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## Research paper

# Antimetastatic effects of electrochemotherapy and of histoincompatible interleukin-2-secreting cells in the murine Lewis lung tumor

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**Murine Lewis lung (3LL) tumors are characterized by the appearance of lung metastases after a regular period following their s.c. transplantation. We tested the respective efficiencies of various antitumor treatments: (i) electrochemotherapy (ECT), i.e. the systemic injection of bleomycin associated with electric pulses, locally delivered, that permeabilizes the tumor cells; (ii) intratumoral injection of histoincompatible cells that have been engineered *in vitro* to secrete high amounts of interleukin-2; and (iii) the combination of these two treatments. The growth of the s.c. transplanted tumors was followed up and the number of lung metastases was counted 14 days after the treatment. ECT alone resulted in the reduction of both the size of the tumor and the number of lung metastases. This latter effect can be partially explained by the effects of ECT on the s.c. tumor mass from which 3LL cells escape to colonize the lungs. The injection of IL-2-secreting cells alone had no effect on the s.c. mass and only a limited effect on the number of lung metastases. However, the combined treatment ECT plus IL-2-secreting cells resulted in antimetastatic effects potentiation that could result from stimulation of a non-specific immune response through an increase of NK activity. [© 1998 Lippincott-Raven Publishers.]**

*Key words*: 3LL tumor, bleomycin, electrochemotherapy, interleukin-2, lung metastases.

## Introduction

Electrochemotherapy (ECT) is a new antitumor treatment based on the large potentiation of the antitumor effect of bleomycin (a cytotoxic non-permeant drug) by the local delivery of permeabilizing electric pulses.[1-3] These electric pulses result in the electropermeabilization (also termed electroporation) of the cells in the tissue, allowing an increased drug delivery inside the tumor cells.[4-6] Consequently bleomycin antitumor effects are greatly enhanced in the electric pulses-treated tumor, in the absence of side effects. We have described the bases of electrochemotherapy *in vitro*, including the mechanisms of cell death induced by bleomycin.[7,8] Using external electrodes, we showed ECT effectiveness on s.c. tumors in mice,[1,2] rabbits[9] and cats,[10] as well as its clinical feasibility on patients affected by permeation nodules of head and neck squamous cell carcinoma.[11,12] Other types of tumors have also been treated in patients.[3,13]

In previous experiments, we demonstrated that the host's immune system plays a central role in the long-term control of tumor growth and cure after ECT, and that the systemic injection of interleukin-2 (IL-2) results in an increase of the cure rate in mice.[14] We also reported the therapeutic benefits in mice of the local and continuous presence of IL-2 obtained by the injection of histoincompatible IL-2-secreting cells[15] in the peritumoral edema which appears after ECT.[16,17] We showed that the combination of ECT with IL-2-secreting histoincompatible cells can be almost 100% effective in causing the regression and cure of a locally treated, established immunogenic fibrosarcoma.[16] It also caused regression and cure of a smaller, contralateral, untreated established tumor of the same histological origin.[17] Finally, cured animals acquired a long-term memory and appeared to be protected against a later challenge by the same tumor cells. These effects can transform ECT from a local antitumoral treatment to a systemic anticancer treatment, that might also act on metastases.

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In this context, we analyzed the antitumor and antimetastatic effects of these treaments on a classical tumor model, the 3LL Lewis lung carcinoma[18] prone to regularly generate lung metastases. The 3LL murine model has been used to study the effects of surgery,[19] of chemotherapy[20] and of various biological response modifiers[21,22] on metastases development. The effects of the combination of conventional chemotherapy with immunotherapy have also been analyzed in the 3LL murine model.[23,24] The specific purposes of the work here presented were to evaluate the effects of ECT, of injections of IL-2-secreting cells, and of their combination on the growth of transplanted 3LL tumors and on the number of spontaneously arising 3LL lung metastases.

## Materials and methods

### Tumor model

3LL tumors were maintained by serial passages in C57Bl/6 mice (R Janvier, Orléans, France). Seven days before the ECT, tumors were explanted, mechanically dissociated and $10^6$ 3LL cells were injected in the left flanks of female C57Bl/6 mice 6–8 weeks old. Each experimental group comprised eight to 10 mice with tumors of 100–300 $mm^3$ on average.

