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Electrochemotherapy of Spontaneous Mammary Tumours in Mice

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Electrochemotherapy delivers external electric pulses to the tumour site to induce local potentiation of the antitumour activity of intramuscular injections of bleomycin. C3H/Bi mice with spontaneous mammary carcinomas received weekly injections of 50 μg bleomycin followed by electric pulses 30 min later. All the 38 tumours treated exhibited at least a partial regression. 23 complete remissions were observed, 3 of which were cures. One difficulty in assessing the cure rate in this model is that frequent parallel or sequential tumours cause early death. Electrochemotherapy appears similarly efficient in spontaneous tumours as in previously studied transplanted tumours.

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INTRODUCTION

In vitro, bleomycin's cytotoxicity is significantly enhanced in cultured cells by the delivery of electric pulses [1]. *In vivo* in C57B1/6 mice bearing transplanted tumours, electric pulses applied at the tumour site 30 min after administration of bleomycin lead to tumour regression and even cure in at least 30% of the treated mice [2]. We have termed this potential new anticancer treatment electrochemotherapy [2].

The potentiation of bloemycin's cytotoxicity, at least *in vitro*, relies on the fact that electric pulses make the cells transiently permeable [1, 3–5]. Consequently, drug uptake is not restricted by the plasma membrane. *In vivo*, the mechanisms involved are under investigation. To exclude the possibility that the previous findings were the result of particular relations between the transplanted tumours used and the host, we investigated the effect of electrochemotherapy on spontaneous mouse mammary tumours.

MATERIALS AND METHODS

Spontaneous mammary tumours of C3H/Bi mice

A colony of C3H/Bi mice, naturally infected by the Bittner murine mammary tumour virus [6], is maintained at the Institut Gustave-Roussy. Females are mated two to three times as soon as they are adult. Mammary tumours appear in these multiparous mice at 5–11 months of age. In most of the animals additional tumours appear at different sites and at various times after their initial tumour(s). Untreated animals die within 1–2 months after the detection of their first tumour(s).

Electrochemotherapy

Electrochemotherapy [2] was repeated every week until 1 week after the nodule was no longer palpable. Briefly, bleomycin (Laboratoires R. Bellon) was injected intramuscularly in the thighs of 10 mice (50 μg in 50 μl saline) 30 min before the delivery of square-wave pulses with a commercially available generator (Bioblock) [1, 3]. The distance between the electrodes (two stainless steel strips, 10 mm in width) was 6.7 mm. 6 randomly assigned mice received bleomycin alone as controls. In our study of several other tumour models [2], the electric pulses had no effect on tumour course. Moreover, similar electric pulses in the absence of bleomycin were not cytotoxic *in vitro* [1, 3].

In "small" tumours, only one run of electric pulses was delivered. A run of electric pulses consisted of 8 pulses of 100 μs and of 1500 V/cm at 1 Hz. In "large" tumours, we applied several runs (2–5) of electric pulses at adjacent positions to cover the entire exposed tumour surface.

Assessment of response

The tumour's longest diameter (a) and the next longest diameter (b) perpendicular to a were measured with a caliper at regular times. The mean diameter of individual tumours was calculated as $0.5\,(a + b)$. Tumour volume was calculated [7] by $V = ab^2\pi/6$, derived from the formula developed by Auerbach *et al.* [8].

Since the mice frequently develop more than one tumour, we scored each tumour individually. The course of the individual tumours after electrochemotherapy was scored as: (i) partial regression (PR); (ii) complete regression (CR); and (iii) cure. According to WHO guidelines, the therapeutic result was termed PR if the initial tumour volume decreased by at least 50% and CR if the tumour became unpalpable. The result was termed cure if the CR achieved at a given tumour site was maintained with recurrence until the animal's death at least 60 days after the first treatment of that tumour.

Because we searched for new tumours in our stock of old multiparous C3H/Bi females weekly, tumours had often reached a size that could not be entirely encompassed by our electrodes. Before this series of experiments, as a rule, electrochemotherapy was applied to tumours of less than 6.7 mm in mean diameter, which corresponded roughly to the maximum distance between the two electrodes. Tumours included in the present study were classified according to their mean diameter on the day of first treatment: small if less than or equal to 6.7 mm, and large if greater than 6.7 mm.

