancer drug on *in vivo* growing tumors. *Jpn J Cancer Res* 1987, **78**, 1319–1321.
16. Bazile D, Mir LM, Paoletti C. Voltage dependent introduction of a d(alpha)octothymidylate into electropermeabilized cells. *Biochem Biophys Res Commun* 1989, **159**, 633–639.


**Acknowledgements**—We thank Dr C. Micheau for histological analysis, Monique Thonier for tissue culture, Marie-Thérèse Maunoury for statistical analysis and drawing of figures, Charles Gosse and Patrice Ardouin for animal house facilities and Lorna Saint-Ange for linguistic revision of our manuscript.