Cystemustine Induces Redifferentiation of Primary Tumors and Confers Protection against Secondary Tumor Growth in a Melanoma Murine Model

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ABSTRACT

N’-(2-Chloroethyl)-N-(2-(methylsulfonyl)-ethyl)-N’-nitrosourea (cystemustine) is a chloroethylnitrosourea that has been used in the treatment of human melanoma. Its main antitumor effect is DNA damage to malignant melanocytes. Although unreported at present, other effects may also account for its cytotoxicity, some of them could be more or less delayed with respect to its administration. In this report, we have developed a model of secondary tumor with B16 melanoma in syngeneic C57Bl6 recipients to investigate the impact of cystemustine treatment of primary B16 melanoma tumors on the fate of secondary implanted untreated tumors. The data presented in this report indicate that cystemustine-treated cells or the administration of cystemustine provoke an important growth delay of primary melanoma tumors, together with an increase in cell pigmentation and cell morphology changes. Data also show that prime treatment induces a dramatic decrease in tumor weight of secondary untreated tumors accompanied by an increase in melanin content and an alteration of cell morphology. Finally, 1H-NMR spectroscopy was performed on treated B16 cells, showing an alteration in the phospholipid derivatives of melanocytes, suggesting subsequent modifications of membrane phospholipid composition. In conclusion, the data highlight two important findings: (a) cystemustine produces modifications other than DNA damage, i.e., cell morphology changes, pigmentation, and phospholipid metabolism alterations, indicating an interference with cell cycle, cell redifferentiation, and proliferation programs; and (b) cystemustine-treated tumors appear to confer a protective effect against the development of secondary untreated tumors that may be mediated by cytokines or an immune response.

INTRODUCTION

Tumor cytotoxicity of chloroethylnitrosoureas is known to involve DNA damage, particularly interstrand DNA cross-linking (1, 2). However other mechanisms are likely involved in their cytotoxicity, such as heat shock protein, cytokines, or an immune response (3–5).

Cystemustine is a chloroethylnitrosourea agent that has been used in the treatment of melanoma in patients. Its main antitumor target has been demonstrated to be DNA, involving O6-chloroethylguanine generation and cross-linking with cDNA strands in malignant melanocytes (6, 7).

However, cystemustine induces other effects on tumor cells: (a) Buchdahl et al. (8) have reported that the exposure of murine melanoma B16 cells to cystemustine in vitro induced an arrest of growth in the G2 phase and stimulated the expression of differentiation markers of B16 melanoma cells, such as increased tyrosinase activity, and enhanced production of melanin. These modifications suggest that cystemustine interferes with cell cycle and cell differentiation programs. Others reports have shown that cystemustine induced apoptosis in human melanoma cell lines in vitro (9).

(b) In a clinical trial of cystemustine, some patients who were unresponsive after the therapeutic protocol underwent a delayed and sustained favorable response. The mechanism of that response remains unelucidated (10).

Indeed, a secondary improvement in tumors may be related to an indirect effect of cystemustine on malignant melanocytes that could involve cytokines and/or an immune response (11). In this report, we propose that cystemustine modifies plasmatic and cell melanocyte membranes. Proteins as heat shock proteins or phospholipids expressed at the plasmatic membrane can be detected as immunogenic (12, 13).

To this aim, we have developed a model of secondary tumor with B16 melanoma in syngeneic C57Bl6 recipients as a means of demonstrating a delayed effect triggered by cystemustine. Such a procedure was proposed in the past in epidermal graft studies (14). Our double tumor model involved a treated primary tumor and a secondary untreated tumor, the fate of which was followed according to the primary tumor processing.

To compare with the double tumor model, we studied the effects of cystemustine treatment on the growth of primary tumors, depending on conditions for its administration. Our therapeutic models were evaluated using tumor morphometry and histopathology with a contribution of NMR spectroscopy.

The data presented in this report indicate that not only does cystemustine provoke an important GD of primary melanoma tumors, but also cystemustine prime treatment induces a dramatic decrease in tumor weight of secondary untreated tumors. These data suggest a delayed and remote effect triggered by cystemustine in these recipients.

MATERIALS AND METHODS

Cell Culture. The transplantable B16 melanoma cells originating from C57Bl6/6J Ico mice were obtained from ICIG (Villejuif, France) and adapted to grow in culture (12). The melanocytes were maintained as monolayers in 75-cm2 culture flasks using culture medium consisting of Eagle’s MEM-glutaMAX medium (Life Technologies, Inc.) supplemented with 10% FCS (Boehringer) 1× vitamin solution (Life Technologies, Inc.), 1 mM sodium pyruvate (Boehringer) 1× nonessential amino acids, and 4 μg/ml gentamicin (Boehringer). The melanocytes were grown in a humidified atmosphere at 37°C under 5% CO2.