### Treatments

*Electrochemotherapy.* BLM (10 μg) was injected i.v. and 4 min later, eight electric pulses of 1300 V/cm and 100 μs were applied at a frequency of 1 Hz through external electrodes located at each side of the tumor. The electric pulses were generated by a PS-15 cell electropulsator (Jouan, Saint-Herblain, France) and controlled using a digital storage oscilloscope VC-6025 Hitachi (Tokyo, Japan).

*Immunotherapy with histoincompatible, xenogeneic, IL-2-secreting cells.* Chinese hamster ovary CHO(IL-2) cells were cultured under the usual cell culture conditions in MEM culture medium supplemented with 8% fetal calf serum and antibiotics (Gibco, Cergy-Pontoise, France). Before their inoculation, cells were resuspended in MEM medium in the absence of serum and 100 μl aliquots containing $10^6$ CHO(IL-2) cells were injected in the peritumoral edema after ECT, or inside or close to the left flank tumor in the absence of ECT, at various times after the day of ECT.

### Follow-up

Tumors longest ($a$) and perpendicular largest ($b$) diameters were measured at regular intervals with a calliper-square. Volume was calculated by the formula $V=\pi ab^2/6$. Mean tumor volume is the mean of all the volumes, including 0 for complete regressions.

### Determination of the metastatic burden in the lungs

Animals were sacrificed 14 or 15 days after the day of the treatment of the ECT-treated groups. India ink was injected through the trachea to stain the normal lung tissue, leaving the metastases as uncolored spots that can be counted using a binocular microscope. After the India ink injection, lungs were removed and immersed in Bouin's fixative solution. After lung fixation, the number and the individual size of the metastases were determined.

### Statistical analysis

The statistical significance of differences between the groups was determined by Student's unpaired $t$-test.

## Results

### Effects of the various treatments on the growth of the s.c. transplanted tumors

Untreated tumors grew regularly and some untreated mice died before the end-point of the experiment (Figure 1). ECT produced a transient slight reduction of the mean tumor volume at day 2 and tumor growth was significantly delayed (Figure 1 and Table 1). Contrary to untreated group, no mouse died during the period between ECT and mouse sacrifice (Figure 1). The effects of the CHO(IL-2) cells alone on tumor growth were much less, both at day 2 and at day 14 after the beginning of the injections (Figure 1 and Table 1). Whatever the number of injections of the CHO(IL-2) cells (five injections, as in Figure 1 and in Table 1, experiments 1 and 2, or three injections, Table 1, experiment 3 and other data not shown), only a slight slow down of tumor growth was obtained. The combination of ECT and of injections of CHO(IL-2) cells resulted in significant antitumor effects in contrast with tumor growth in the untreated control mice (Table 1 and Figure 1). However, this combination did not significantly modify the tumor growth

with respect to ECT alone, except in experiment 1 (Table 1).

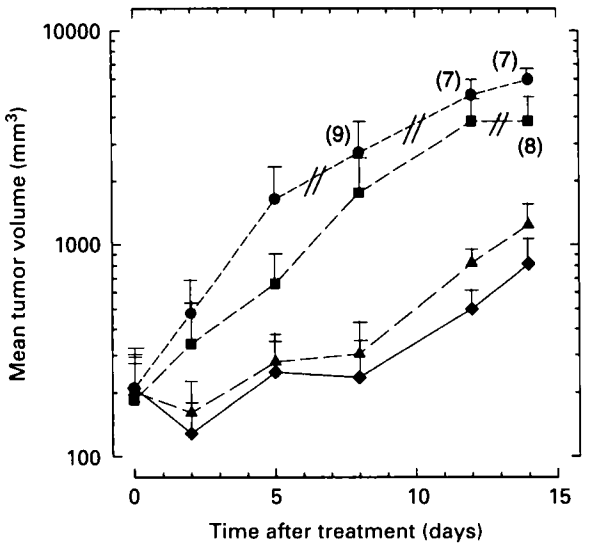


**Figure 1.** Tumor growth in mice treated by ECT or by injections of CHO(IL-2) cells or by their combination. Circles, controls (10 mice at day 0; numbers between brackets indicate the number of mice alive, showing that deaths occurred before the experiment end-point in mice with the largest and deepest tumors); squares, CHO(IL-2) cells alone (10 mice at day 0); triangles, ECT alone (nine mice at day 0); diamonds, ECT + CHO(IL-2) cells (nine mice at day 0).

