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Charcoal tattooing of mammary carcinoma in mice

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Summary

We describe a step in the development of a charcoal suspension for tattooing human mammary carcinomas prior to surgery and chemotherapy. Two vehicles (injectable water and 0.9% NaCl) were compared. Migration of the charcoal particles was studied in vitro (gel model) and in vivo (intratumoral injection into nude mice). The charcoal suspension in injectable water was more effective in the detection of tumors.

The latest protocols for treatment of breast cancers include pre-operative chemotherapy for both inflammatory and bulky (> 3 cm) tumors. A major aim of such chemotherapy is to achieve a reduction in the size of the tumor (achieved in > 50% of cases), thereby rendering it accessible to conservative treatment.

However, in some instances where a good response is obtained, the residual tumor is no longer clinically detectable; therefore, the use of a tattooing technique is necessary in order for the surgeon to be able to operate with precision. For this purpose, intratumoral injections of charcoal suspensions have been proposed as a suitable protocol. The particles should undergo diffusion to the smallest possible extent and should not migrate into the surrounding tissues.

This paper reports the results of our in vitro (gel diffusion) and in vivo examination of two galenic forms of charcoal suspensions in order to ascertain an effective means of pre-operative tattooing of mammary tumors in nude mice.

Suspensions were prepared using peak charcoal (Norit 93153, Le Blanc Mesnil) micronized with the aid of a Jet ô Mizer (Lab-service, Macon, France) (Arriagada et al., 1990). The two vehicles employed were injectable water and 0.9% NaCl (Pharmacie Centrale des Hôpitaux, Paris). The charcoal was dispersed at a concentration of 4% using a swirling mixer (200 rpm, 20 min). The suspensions were sterilized by heating for 20 min at 120°C. Three pH measurements were per-

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formed on each suspension using a Multiparametric pH meter (P-407 MCNS II, Avantec-Bioblock). Particle size was established with a Coulter counter (Coulter TA II, Coultronics France SA, 95 Margency). Particle charge (ζ-potential) was determined using an Acoustophoretic analyzer (Penkem 7000, Noviprofibre, Eybens).

Gels were developed on the basis of the physical and chemical characteristics of human breast cancers, with analysis of the two variables (conductivity and pH) being carried out by means of the Multiparametric analyzer (P-407 MCNS II) equipped with a conductivity cell (YSI 3417, Bioblock).

Aqueous muscle-equivalent gels for use as in vitro hyperthermic cancer models were prepared according to the method of El Akoum and Gautherie (1988) with modifications as follows: 5 g methylmethacrylate-methacrylic acid polymer (Eudispert hv) (Rôhm Pharma), 1.48 g NaOH (Prolabo), 15 g polyethylene glycol 4000 (Prolabo) and 78 ml injectable water.

Square-shaped crystalline polystyrene petri dishes (BPC 12OE, Gosselin Plastique) were filled with 120 ml of gel and 1 ml of each suspension was injected into the gel via a tuberculin syringe fitted with a Térumo Luer 12 (22 gauge needle $\times$ $1\frac{1}{2}$, 0.7×30, 30×30, Leuven, Belgium).

Diffusion of charcoal particles within the gel was determined as the product of the maximum length and breadth of the peripheral diffusion area after 24 h at room temperature. Each suspension was tested three times. The results were expressed in cm^2 and analysed using an unpaired t-test; $P < 0.05$ was considered statistically significant.

Nude mice were grafted with tumors occurring spontaneously in nude mice and maintained monthly in the animal house of the Institut Gustave-Roussy. A 0.5 ml volume of filtered tumoral cell suspension was injected subcutaneously, the mice subsequently developing tumors of 1–2 cm within 3 weeks. Four batches of 10 mice were used. The first was treated with a 4% aqueous suspension of charcoal in injectable water, the second with injectable water, the third with 4% charcoal in 0.9% NaCl and the fourth with 0.9% NaCl alone.