RESULTS

Small tumours

Almost all the tumours regressed, at least partially, within 2–3 weeks (Table 1, Fig. 1). As early as 7 days after the first

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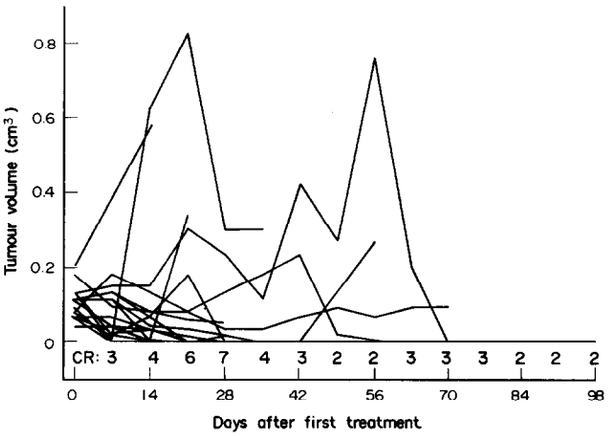

Fig. 1. Growth curves of small tumours treated with electrochemotherapy. Day 0 = day of first treatment. Overlapping curves have been omitted.

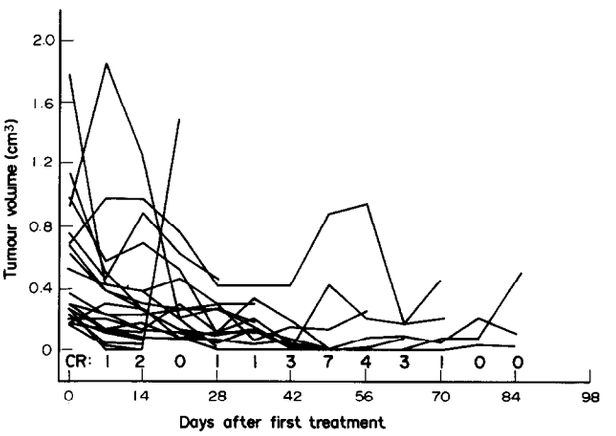

Fig. 2. Growth curves of large tumours treated with electrochemotherapy. Note different scale on y-axis.

treatment, 6 tumours showed PR and 3 CR. Among the 9 remaining tumours, 7 achieved PR or CR later, 1 decreased to 80% of initial volume and only 1 continued to grow. Among the 10 CRs, only 3 recurred. For the remaining 7 CRs, follow-up was not long enough to score the CR as a cure because the animals died within 60 days after the first treatment of the tumour concerned.

In fact, follow-up was often limited in time because most of the mice developed new tumours at various sites during the treatment of their initial tumour(s). For this reason electrochemotherapy was not interrupted when the initial tumour was in CR because these animals were treated continuously at other tumour site(s). Thus, after 10–12 successive treatments, the weekly administration of bleomycin sometimes led to death from cumulative toxicity. Thus, since long-term follow-up of most of the CRs was not possible, the number of cures is probably an underestimation. Despite the fact that follow-up for more than 60 days was only possible in 5 of the 18 treated small tumours, as many as 3 cures were achieved.

An unusual course in one of the treated small tumours is worth mentioning. After three treatments, this tumour showed a growth arrest only and after the fourth treatment, electrochemotherapy was stopped. The size of the tumour remained constant for 2 further weeks and only later decreased. CR was observed 6 weeks after treatment stopped and no additional tumours appeared during this period. Thus, after the four treatments, although regression was not immediately evident, the tumour tissues seemed to be efficiently inactivated.

Large tumours

Most of the large tumours rapidly achieved PR or CR (Table 1, Fig. 2). Obviously, the complete disappearance of palpable nodules was later than that for small tumours. Again, the evaluation of cures was not easy since the progressive appearance of new tumours in the animals under treatment and the constraint of a continuous weekly treatment leading sometimes to cumulative bleomycin toxicity did not allow a substantial gain in survival. Among the 10 CRs, 1 was in an animal which died without signs of recurrence at day 58 (i.e. 2 days before limit for classification as a cure). 3 other tumours were in CR at day 60 but they were not considered as cures because recurrences or new equivocal tumours located close to the initial tumour sites appeared at day 77 (Fig. 2).

Some large tumours showed a distinct course. They stopped growing but showed only limited regression. After 5–6 weeks of treatment, a scab was visible at the tumour site. Below the scab, the consistency of the tumour mass was not firm. During manipulation of these mice, the scab broke and a semi-solid necrotic substance was spontaneously extruded. By gently pressing the site, it was possible to remove the necrotic tumour mass almost completely. Thus, even if no reduction was observed in tumour size, necrosis supervened.