Chemicals and Drugs. Cystemustine was synthesized according to the procedures described previously by Madelmont et al. (7). It was then dissolved (5 mM) in 0.9% NaCl before being used in vitro in fresh culture medium or in vivo by injection to mice under several therapeutic protocols.

Cell Treatments. B16 melanocytes were harvested by trypsinization and plated 20 h before treatment for 2 h with 10, 50, and 200 μM cystemustine. After treatment, melanocytes were then rinsed in PBS (Boehringer) and maintained in fresh culture medium for 3 days before being inoculated into mice.

Animals and Treatment Protocols. Male C57Bl6/6J mice, 6–8 weeks of age, were purchased from IFFA CREDO (L’Arbresle, France). Mice were shaved before receiving s.c. injections into the right flank of 5 × 106 B16 melanoma cells, and cystemustine was given at a 15 μg/dose by i.v. or injected directly into the tumor (i.t.). In some experiments, to determine the
effect of *in vitro* treatment of cystemustine on the growth rate of tumors, melanocytes were pretreated with cystemustine for 2 h at 37°C at various doses and then washed and maintained in culture during 3 days in cystemustine-free medium. Following this incubation, $5 \times 10^6$ cells were inoculated s.c. to syngeneic mice. In others experiments, three treatment protocols were tested. In the first one, cystemustine was injected i.v. into the tail vein, starting before tumor growth, at days 1, 5, and 9 after B16 cell inoculation (protocol IV-1). In the other protocols, cystemustine was given after tumors become measurable either i.v. or i.t. at days 11, 14, and 19 after inoculation (protocols IV-2 and IT, respectively). Control mice received sham injection of physiological serum. At the end of each experiment, mice were sacrificed according to institutional guidelines for the animals’ welfare, and suitable conduct for experiments was followed.

**Histopathology.** Histological examinations of melanoma tumors were performed on untreated tumors, tumors derived from cells pretreated *in vitro*, and tumors treated with cystemustine i.v. and i.t. A piece of tumor was fixed in formal saline. Paraffin-embedded sections were cut into 4-μm sections, and tissue sections were prepared for H&E staining and routine pathological analysis.

**Challenge with B16 Melanoma Cells in Double Tumor Experiments.** Mice received injections of cystemustine-treated cells or were treated with cystemustine i.v. or i.t. to assess the effect of prime cystemustine treatment on the fate of secondary untreated B16 melanoma. On day 30, primed mice were challenged s.c. in the left flank with $5 \times 10^6$ B16 melanoma cells. Secondary tumor development and growth were evaluated twice a week.

**Morphometry: Tumor Growth Curve Analysis.** Eight days later, tumor surface areas were measured with a caliper square, and tumor weight was calculated using the formula by Dolan et al. (15): $W (g) = \frac{1}{2} \left( \frac{\text{width in cm}}{2} \right) \times \left( \frac{\text{length in cm}}{2} \right) \times \text{(width in cm)}^2$. Tumor dimensions were determined every 2 days. Mean tumor growth curves were fitted to the Gompertz function, as described by Spang-Thomsen et al. (16). For the determination of the growth curve parameters, the Gompertz function was linearized according to: Transformed tumor weight = $\ln [\ln (W_{max}) - \ln (W(t))] = \ln (B(t)) - \alpha \times t$, where $\alpha$ and $\beta$ are growth rate parameters, $W_{max}$ is the theoretical maximum tumor weight, and $W(t)$ is the tumor weight at growth time $t$. For treated tumors, the Gompertz fit followed a GD modeled as a flat growth period, and a consequence on the model was to replace $\beta$ by $t \cdot GD$. $W_{max}$ was determined in addition to $\alpha$ and $\beta$ parameters according to the best fit criterion.

Thus, the growth curve was established following the Gompertz function using two growth rate parameters: $\alpha$, which correlates with the starting growth rate of the tumor; and $\beta$, which is a measure of the intensity of growth slowing down during tumor evolution (17). The apparent slope of the Gompertz function thus accounts for both $\alpha$ and $\beta$ rates.