TABLE 1

Properties of micronized suspensions of charcoal

	Injectable water	0.9% NaCl
Mean diameter (μm)	6.5 ± 0.6	5.7 ± 0.1
1.0–2.1 μm (%)	23 ± 0.1	32 ± 1.2
2.1–5.4 μm (%)	62 ± 0.2	57 ± 1.2
5.4–10.8 μm (%)	15.1 ± 0.2	10.6 ± 0.1
pH	5.38	7.17
Zeta potential	negative	0

A 0.1 ml volume of each preparation was injected into the tumors 15 days after grafting, by which time the mice had reached the age of 3 months and a mean weight of 24 g. One mouse was killed every 3 days and the tumor fixed in Bouin's solution, followed by inclusion in paraffin. Two further mice from each batch were killed 17 days after injection of the suspensions. The diffusion of charcoal particles was assessed macroscopically (tumor), and histologically (tumor, kidney, liver, spleen, lung, heart).

The mean diameter of the micronized charcoal particles in water was 6.5 μm, none being larger than 10 μm (Table 1). The mean diameter was smaller in 0.9% sodium chloride (5.7 μm), with a significant increase in the proportion of particles measuring between 1 and 2 μm. The pH of the suspensions was 5.38 in water and 7.17 in NaCl. At these pH values, the respective potentials were negative in water and zero in sodium chloride solution.

The pH of the gel was 7.3 and the conductivity 9 mS, these values corresponding to those reported previously for human breast cancers (Van der Berg et al., 1982; Surowiec et al., 1988). 24 h after injection the peripheral diffusion diameters were found to be significantly greater for the suspensions in NaCl (13.3 ± 2.4 cm^2) as compared to those in injectable water (8.2 ± 0.9 cm^2) (Table 2). The degree of migration observed with the suspension in sodium chloride was greater and appeared to involve smaller particles. Photographs of the gel are shown in Fig. 1.

With injectable water as vehicle, micronized charcoal was macroscopically evident in seven of

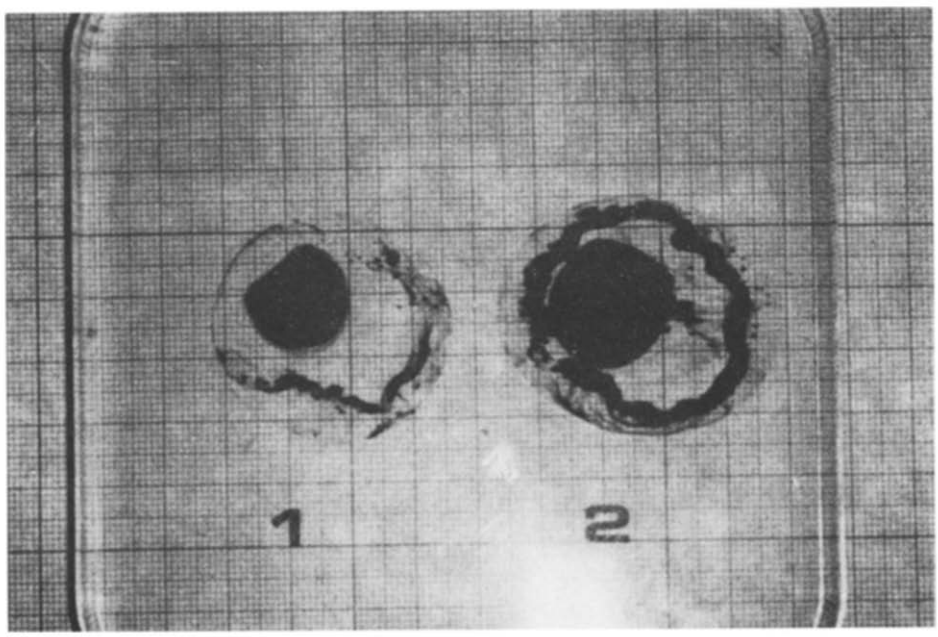

Fig. 1. In vitro diffusion study within the gel. (1) Charcoal in injectable water. (2) Charcoal in 0.9% NaCl.