Furthermore, in two of these large tumours, growth persisted under the scab and necrotic mass. After 2–3 weeks, this led to a column-shaped nodule consisting of clearly necrotic tissue except at the base. We think that the scab and the necrotic tissue

Table 1. Effect of electrochemotherapy

Size	No. of tumours	PR	CR	Cures
Small	18	3 (17%)	10 (56%)	3 (17%)
Large	20	10 (50%)	10 (50%)	0

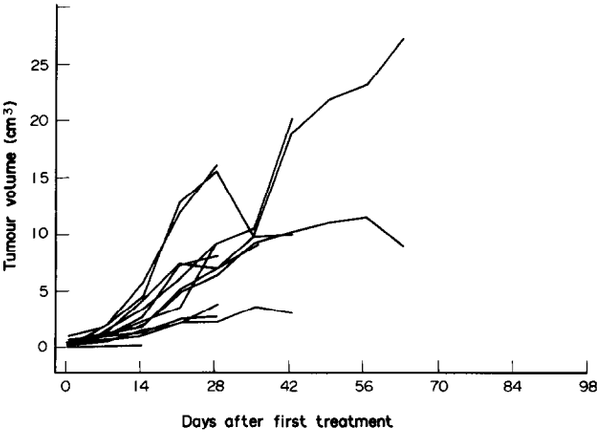

Fig. 3. Growth curves of control tumours. Note different scale on y-axis.

*Table 2. Age, survival and time of appearance of additional tumours**

	Age (mo)	Survival (days)	Appearance of additional tumours (days)
Treated			
1	10	123	21 28 63
2†	9	39	14
3†	10	121	49 55 63
4†	9	65	14 41 41 49
5†	7	82	7 28
6	11	29	—
7	11	48	24
8	5	219	120 157
9	8	108	61 70 70 89
10†	8	73	7 49
Median	9.2	82	
Control			
1	8	67	—
2	9	42	—
3	9	29	21
4	6	68	—
5	8	47	4 7 14 26
6	9	45	14 16 16
Median	8.7	47	

*Age at time of detection of first tumour; number of days of survival after detection of the initial tumour; time of detection of each new additional tumour after start of treatment of initial tumour(s).
†More than 1 tumour at beginning of treatment.

had formed a structure limiting the access of the electric field to the part of the tumour where growth persisted. Thus, removal of part of the necrotic mass might allow deeper penetration of the field.

Control tumours

Neither PR nor even growth arrests were observed in control mice (Fig. 3). Only a slight reduction in tumour growth rate was detected after six to seven bleomycin injections, which was probably related to the large tumour size reached at that time and not directly to drug activity. Moreover, it was sometimes difficult to measure tumour size (e.g., if tumours on the same animal became confluent). Given the limited number of tumour bearing animals available for study, we omitted a control group treated by electric pulses without bleomycin.

Survival

There was an overall increase in survival of treated mice from the day of the detection and first treatment of their initial tumour(s) compared with controls (Table 2). The median survival of the treated mice (82 days) was nearly double that of the controls (47 days). However, this increase was not significant ($P = 0.06$, Wilcoxon rank-sum test).

The reliable detection of new additional tumours in control animals was only feasible within the first month after the detection of the first tumour of each mouse, when initial tumours were not yet too large. During this period, 8 new tumours were detected in the 6 control mice and 8 in the 10 treated mice (Table 2). This situation contrasts with the data on the overall appearance of additional tumours (22 in treated and 8 in controls). The increased survival of the treated mice may explain the fact that more additional tumours were detected in treated mice than in controls. At the time of detection of the first tumour, the median age in both groups of mice was similar. When mice were examined individually, no correlation was noted between tumour occurrence and age.

DISCUSSION

Electrochemotherapy effectively treated these spontaneous tumours, even though the C3H/Bi mouse spontaneous mammary carcinoma did not always allow long-term follow-up and hence assessment of cure. Nevertheless, the main features reported with electrochemotherapy in transplanted tumours [2] were also observed in this spontaneous tumour model—i.e. PRs in most tumours followed by approximately 60% CR and cure of the tumour site in at least 3 of the 38 treated tumours. We newly observed massive necrosis with complete sterilisation of the tumour *in situ* which did not necessarily result in the rapid resorption of the necrotic tissue.

A tendency towards increased survival was noted after electrochemotherapy compared with controls treated with drug alone, and this was independent of the course of the individual tumours. However, this gain in survival was limited by the appearance of additional tumours in some animals, a characteristic inherent to this particular tumour model.

The initial sizes of the small tumours analysed were similar to those of the transplanted tumours treated in our previous study [2]. However, it was difficult to make an accurate comparison of the rate of definitive cures between these transplanted tumours and the spontaneous small tumours because long-term follow-up was not feasible. Indeed, the cure rates of individual spontaneous tumours might have been higher but a direct comparison of spontaneous and transplanted tumours was impossible, essentially because the transplanted tumours had never received more than 1–3 treatments and had never been treated continuously. A comparison is nevertheless possible between the small mammary carcinomas of similar sizes to the transplanted tumours. On the whole, results were similar between the spontaneous and the transplanted tumour model.