**Table 1.** Tumor volumes 14 days after ECT or after injections of CHO(IL-2) cells or after their combination

| Experiment | Control | CHO (IL-2) | ECT | ECT+CHO (IL-2) |
|---|---|---|---|---|
| 1 | 100 ($n=10$) | – | $39\pm12^{b}$ ($n=10$) | $16\pm10^{b/c}$ ($n=12$) |
| 2 | 100 ($n=10$) | $65\pm30^{a}$ ($n=10$) | $27\pm18^{b}$ ($n=10$) | $15\pm8^{b}$ ($n=10$) |
| 3 | 100 ($n=7$) | $66\pm29^{a}$ ($n=8$) | $21\pm10^{b}$ ($n=9$) | $14\pm9^{b}$ ($n=9$) |

Results of three representative independent experiments are reported. In the first and second ones, the treatment using CHO(IL-2) cells consisted of five injections of $10^6$ cells at days 0, 1, 2, 3 and 4; in the third experiment, only three injections were performed at days 0, 1 and 2. Treatment efficacy was evaluated 14 days after the treatment. Results are reported as the percentage ($\pm$SD) of the mean of tumor volumes in the experimental group with respect to the mean of tumor volumes in the control group. Absolute values of the control tumors mean volumes at day 14 were: $3600\pm900$, $4400\pm1300$ and $6100\pm900$ mm$^3$ for experiments 1, 2 and 3, respectively. Significance of the differences in tumor volumes at day 14 is reported in the table ($^{a}p<0.05$ and $^{b}p<0.001$ with respect to the control group; $^{c}p<0.05$ with respect to the ECT alone group).

## Effects of the various treatments on the number of lung metastases

The effects of the various treatments on the small (diameter less than 1 mm) or on the large (diameter greater than 1 mm) metastases were different.

For the small metastases (Figure 2 and Table 2), injections of CHO(IL-2) cells alone did not result in a significant reduction in the number of metastases with respect to the untreated control group. ECT alone produced a significant reduction in the number of small metastases ($p<0.001$ in three separate experiments). When both treatments were simultaneously applied to mice, a significant reduction was obtained in the number of small metastases with respect to the treatment by ECT alone in some experiments as well as when all the data were analyzed together ($p<0.01$).

For the large metastases (Figure 3 and Table 2), the injections of CHO(IL-2) cells alone, the ECT alone as well as their combination displayed significant effects in some experiments ($p<0.05$) as well as when the data were analyzed together ($p<0.01$). However, the combined treatment did

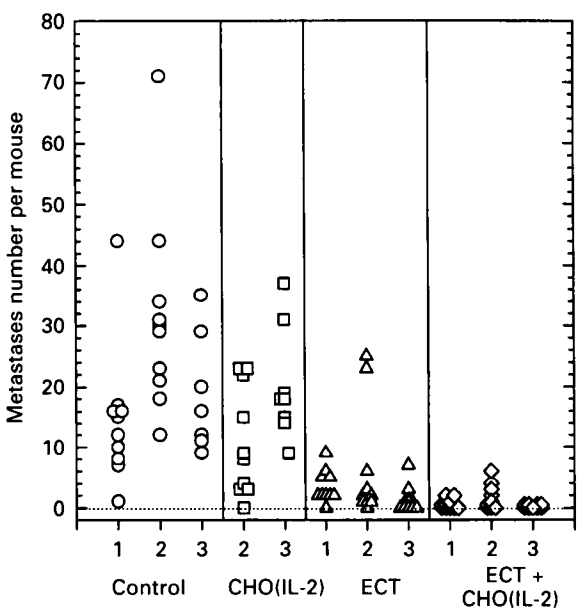


**Figure 2.** Number of small metastases, with an apparent diameter less than 1 mm, in mice treated by ECT or by injections of CHO(IL-2) cells or by their combination. Metastases numbers and sizes were determined as described in Materials and methods on groups of 27 (control), 18 (CHO(IL-2) cells), 29 (ECT) and 31 (ECT + CHO(IL-2) cells) mice corresponding to the mice entered in the three independent experiments of Table 1.