TABLE 2

In vitro and in vivo diffusion of charcoal particles: effects of suspension vehicle

	Injectable water	0.9% NaCl
In vitro studies		
Peripheral diffusion area (cm^2)	8.3 ± 0.9	13.3 ± 2.4 [a]
In vivo studies		
Macroscopically visible charcoal	7/8	4/8
Histologically visible charcoal	black band 2–4 mm in diameter around the tumor, rarely within cells; sometimes within histiocytes	edge of the tumor in the form of clumps within histiocytes; areas of tumoral necrosis (4/8) within a few cells
Charcoal diffusion	very low	low

[a] $p < 0.05$.

the eight tumors studied and appeared as a black band of diameter between 2 and 4 mm. Histologically, particles were observed at the edges of the tumor in the form of clumps, frequently surrounded by fibrosis and occasionally within histiocytes (Fig. 2). The particles were often found in areas of necrosis, and in one case within a few tumoral cells.

With the sodium chloride suspension, particles were macroscopically evident within four of the eight tumors studied. Histologically, they were observed at the edges of the tumor within isolated histiocytes, but only rarely were they seen to be extracellular (Fig. 3). Particles were also found within areas of tumoral necrosis and, in four cases, within the cytoplasm of a few tumoral cells. The distribution of charcoal particles did not differ according to the day of killing nor did any acute inflammatory reaction occur around the particles. The degree of diffusion (histologic and macroscopic) was greater with NaCl as excipient, although remaining low. Diffusion into other organs in animals examined at day 17 did not take place, regardless of the excipient. In addition, the organs were histologically normal, without any

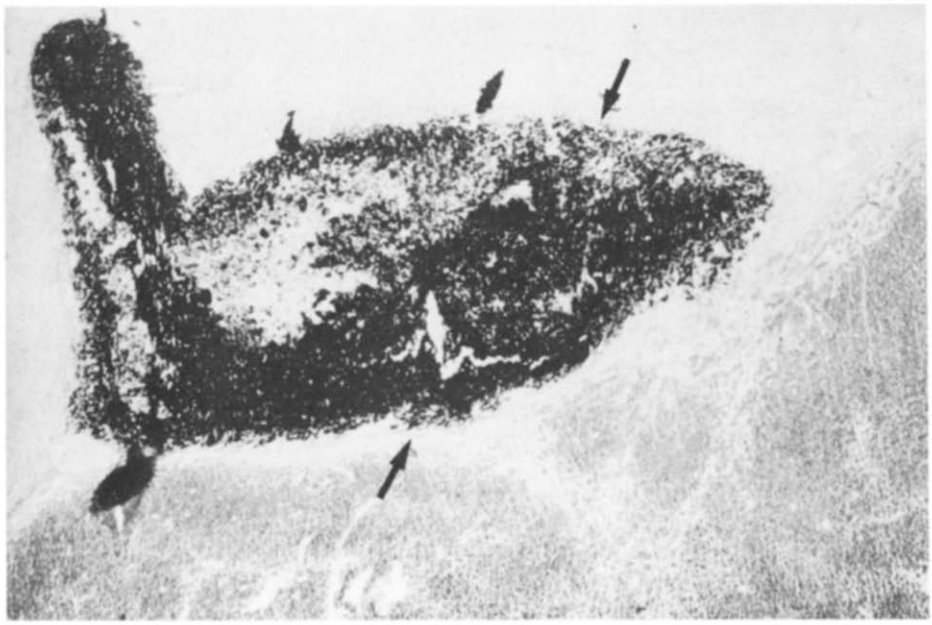

Fig. 2. Micronized charcoal with injectable water found in the form of a large clump. Particles are extracellular, or sometimes at the periphery within histiocytes (hematoxylin-eosin saffron, ×80).