The results for the treatment of large tumours also indicated that it is possible to treat tumours whose size exceeds the distance between the electrodes, this distance being imposed by the output voltage characteristics of our generator. Access of the electric field to the central core of the tumour was the main theoretical problem for these large tumours. However, a step-by-step treatment at the periphery and the top of the tumour mass resolved this problem. The use of a more powerful generator with appropriate electrodes should facilitate the use of electrochemotherapy for the treatment of even larger tumours in larger animals and in man.

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Relative Role of Bone and Kidney in the Hypercalcaemia Associated with the Rat Walker Carcinosarcoma 256

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The rat Walker carcinosarcoma 256 is an animal model for humoral hypercalcaemia of malignancy (HHM). In this model, the relative contribution of bone and kidney in the hypercalcaemia of tumour-bearing rats was investigated. Daily administration of pamidronate, a bone resorption inhibitor, for 2 days prevented the increased fasting Ca^{2+} excretion observed in the hypercalcaemic rats, although serum Ca^{2+} remained high. However, the high serum Ca^{2+} normalised after the acute injection of ethiofos, an inhibitor of renal Ca^{2+} reabsorption, which was associated with a marked increase of Ca^{2+} excretion. Changes in Ca^{2+} were accompanied by similar changes in Mg^{2+}. The results indicate that altered renal Ca^{2+} handling has a key role in the hypercalcaemia associated with this HHM model.
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INTRODUCTION

THE WALKER carcinosarcoma 256 implanted in the rat is one of the few known models for humoral hypercalcaemia of malignancy (HHM) [1]. Both increased bone resorption and stimulation of renal calcium (Ca^{2+}) reabsorption appear to contribute to the hypercalcaemia associated with this tumour [2–4]. A factor(s) which interacts with adenylate cyclase-coupled parathormone (PTH) receptors in bone cells has been partly purified from Walker tumour cells [5]. In addition, we have isolated from this tumour a chromatographic fraction displaying PTH-like and growth factor activities for renal cells (ref. 6 and unpublished). However, the relation between these activities and either the increased bone resorption or the stimulated tubular Ca^{2+} reabsorption is unclear [7].

The present study was done to evaluate the relative contribution of bone and kidney in the pathogenesis of the hypercalcaemia associated with this tumour. We studied the efficacy of pamidronate, a known inhibitor of bone resorption [8], and of ethiofos, a compound that inhibits renal Ca^{2+} reabsorption [9] in the treatment of hypercalcaemia in Walker tumour bearing rats.

MATERIALS AND METHODS

The Walker tumour 256, supplied by Prof. A.J.S. Davies (Institute of Cancer Research, London), was continuously transplanted into female Wistar rats (200 g) [3]. On day 8 after tumour implantation, the animals were put into restrictive cages. 3 days later, one group received pamidronate 21 μmol/kg (a gift from Ciba-Geigy, Basel) or saline daily for 2 days subcutaneously. This dose inhibits bone resorption in the rat [8]. On days 10 and 13, rat urine and plasma were collected after a 24 h fast [3].

Ethiofos, supplied by Dr M. Attie and Dr J.P. Bonjour (National Cancer Institute, Bethesda and University Hospital of Geneva, respectively) was dissolved in saline and adjusted to pH 7.0 with 1 mol/l sodium bicarbonate. On day 14 after tumour implantation, 5 ml 20% mannitol solution was administered intraperitoneably to promote an adequate urine flow. Urine was collected over 2 h, and blood was then taken by cardiac puncture under light ether anaesthesia. Then, ethiofos 0.7 mmol/kg or its solvent was given subcutaneously. This dose is adequate in the acute control of the hypercalcaemia associated with the Leydig cell tumour in the rat [10]. Blood and urine were again collected 2 h later. In some animals, ethiofos was administered after pamidronate treatment. Blood and urine were taken before and 2 h after ethiofos injection. On days 12 and 13 after tumour implantation, all the animals were given 10 ml saline intraperitoneally. This amount was sufficient to maintain normal sodium (Na^+) excretion [3].

Ca^{2+} and magnesium (Mg^{2+}) were measured by atomic absorption spectrometry. Inorganic phosphate (Pi) and creatinine were measured by colorimetry [11, 12]. Urinary Ca^{2+}, Mg^{2+} and Pi excretions were expressed as a molar ratio relative to urine creatinine.

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