**Table 2.** Number of lung metastases 14 days after ECT or after injections of CHO(IL-2) cells or after their combination

| | Control | CHO(IL-2) | ECT | ECT+CHO(IL-2) |
|---|---|---|---|---|
| Mean number of metastases with diameter <1 mm | 22±15 | 15±10 | 3.9±6.0[b] | 0.7±1.4[b/c] |
| Mean number of metastases with diameter >1 mm | 7.7±6.3 | 3.1±3.2[a] | 3.0±3.7[a] | 1.7±2.7[a] |
| Number of mice without metastases | 0/27 | 0/18 | 5/29 (17%) | 16/31 (52%) |

The results of the three experiments separately reported in Table 1 have been pooled. The number and the size of lung metastases was determined after necropsy of the mice at day 14, staining of the lungs and visual determination of the presence of metastases, as described in Materials and methods, on groups of 27 (control), 18 (CHO(IL-2) cells), 29 (ECT) and 31 (ECT + CHO(IL-2) cells) mice. Values are mean ± SD. Significance of the differences in metastases numbers is reported in the table ([a]$p<0.01$ and [b]$p<0.001$ with respect to the control group; [c]$p<0.01$ with respect to the ECT alone group).

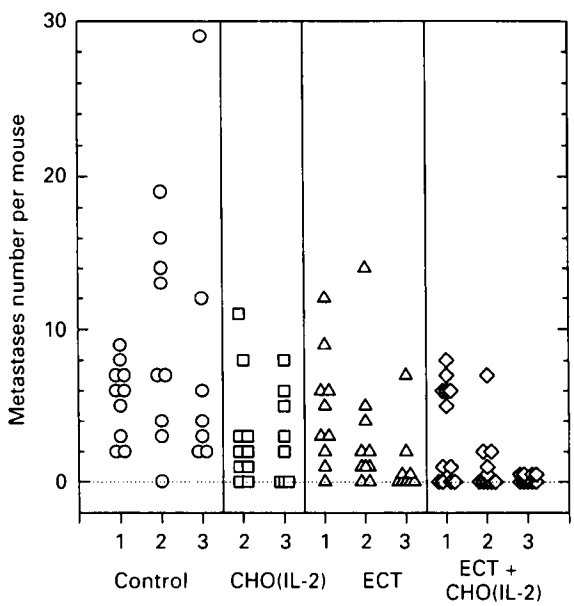


**Figure 3.** Number of large metastases, with an apparent diameter greater than 1 mm, in mice treated by ECT or by injections of CHO(IL-2) cells or by their combination. Metastases numbers and sizes were determined as described in Fig. 2.

not display a significant reduction of the number of large metastases with respect to the ECT alone.

These results can also be expressed according to the number of mice without metastases. All the mice of the control and CHO(IL-2) cells alone groups showed lung metastases at the necropsy. After ECT alone, 17% of the mice did not display lung metastases at the necropsy. This result was clearly improved in the case of the combined treatment, that resulted in more than 50% of mice without metastases (Table 2).

## Discussion

### Antitumor effects of ECT

Our results show that Lewis lung tumor treatment by ECT transiently slowed down or arrested the tumor growth and reduced the number of metastases counted at necropsy on a fixed day. Therefore, ECT had clear antitumor effects even if, contrary to situations previously reported with other murine tumors,[1,25,26] no complete regression nor cures of animals was achieved. 3LL has been a tumor model frequently used in the past.[19–24,27] It was already known that it was characterized by a poor immunogenicity.[22] Since we demonstrated the role of the host immune response to obtain cures after ECT,[14,17] the poor immunogenicity of the 3LL carcinoma can be related to the findings reported here that the 3LL tumors are less responsive than other immunogenic murine tumor models previously used.[1,25,26]

The local antitumor effects of ECT here reported should be due, as in other situations histologically documented, to a large tumor cell killing in the tumor mass.[9,10] This should result in a decrease in the shedding of metastatic cells that could explain the reduction in the number of lung metastases in the ECT-treated mice.