**NMR Spectroscopy.** $^1$H-NMR spectra were obtained on B16 cells. B16 cells were washed with PBS and then centrifuged at $400 \times g$. About $2 \times 10^6$ cells were freshly collected inside small NMR tubes (50 μl) designed to be spun at 4 kHz in an high-resolution magic angle spinning accessory (18) of a 500 MHz magnet (Bruker, Karlsruhe, Germany). The combination of a high field with the high-resolution magic angle spinning facility allowed us to obtain high-resolution spectra of small amounts of B16 cells, also to minimize susceptibility effects, e.g., because of air bubbles trapped inside the B16 cell suspension. B16 cells were resuspended in three D$_2$O saline drops to allow for locking the spectrometer. Thirty-two $^1$H free induction decays were collected to improve the signal-to-noise ratio in conditions of full relaxation of spins (repetition time, 10 s) and water suppression at low power, with a spectral width of 10 ppm and a sampling number of 8 K. $^1$H spectra were generated after Fourier transformation. No baseline correction was required in these experiments.

**Statistical Analysis.** Comparisons between parameters of control and treated groups were performed using Student’s $t$ tests.

**RESULTS**

**Tumors Developed from Inoculation of Cystemustine-pretreated B16 Cells.** Control and cystemustine-pretreated B16 cell tumor growth curves are shown in Fig. 1. Whereas untreated cells induced vigorous tumor growth (Fig. 1), tumors derived from cystemustine-pretreated cells were significantly reduced in size. In the control group, tumors became measurable at days 8–10, and the control tumor reached a maximum weight ($W_{max}$) of 5.24 ± 0.91 g, whereas for the groups inoculated with *in vitro* pretreated cells at 50 or 100 μM cystemustine, the tumors became measurable from days 14 to 18, and $W_{max}$ was reduced (3.32 ± 0.48 and 2.92 ± 0.94 g, respectively; Table 1). The pretreatment of B16 cells *in vitro* induced a significant tumor growth inhibition. However, when melanocytes were pretreated at 200 μM, we observed a strong decrease in $W_{max}$ or a long GD of 21 days. Tumors appeared after 40 days or later.

In these experiments, we had a roughly sigmoid dose-dependent response. As the cystemustine dose increased, the rate of tumor growth ($\alpha$ parameter) decreased nonlinearly, together with a decrease in $W_{max}$ and a prolongation in GD. At the highest dose, $W_{max}$ was $>50$ times less than the control one ($0.10 \pm 0.24$ versus $5.24 \pm 0.91$ g; $P < 0.001$), and the GD was 21 days. The small tumor growth after 40 days post cell inoculation indicates that the viability of inoculated B16 cells was preserved by the treatment at 200 μM.

Histological modifications were more contrasted on tumors derived from cells pretreated at 200 μM. The examination of these biopsy sections indicated that melanin content was increased in comparison with untreated tumors or tumors treated at lower doses. In addition, in some treated tumors, thrombosis was observed (Fig. 2, A–C). From dose-response findings, additional experiments were conducted with cells pulsed at 200 μM cystemustine, including experiments designed to follow the fate of secondary untreated tumors.

**Tumors during the IV-1 Protocol.** In the first experiment *in vivo*, we analyzed the effect of cystemustine given i.v. at days 1, 5, and 9 after cell inoculation. Fig. 3 represents the means of triplicate experiments and shows the growth curve of tumors treated with i.v. injections as compared with control (untreated) tumors. Treatment by cystemustine induced a GD of 17.9 days, a mild decrease in $W_{max}$ was $>50$ times less than the control one ($0.10 \pm 0.24$ versus $5.24 \pm 0.91$ g; $P < 0.001$; Table 1).

Histological examinations of IV-1 protocol tumors at day 40 after cell inoculation showed two areas (Fig. 2, D and E). The peripheral area exhibited alteration in cell morphology and increase in melanin content. In contrast, the core area looked like untreated tumors with regard to cell morphology, number of mitoses, and melanin content. From these observations, it appeared that when cystemustine was
and 5). When comparing IV-2 i.t.-treated tumors, GD was of 17.5 days, primary tumors and secondary tumors implanted 21–30 days later. In a first step, we conducted a double tumor experiment with untreated treated tumors. Illustrated in Fig. 2, of mitoses was strongly reduced by 10-fold. Cell morphology alteration of the microscopic field was about 90–120, whereas in treated tumors the number of mitoses was strongly reduced by 10-fold. Cell morphology alteration was homogeneous and concerned all of the treated tumors, as illustrated in Fig. 2, F and G. The melanin content of treated tumors was three times below that of the control. The melanin content of treated tumors was three times below that of the control.

