Growth Support of Small B16 Melanoma Implants with Nitrosourea-sterilized Fractions of the Same Tumor

D. J. Dykes, D. P. Griswold, Jr., and F. M. Schabel, Jr.
Kettering-Meyer Laboratories, Southern Research Institute, Birmingham, Alabama 35205

SUMMARY

B16 melanoma cells sterilized in vitro with 1,3-bis(2-chloroethyl)-1-nitrosourea or in vivo with trans-1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea, have been used to enhance the percentage of tumor takes with small s.c. implants of viable cells and to reduce the latent period between tumor implantation and palpability. The admixture of trans-1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea-inactivated cells with viable cell implants reduced the number of cells required to produce tumors in 50% of the animals by approximately 3 log<sub>10</sub> units and markedly reduced the time of tumor appearance from implants of up to 10<sup>6</sup> cells. Similar results were obtained with 1,3-bis(2-chloroethyl)-1-nitrosourea-sterilized cells. The growth-supporting effect obtained with the nitrosourea-inactivated cells appeared to be as pronounced as that previously reported, for this tumor system, with radiation-inactivated cells.

INTRODUCTION

THE GSE<sup>3</sup> of lethally irradiated cells on VC was first reported by Puck and Marcus (9) in an in vitro cell culture system of human cervical carcinoma (HeLa) in 1955. In 1958, a GSE resulting from the admixture of radiatoinactivated cells with VC implants was reported to occur in several in vivo model tumor systems by Révész (10).

The application of this phenomenon to the study of small tumor cell populations has been routinely used by us (2, 3) and others (6, 14) for purposes of assaying for small numbers of cells, determination of tumor growth rates, and measurement of the effect of chemotherapy on small tumor cell populations. In many instances tumor growth cannot be initiated from small numbers of cells, say 10<sup>4</sup> or less, without use of lethally irradiated cells. One of us (12) has recently reported our control results with a variety of in vivo tumor systems. The interest resulting from this experiment has encouraged the study of a chemically induced GSE and its possible influence on chemotherapy.

It has been shown that, for many model tumor systems, implants of 10<sup>4</sup> to 10<sup>6</sup> tumor cells are required to initiate tumors (12). It might seem logical to assume that, in these solid tumor systems, chemical reduction of larger tumor cell implants to below the tumor "take" size should result in a high percentage of "cures." However, treatment 24 hr after implant of 10<sup>4</sup> to 10<sup>6</sup> tumor cells with even the best chemotherapeutic regimens seldom results in appreciable numbers of cures.

This failure to cure relatively small tumors has led us to suspect chemically inactivated tumor fractions as growth-supporting influences in the persistent recurrence of primary and metastatic tumors.

MATERIALS AND METHODS

The B16 melanoma originated on the ear of a C57BL/6J mouse as a spontaneous tumor (4). In our laboratories, the tumor has been maintained by serial passage in the host of origin; however, for all experimentation C57BL × DBA/2 F<sub>1</sub> mice were used.

Preparation of Monodispersed VCS. Approximately 1.0-g tumors were minced very finely in Locke's solution plus 0.5% glucose (with added penicillin, 20,000 units/100 ml, and streptomycin, 21 mg/100 ml) dissociating the cells by gentle flushing through a 5-ml glass syringe. The resulting cell suspension was then strained through a 420-μm mesh sieve and centrifuged in graduated tubes for a time sufficient to pack the cells gently. The supernatant was removed and the packed cells resuspended in 4 parts of Locke's solution, resulting in a 20% w/v cell suspension. Cell concentration was determined at this point by hemocytometer counts.

Preparation of ICS. A VCS, prepared as described above, was incubated in a water bath with BCNU, 80 μg/ml, for 1 hr at 37°, after which the suspension was centrifuged, washed 3 times, and resuspended in 4 parts of Locke's solution. Alternatively, a cell suspension was prepared, as described for VCS, from tumor harvested from animals treated 24 or 48 hr earlier with a supralethal dose of MeCCNU (160 mg/kg). This dosage, however, allowed the animals to live to the time of tumor harvest for implant preparation.