### Effects of the IL-2-based immunotherapy alone or in combination with the ECT

The IL-2-based immunotherapy alone did not result in net antitumor effects, neither on the volume of the transplanted mass nor in the number of metastases. However, compared to ECT alone, the combination of

ECT with the injections of CHO(IL-2) cells provoked a decrease of the metastases number and an increase in the number of mice without lung metastases. It is noteworthy that these beneficial effects were observed even if the combined treatment did not result in increased local antitumor effects on the s.c. transplanted tumor. Thus the reduction in metastases number observed after the combined treatment cannot be merely due to a decrease in the shedding of metastatic cells as a result of tumor cell death in the treated volume. Consequently, this reduction in metastases number could be due, at least in part, to stimulation of the host immune response by IL-2 in the context of the ECT-treated tumor. This host's anti-3LL response could be due to the stimulation of the NK activity since the role of the non-specific immune response and, in particular, the role of the IL-2-stimulatable NK cells has already been reported in the 3LL model.[27]

CHO(IL-2) cells seem to be an interesting immunotherapeutic agent when combined to ECT because, on an immunogenic fibrosarcoma, the prolonged presence of the IL-2 secreted by these xenogeneic cells may potentiate the host's immune response up to the effects previously reported.[17] In the weakly immunogenic 3LL tumor, we now report that the combination of ECT with the continuous presence of large amounts of IL-2 in the vicinity of the tumor (secreted by the intratumorally or peritumorally injected CHO(IL-2) cells) results in a clear decrease in the number of countable metastases. Therefore, in conclusion, our results suggest that it seems beneficial to combine ECT with a locally delivered IL-2-based immunotherapy not only in the case of highly immunogenic tumors but also in the case of weakly immunogenic tumors.