### Tumors during the IV-2 and IT Protocols.

Three groups of mice were used. One group was left untreated and represented the control group. Another one was treated with cystemustine by i.v. injections (IV-2 group). In a third group, cystemustine was directly delivered into the tumor (IT group). Cystemustine was given at days 11, 14, and 19 after B16 cell inoculation either i.v. or i.t. In all experiments, no important toxicity was observed in any of the animals, as assessed by weight loss.

In IV-2-treated tumors, GD was 11.8 days; α was similar to that of control (0.169 ± 0.020 versus 0.158 ± 0.011 day⁻¹; P = not significant; Table 1), although Wmax was 2.5 times below that of the control curve (1.92 ± 0.82 versus 4.91 ± 0.46 g; P < 0.001). In i.t.-treated tumors, GD was of 17.5 days, α was similar to that of control (0.132 ± 0.012 versus 0.136 ± 0.007 day⁻¹; P = not significant; Table 1), although Wmax was three times below that of the control curve (1.57 ± 0.33 versus 4.82 ± 0.72 g; P < 0.001; Figs. 4 and 5). When comparing IV-2 versus IT protocols, the i.t. treatment was more efficient on GD (P < 0.001).

In both treated tumors, the inhibition of growth was accompanied by several histopathological modifications. Cystemustine treatment induced deep alterations concerning cell morphology, cell cycle, and melanin content. In control tumors, the number of mitoses/microscopic field was about 90–120, whereas in treated tumors the number of mitoses was strongly reduced by 10-fold. Cell morphology alteration was homogeneous and concerned all of the treated tumors, as illustrated in Fig. 2, F and G. The melanin content of treated tumors was strongly increased and appeared similar in IV-2 and i.t. protocol-treated tumors.

### Fate of Secondary Tumors in Primed Cystemustine Recipients.

In a first step, we conducted a double tumor experiment with untreated primary tumors and secondary tumors implanted 21–30 days later. Initial growth curves of both tumors over 2 weeks after B16 cell inoculation were similar. The growth curve of secondary tumors could not be followed any longer, because the mice died (data not shown). Double tumor experiments with treated primary tumors were performed as follows. Thirty days after the cessation of exposure of primary treated tumors to cystemustine in protocols IV-1 and IT, each recipient was orthotopically inoculated with 5 × 10⁵ untreated B16 cells.

Secondary tumors showed small GDs of 1.4 and 3.5 days for IV-1 and IT groups, respectively (Figs. 6 and 7). Also α of secondary tumors was similar in both IV-1-treated and control groups (0.171 ± 0.010 versus 0.163 ± 0.008 day⁻¹; P = not significant; Table 1). Only the IT group exhibited a small, nonsignificant decrease in α parameter. However, Wmax of secondary tumors were 6- and 15-fold lower than the control one for the IT (0.76 ± 0.40 versus 4.78 ± 0.49 g; P < 0.001) and the IV-1 (0.28 ± 0.07 versus 4.78 ± 0.49 g; P < 0.001) groups. The fate of secondary tumors was thus mainly reflected by Wmax rather than by GD or α. Data are summarized in Table 1. Similar results were obtained when primary tumors were treated with cystemustine in the IV-2 protocol as well as when cells were treated with cystemustine in vitro before inoculation (data not shown).

The second inoculation of untreated cells did not break the contemporary growth of primary tumors; in others words, the secondary inoculation did not promote an accelerated growth of the primary tumor. Although secondary untreated tumors had never been in contact with cystemustine, histopathological analysis of these tumors revealed an increase in melanin content and a strong necrosis of the tumor, with large and numerous necrotic areas corresponding to >50% of the tumor (Fig. 2, H and I).

### 1H NMR Spectroscopy of Melanocyte Cell Cultures.

1H NMR spectra showed that the main change in B16 cell spectrum 3 days after pulse exposure to cystemustine was an increased signal in the 3.22–3.23 ppm area. This signal corresponds to phospholipid derivatives, i.e., GPC, phosphocholine, and phosphoethanolamine (Fig. 8). More detailed analysis of this strong modification in treated cell cultures and in tumors in vivo is reported elsewhere.4

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4 Submitted for publication.
DISCUSSION

In the present study, a set of murine tumor models involving different procedures of administration of cystemustine allowed us to demonstrate that: (a) cystemustine produces modifications other than DNA damage (1, 2), which may participate in its cytotoxicity (3, 4); and (b) cystemustine prime treatment interferes with the fate of secondary untreated B16 melanoma in the same recipient, thus appearing to confer some protective effect.