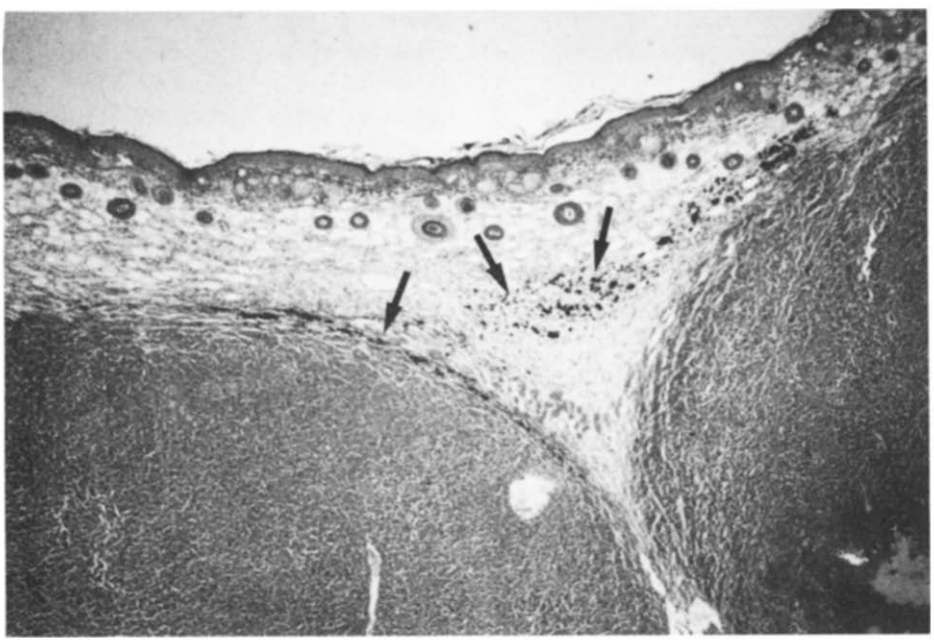

Fig. 3. Micronized charcoal with sodium chloride suspension was less visibly evident. Particles are found in the edge of the tumor essentially within histiocytes (hematoxylin-eosin saffron, ×80).

visible lesions. For the control mice, spleen, lung, kidney, heart and liver were normal.

A larger quantity of charcoal particles was found within tumors for the suspension in injectable water. The difference in the degree of diffusion between the two excipients could be explained as being due to the effect of osmosis. Indeed, the injection of a non-physiologic vehicle can lead to local tissue destruction which favors particle deposition and limits diffusion. The higher degree of diffusion observed with the suspension in sodium chloride solution could also be due to the slightly greater proportion of small particles with this excipient. Charcoal particles were seldom found within tumor cells, however, they were more frequently observed within histiocytes. This phenomenon occurred with both excipients but more markedly so with sodium chloride. This may be due to the charge on the charcoal particles.

Lampidis et al. (1985) have reported that a cell line derived from human breast epithelioma (MCF7) has a higher negative charge (−83 mV) than cells derived from monkey kidney tumor (CV1) (−48 mV) which are characterized by a phenotype of non-transformed cells. This could also in part explain the greater cytotoxic efficacy of lipophilic cationic substances.

In our model, the higher degree of charcoal-particle uptake by both tumoral cells and histiocytes with the sodium chloride suspension could be ascribed to the fact that the particles are uncharged, whereas in water they are negatively charged.

Preliminary studies demonstrated a strong correlation between the in vitro and in vivo diffusion data, suggesting that further investigations could be conducted using gels, thereby reducing the need for animal experimentation. The charcoal particles appeared to be well tolerated, since neither acute inflammatory responses nor foreign-body reactions were observed, in addition to diffusion into other organs not occurring.

The present results concerning the pre-operative tattooing of mammary tumors in nude mice show that tumors become detectable when a 4% suspension in injectable water is used. Investigations of toxicity are now required in order to determine whether such a suspension is suitable for tattooing human breast tumors prior to chemotherapy and surgery.

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