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## References

1. Mir LM, Orlowski S, Belehradek J Jr, Paoletti C. Electrochemotherapy: potentiation of antitumor effect of bleomycin by local electric pulses. *Eur J Cancer* 1991; **27**: 68–72.
2. Belehradek J Jr, Orlowski S, Poddevin B, Paoletti C, Mir LM. Electrochemotherapy of spontaneous mammary tumors in mice. *Eur J Cancer* 1991; **27**: 73–6.
3. Mir LM, Orlowski S, Belehradek J Jr, *et al.* Biomedical applications of electric pulses with special emphasis on antitumor electrochemotherapy. *Bioelectrochem Bioenerg* 1995; **38**: 203–7.
4. Neumann E, Sowers AE, Jordan CA. *Electroporation and electrofusion in cell biology*. New York: Plenum Press 1989.
5. Orlowski S, Mir LM. Cell electropermeabilization: a new tool for biochemical and pharmacological studies. *Biochim Biophys Acta* 1993; **1154**: 51–63.
6. Belehradek J Jr, Orlowski S, Ramirez LH, Pron G, Poddevin B, Mir LM. Electropermeabilization of cells in tissues assessed by the qualitative and quantitative electroloading of bleomycin. *Biochim Biophys Acta* 1994; **1190**: 155–63.
7. Tounekti O, Pron G, Belehradek J Jr, Mir LM. Bleomycin, an apoptotis-mimetic drug that induces two types of cell death depending on the number of molecules internalized. *Cancer Res* 1993; **53**: 5462–9.
8. Mir LM, Tounekti O, Orlowski S. Bleomycin: revival of an old drug. *Gen Pharmacol* 1996; **27**: 745–8.
9. Ramirez LH, Orlowski S, An D, *et al.* Electrochemotherapy on liver tumors in rabbits. *Br J Cancer* 1998; in press.
10. Mir LM, Devauchelle P, Quintin-Colonna F, *et al.* First clinical trial of electrochemotherapy for the treatment of cat soft tissue sarcomas. *Br J Cancer* 1997; **76**: 1617–22.
11. Belehradek M, Domenge C, Luboinski B, Orlowski S, Belehradek J Jr, Mir LM. Electrochemotherapy, a new antitumor treatment: first clinical phase I–II trial report. *Cancer* 1993; **72**: 3694–700.
12. Domenge C, Orlowski S, Luboinski B, *et al.* Antitumor electrochemotherapy: new advances in the clinical protocols. *Cancer* 1996; **77**: 956–63.
13. Mir LM, Glass LF, Serša G, *et al.* Effective treatment of cutaneous and subcutaneous malignant tumors by electrochemotherapy. *Br J Cancer* 1998; **77**: in press.
14. Mir LM, Orlowski S, Poddevin B, Belehradek J Jr. Electrochemotherapy tumor treatment is improved by interleukin-2 stimulation of host's defenses. *Eur Cytokine Netw* 1992; **3**: 331–4.
15. Roth C, Mir LM, Cressent M, *et al.* Inhibition of tumor growth by histoincompatible cells expressing interleukin-2. *Int Immunol* 1992; **4**: 1429–36.
16. Mir LM, Roth C, Orlowski S, *et al.* Potentiation of the antitumoral effect of electrochemotherapy by an immunotherapy with allogeneic cells producing interleukin-2. *C R Acad Sci (Paris) Sér III* 1992; **314**: 613–8.
17. Mir LM, Roth C, Orlowski S, *et al.* Systemic antitumor effects of electrochemotherapy combined with histoincompatible cells secreting interleukin-2. *J Immunother* 1995; **17**: 30–8.
18. Sugiura K, Stock CC. Studies in a tumor spectrum. III The effect of phosphoramides on the growth of a variety of mouse and rat tumors. *Cancer Res* 1954; **15**: 38–51.
19. Gorelik E, Segal S, Feldman M. Control of lung metastasis progression in mice: role of growth kinetics of 3LL Lewis lung carcinoma and host immune reactivity. *J Natl Cancer Inst* 1980; **65**: 1257–64.
20. Ishikawa T, Ura M, Yamamoto T, Tanaka Y, Ishitsuka H. Selective inhibition of spontaneous pulmonary metastasis of Lewis lung carcinoma by 5'-deoxy-5-fluorouridine. *Int J Cancer* 1995; **61**: 516–21.

21. Katz A, Shulman LM, Revel M, Feldman M, Eisenbach L. Combined therapy with IL-6 and inactivated tumor cells suppresses metastasis in mice bearing 3LL lung carcinomas. *Int J Cancer* 1993; **53**: 812–8.
22. Ohe Y, Podack ER, Olsen KJ, *et al.* Combination effect of vaccination with IL2 and IL4 cDNA transfected cells on the induction of a therapeutic immune response against Lewis lung carcinoma cells. *Int J Cancer* 1993; **53**: 432–7.
23. Tentori L, Leonetti C, Lozupone F, Bonmassar E. Antitumor and antimetastatic effects of dacarbazine combined with cyclophosphamide and interleukin-2 in Lewis lung carcinoma (3LL). *Cancer Immunol Immunother* 1995; **41**: 375–83.
24. Mastino A, Favalli C, Grelli S, *et al.* Combination therapy with thymosin alpha 1 potentiates the anti-tumor activity of interleukin-2 with cyclophosphamide in the treatment of the Lewis lung carcinoma in mice. *Int J Cancer* 1992; **50**: 493–9.
25. Heller R, Jaroszeski MJ, Leo-Messina J, *et al.* Treatment of B16 mouse melanoma with the combination of electropermeabilization and chemotherapy. *Bioelectrochem Bioenerg* 1995; **36**: 83–7.
26. Šersa G, Cemazar M, Miklavcic D, Mir LM. Electrochemotherapy: variable antitumor effect on different tumor models. *Bioelectrochem Bioenerg* 1994; **35**: 23–7.
27. Gorelik E, Fogel M, Feldman M, Segal S. Differences in resistance of metastatic tumor cells and cells from local tumor growth to cytotoxicity of natural killer cells. *J Natl Cancer Inst* 1979; **63**: 1397–404.

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