Our tumor weight data have been fitted using the Gompertz function. This processing has proved efficient for the quantitative determination of therapy-induced cell destruction. The recording of growth data for 3 weeks after B16 melanoma cell inoculation provides sufficient bases for determining the course of growth for untreated tumors and thereby provides an unequivocal reference for the individual treated tumors (16, 17).

We investigated the cystemustine effect on melanoma tumor growth in a syngeneic murine model. We demonstrated in these experiments that cystemustine induced a tumor weight ($W_{max}$) decrease and a significant GD whatever the therapeutic protocol, inoculation of cystemustine pretreated cells or cystemustine administered i.v. and i.t. A hierarchy between treatment protocols can be established when considering GD and $W_{max}$ parameters, yielding to a gradient of effectiveness for cystemustine treatment according to the used protocol. We may classify the efficiency of protocols on tumor growth as: in vitro pretreatment $\geq$ IV-1 $\geq$ IT $> IV-2$.

Usually, the IV-2 protocol is used in clinics, because cystemustine is administrated i.v. every 2 weeks during 2 months. The IV-1 protocol would correspond to a rare condition in humans, because it would model the treatment of an early, even infraclinical, tumor.

Fig. 2. Morphological features of B16 melanoma tumors developed in C57BL6 recipients from days 20 to 40 after cell inoculation. A, control tumor. Histology shows that 30 days after cell inoculation, the number of mitoses was elevated, and cells appear aggressive and have a weak melanin content ($\times 200$). B and C, tissue from tumors developed from B16 cells pretreated with 200 $\mu$g cystemustine in vitro prior to inoculation. Thirty-five days after B16 inoculation, B shows that melanin expression is increased, and C shows thrombosis 35 days after cell inoculation (B and C, $\times 80$). D and E, tumors treated using IV-1 protocol. D shows that the treated tumor has two distinct areas ($\times 20$). E shows a peripheral area with a strong increase of melanin content associated with cell morphology alteration, and a core area that looks unaffected by cystemustine treatment ($\times 80$). F and G, tumors treated using IV-2 ($\times 200$) and IT ($\times 320$) protocols, respectively, showing a striking homogeneity in the increase of cell pigmentation. Cells have a modified morphology with a large cytoplasm, and appear less aggressive as compared with the control. IV-2 and IT protocols displayed similar changes 27 days after cell inoculation. H and I, morphological features of B16 melanoma secondary tumors in IV-1-pretreated recipients, 20 days after challenge with untreated B16 cells. H reveals intense pigmentation of the secondary tumor, which has never been in contact with cystemustine ($\times 80$), I shows large necrotic areas, in a secondary tumor partly destroyed. Similar histological findings were obtained when recipients were primed in IT protocols.

Fig. 3. Growth curves of control versus IV-1 protocol. Cells ($0.5 \times 10^5$) were inoculated s.c. at day 0 in both groups. In the IV-1 group, the cystemustine dose (15 $\mu$g/g) was injected i.v. at days 1, 5, and 9 after cell inoculation. Curves show a strong GD in the IV-1-treated group and a large difference in $W_{max}$ between both groups. Bars, SD.

Fig. 4. Growth curves of control versus IV-2 protocol. Cells ($0.5 \times 10^5$) were inoculated s.c. at day 0 in both groups. In the IV-2 group, the cystemustine dose (15 $\mu$g/g) was injected i.v. at days 11, 14, and 19 after cell inoculation. Curves show a strong GD in the IV-2-treated group and a large difference in $W_{max}$ between both groups. Bars, SD.
We found that the IT protocol improved the regression of melanoma tumors. Thus, delivering cystemustine directly into the tumor could represent a good strategy to treat patients, although it will depend on tumor accessibility and safety of the procedure (19). The comparison between IV-2 and IT protocols indicates that the efficiency of drugs depends on the route of cystemustine treatment.

The pretreated melanoma model proceeds from a different approach. It may be a model for secondary prevention in patients who are at risk of developing a cancer, in whom the administration of a low dose of cystemustine could impede a tumor development, or it could be a model for cell therapy aimed at providing protection against tumor growth.

The in vivo administration of cystemustine produced similar changes as those described in vitro (8). More recently, similar observations on B16 melanoma cells were obtained using as an agent a microbial extracellular glycolipid, which inhibited the growth of B16 cells by inducing apoptosis and increasing tyrosinase activity and melanin content (20).

