Relationship of Antigenicity of Melanoma Cells Grown in 5-Bromodeoxyuridine to Reduced Tumorigenicity

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SUMMARY

B16 mouse melanoma cells (clone B5.59) grown in 1 to 3 µg of 5-bromodeoxyuridine (BUDR) per ml form few or no tumors in C57BL/6J adult mice when injected subcutaneously in doses at which untreated cells always form tumors. Injections of BUDR-grown cells protect adult mice against melanoma. Melanoma cells grown in 1 µg of BUDR per ml for almost 1 year (clone C471) retain a plating efficiency similar to that of untreated cells. When C471 cells are injected into C57BL/6J mice treated with antithymocyte serum or into neonates, tumors grow and kill all the mice. Melanoma cells grown in 3 µg of BUDR per ml for 14 days form no tumors in normal adult mice, but form tumors and kill 72% of antithymocyte serum-treated adults and 21% of neonates inoculated with 10^6 cells. Although their plating efficiency is reduced from control levels, the ability of BUDR-treated melanoma cells to grow in vivo is proved by tumor formation in immunologically compromised mice. Growth in both concentrations of BUDR greatly increases production of a C-type virus. C471 cells express Gross cell surface antigen, undetectable in control cells, and increase their expression of H-2^b antigen. The ability of BUDR-grown cells to protect against melanoma and to form tumors in neonates and antithymocyte serum-treated adults, in contrast to normal adults, and their increased production of virus and of cell surface antigens, all make it likely that one component of the loss of tumorigenicity is a change in antigenicity of these cells.

INTRODUCTION

Mouse melanoma cells (clone B5.59 derived from B16 melanoma) exhibit diminished or completely suppressed tumorigenicity, lose pigment, and gain "contact inhibition" during growth in 3 µg of BUDR per ml for 2 to 14 days or in

1 µg of BUDR per ml for 2 weeks or longer (11). All these effects are reversed when cells are cultured in RM (9) and appear to be dependent on DNA synthesis and incorporation of BUDR into DNA (9, 11). Cells that produce almost no virus when growing in RM produce greatly increased quantities of C-type virus when grown in BUDR-containing medium. Virus production is most marked when cells are grown in 1 µg of BUDR per ml for several months (10).

Previous studies showed that weekly injections of melanoma cells grown for 4 to 12 months in 1 µg of BUDR per ml (clone C471 or C3471) protected normal C57BL/6J animals against challenge with 10^6 untreated melanoma cells, which produce tumors and death in all unprotected animals (9). Protection against tumor challenge was proportional to the number of preinjections, ranging from 7% with 1 inoculation to 90% with 4 inoculations with C471 cells (9). Other early experiments showed that cells grown for 14 days in 3 µg of BUDR per ml protected 17% of the mice, whereas untreated cells did not protect any. The degree of protection was also proportional to the number of virus particles being produced in the "immunizing" cells (10).

This paper attempts to determine whether the decreased tumorigenicity of BUDR-grown cells is due to a change in their intrinsic capacity to grow progressively and kill the isogenic host, to a change in the antigenicity of BUDR-treated cells which evokes a host response, or to both. An immune response may destroy the BUDR-treated cells before they have an opportunity to grow. Since the in vivo test for malignancy and immunogenicity is the same, our previous results do not distinguish between these alternatives.

In this study we inoculated "nontumorigenic" BUDR-treated cells into immunologically compromised mice. These cells formed tumors in many neonates and mice treated with ATS, showing that they retained the ability to grow. Since we had previously shown that these treated cells were causing a host response in immunologically competent animals (9) and, since it is known that many malignant cells possess antigenic determinants capable of inducing an immune response (7), we also sought measurable antigenic differences between BUDR-treated and control melanoma cells.