For preparation of radiation-inactivated cells, the VCS was placed in the presence of a 60Co source for a time sufficient to receive 10,000 rads. This dosage has been shown to be sufficient to sterilize every cell in very large...
Table 1

Tumor incidence following s.c. implantation of B16 melanoma cells in the presence of the various inactivated cell preparations

<table>
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<tr>
<th>Experiment</th>
<th>Type of ICS preparation</th>
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<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
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* Only ICS was implanted (0.5 ml).
* ICS was prepared 24 hr after treatment.
* The numbers in parentheses indicate the median day on which the tumors reached 200 mg (comparative day of tumor appearance).
* ICS was prepared 48 hr after treatment.
The percentage of tumor takes at the $10^1$ viable cell implant level (a point of reference for comparing the GSE) was 70 and 89%, respectively, for Experiments 1 and 2 and 70 and 90%, respectively for Experiments 3 and 4, an almost identical number of takes from the 24- and 48-hr MeCCNU-ICS preparations. However, the MDTA of the 48-hr preparation appeared to be slightly longer at both at $10^3$ and $10^4$ viable cell implant levels, but the difference (1 to 5 days) was not so great that it could not be attributed to the differences in viability of the viable cell preparations. No inhibitory effect, if any, from residual drug could be determined from our studies. Because of the generally good agreement between the 4 MeCCNU-ICS experiments, the data from individuals in these experiments were pooled and illustrated graphically in Chart 1. The TD$_{50}$ was estimated to be between $10^2$ and $10^3$ viable cells with added MeCCNU-ICS or $^{60}$Co-ICS, whereas, without the presence of ICS, the TD$_{50}$ was estimated to be between $10^2$ and $10^3$ cells. The MDTA from these sets of dilutions was also compared in Chart 1. As may be seen, the significance of the differences in MDTA at the $10^2$ and $10^3$ viable cell levels is high; $p = <0.001$ at both points.

The data of Experiments 5 and 6, in which BCNU-ICS was used, are shown graphically in Chart 2. Because of the obvious difference in viability of the VC preparation, the data were not pooled and are illustrated separately. In Experiment 5, the TD$_{50}$ was estimated to be near $10^3$ VC with added BCNU-ICS or $^{60}$Co-ICS, whereas the TD$_{50}$ for VC alone was between $10^2$ and $10^3$ cells. In Experiment 6 the TD$_{50}$ with added BCNU-ICS or $^{60}$Co-ICS was estimated to be between $10^2$ and $10^4$ VC, and with VC alone it was estimated to be between $10^2$ and $10^3$ cells. In both experiments, there were no differences in the MDTA’s of the ICS and non-ICS groups at the $10^3$ VC implant level, but there was a significant difference at the $10^4$ VC implant level.

**DISCUSSION**

Tumor growth has been shown to occur from much smaller cell implants in the presence of radiation-inactivated cells than otherwise would have occurred (10). A summary of the results from all experiments in which there was $\geq 50\%$ tumor takes was compiled in Table 2. These data show a very similar response from VC dilutions with added MeCCNU-, BCNU-, or $^{60}$Co-inactivated cell suspensions and a clear reduction of 3 to 4 log$_{10}$ units in the number of VC required to produce tumors. The GSE of lethally irradiated cells has been confirmed by many investigators (5, 15–17) including ourselves (12). The clinical implications of this phenomenon have been discussed by Révész (11), who has noted that incomplete cell destruction with radiation therapy (i.e., $>0$ surviving cells) in nonantigenic tumors may not be sufficient to prevent regrowth. Responses of VC to the GSE of MeCCNU-ICS, BCNU-ICS, and $^{60}$Co-ICS indicate similar effect but not necessarily similar mechanism of action. It would appear, as with curative radiation therapy, that chemotherapy trials designed for less than total cell kill would be insufficient for cure, particularly if a GSE occurred as a result of treatment.

The GSE is thought not to be a systemic influence but rather to result from local association of inactivated cells and VC (5, 17) with a criterion for the inactivated cells to be

<table>
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<th>ICS preparation</th>
<th>No. of experiments involved</th>
<th>No. of viable cells implanted</th>
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doomed to die, but metabolically active, for some period of time and to have intact membranes (17). The exact mechanism of action of GSE is not known. Hewitt et al. (5) have considered the possibility of immune suppression but concluded from their data that the effect could not be attributed to immune responsiveness or lack of it. Curiously, it has been shown that certain normal tissues, e.g., brain, as well as lethally irradiated cells from a foreign tumor type may be used to bring about a GSE (1, 5). The GSE has been observed to be increasingly more effective as the viable cell implant decreases in size (15); however, it is known that a naturally doomed to die but metabolically active subpopulation of cells exists in most large tumor cell populations as a result of cells going out of cycle (13). Peters and Hewitt (8) have conjectured that these dead cells may influence the progressive growth as well as the initiation of a tumor. If this is the case, it seems possible that a naturally occurring GSE may exist in large implant sizes due to the existence of nonviable tumor cells, particularly in the case of monodispersed implant suspensions prepared from solid tumors where the degree of viability is difficult to determine. Such is probably the case in Experiments 5 and 6 where neither the BCNU-ICS or 60Co-ICS significantly enhanced growth at the 10^7 VC implant level over the VC implant without added ICS.

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REFERENCES

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