Cystemustine therapy induced a strong decrease in the number of mitoses during the period of GD, an increase in melanin content, and an alteration of cell morphology. Cystemustine might have a vascular effect. When B16 cells were pretreated with cystemustine prior cell inoculation, thrombosis was obvious. This could result from a modulation of endothelial cell functions related to hemostasis and thrombosis. Furthermore, solid tumor growth >2-mm diameter depends on neovascularization, allowing a supply of oxygen and nutrients (21, 22). An effect on neovascularization could also participate in the arrest of tumor growth. More detailed analysis of vascular phenomena is beyond the scope of this article.

For the first time, having used a double tumor model, we demonstrate that prime treatment with cystemustine interferes with the fate of untreated secondary B16 melanoma in the same recipient. The effect of cystemustine cannot be direct; the half life of cystemustine and of its main metabolites in vivo is ≈50 min in humans and ≈30 min in mice (23), and the secondary tumor was implanted 30 days after the cessation of treatment of the primary tumor. Growth curves of secondary tumors show a marked decrease in Wmax. This growth pattern looks characteristic of secondary tumors. This finding is consistent with the observation of a large necrosis in secondary tumors, because it is estimated that necrosis may be an important factor for limiting tumor mass increase and for explaining Wmax (16). In addition, histopathological examination revealed an increased pigmentation of these tumors, despite that they never been in contact with cystemustine. This suggests that melanin changes, and thus increased tyrosinase activity, should result from a nonspecific process related to cell redifferentiation and cell cycle and proliferation programs.

The mechanisms by which the primary tumor impairs secondary tumor growth are not still defined. To our knowledge, there are no
reports to date of the induction of a protective effect of cytemustine or another chemotherapeutic agent against secondary untreated tumors. However, recently a relevant article by Adris et al. (24) demonstrated that treated colon cells with interleukin 12 gene transfer injected into healthy mice provoked an immune response against the parental tumors but also against syngeneic types of other tumors. The method was proposed as a possible vaccine.

The fact that the tumor weight of secondary tumors was markedly reduced could be related to the production of a humoral factor by immune cells such as antibodies, cytokines, or to the generation of specific cytotoxic T cells. It has been reported that DNA-damaging chemotherapeutic drugs, such as cis-diaminedichloroplatinum(II) and etoposide, elicited increased expression of death receptor DR5 to tumor necrosis factor-α in human glioma cells (25). Additional studies are needed to elucidate the precise mechanism(s) of action of primary tumors or other tissues (lymphoid tissue) on the fate of secondary untreated tumors (e.g., CTL activity and cytokine production measurements; Refs. 26 and 27).

We find from NMR spectroscopy measurements that the administration of cytemustine likely modifies cell and plasmatic membranes. NMR spectra show that cytemustine treatment alters phospholipid metabolism with increased expression of phospholipid derivatives such as GPC, phosphocholine, and phosphoethanolamine. The role of phospholipid, GPC, sphingomyelin, and ceramides in apoptosis and in cell cycle arrest has been well established (28–31). Phospholipid alteration under cytemustine treatment may be related to cell cycle, cell proliferation, cell redifferentiation program modification, or to cell signaling. The cytemustine-treated cell protocol has already been published (8). Cell growth arrest occurred from day 3 after cytemustine treatment. During the first 3 days, cells were blocked in the G2 phase. Our NMR measurements at day 3 were performed at this cell cycle stage. Long-term treated cell cultures have been investigated with NMR spectroscopy.2 Phospholipid changes at the plasmatic membrane could be involved in an immune response (12, 32–34).

Regarding a previous report that some patients underwent a delayed and sustained response after cytemustine treatment (10), one possible explanation supported by our double tumor model is that these patients could have developed a defense response against tumor growth that could involve a tumor necrosis factor-like factor or the immune system.

In summary, two major findings emerge from the present study: (a) the cytemustine chemotherapy agent induces in vivo modifications other than DNA damage, consistent with a redifferentiation pattern that may participate in its cytotoxicity; and (b) the challenge with B16 untreated cells into these recipients provokes a dramatic decrease of secondary tumor weight, accompanied by melanin modifications similar to those observed under cytemustine. Thus, cytemustine should elicit a humoral and/or immune cell mediator accounting for a delayed effect on secondary tumor growth. Therefore, cytemustine-treated tumors appear to confer a protective effect against the evolution and the growth of secondary untreated tumors.

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