MATERIALS AND METHODS

Cell Culture. Cells were cultured with or without BUDR (Nutritional Biochemicals Co., Inc., Cleveland, Ohio) in Eagle's...
Antigenicity of Melanoma Cells Grown in BUdR

Charles H. Kohn, B. Lynn Bevan, and William J. McMichael

January 1974

101

Table 1

Characteristics of control and BUdR-grown melanoma cells

Comparison of growth of untreated and treated mouse melanoma cells in vivo (10^4 cells/mouse, s.c.) and in vitro. Methods of determining plating efficiencies and population doubling time are described in Refs. 9 and 11.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% tumor formation in normal adult mice given 10^6 cells</th>
<th>Latent period (days)</th>
<th>Plating efficiency (%)</th>
<th>Population-doubling time (hr)</th>
<th>Protection against challenge with 10^6 B559 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control melanoma B559 cells</td>
<td>100^b</td>
<td>8–20</td>
<td>67–92</td>
<td>~24</td>
<td>None</td>
</tr>
<tr>
<td>BUdR-grown C3471 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 μg/ml for 1 yr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 μg/ml for 1 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 μg/ml for 14 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

By 3 weekly injections. See Refs. 9 and 10.

For 8 × 10^4 cells, 100%; for 5 × 10^4 cells, 50%.

See text.

See Refs. 9 and 11.
cells (3 µg/ml for 14 days) do not form tumors in 6- to 8-week-old control mice that are untreated, or that are given injections of 0.9% NaCl solution or NRS. The same cells formed tumors in 72% of ATS-treated mice. The mean latent period was increased over that for untreated cells from Day 13 to 65.

Clone C471, grown continuously in 1 µg of BUdR per ml for almost 1 year, formed tumors in all immunosuppressed animals with a latent period unchanged from that for untreated cells. Clone C471 formed tumors in 26% (9 of 34) of control mice, but after a longer latent period than for tumors grown from untreated melanoma cells in control mice.

Neonates. We inoculated neonatal mice with 10^4 to 10^6 cells grown in 3 µg of BUdR per ml for varying lengths of time. We had previously shown (11) that such cells gradually decrease in tumorigenicity with increasing exposure to BUdR, becoming essentially nontumorigenic in adult mice after 1 week. Table 3 compares the results of tests of BUdR-grown cells with those for control cells inoculated into neonates. Regardless of length of BUdR incubation, fewer tumors formed than in newborn mice given untreated melanoma cells. However, far more formed than had been demonstrated (11) in adults given similarly treated cells. BUdR-grown cells took longer (p < 0.05) to form tumors (latent period, 27 to 87 days) in neonates than did control cells (latent period, 14 to 26 days). Survival time of animals bearing tumors growing from treated cells was also greater than for those given control cells. None of the latter survived longer than 30 days postinjection. All animals developing tumors from BUdR-grown cells survived beyond that time, with the exception of 1 group, namely, neonates given 10^6 cells that were grown with BUdR for 4 to 5 days. This was also the only group in which tumorigenicity reached 100%. When fewer cells or cells incubated with BUdR for a longer time were inoculated, tumors formed in fewer animals than in controls inoculated with untreated cells. The partial suppression of tumorigenicity of cells treated for more than 1 week with 3 µg of BUdR per ml in newborns is similar to that in adult animals treated with ATS.

Similar results were obtained even when a passage of C3471 cells was tested that was only partially suppressed in tumorigenicity (10). Tumors formed in 95% of neonates (18 of 19) inoculated with IO6 of these cells grown in 1 ng of BUdR per ml, as compared with 68% (17 of 25) of adults given the same cells.

GCRA: Typing of BUdR-grown Cells (C471) and Untreated Melanoma Cells (B59). Absorption of antiserum diluted to near the end point is a sensitive serological method in vitro for detecting cell surface antigens (6). We did 4 experiments, all of which showed a difference between the ability of BUdR-grown cells (C471) and that of untreated melanoma (B59) cells to absorb out cytotoxic activity from standard typing serum against GCRA. Chart 1 shows the results of a typical experiment, indicating that both unabsorbed serum and serum absorbed with B59 cells have retained approximately equal cytotoxic antibody against the standard test cells (EdG2). In contrast, C471 cells of 2 separate passages absorbed out most of the cytotoxic activity of the standard typing serum. In other experiments, known GCSA-negative cells (C57BL/6J primary embryo cells and C57BL/6J radiation-induced leukemia (ERLD) absorbed out similar quantities of cytotoxic activity, as did B59 cells.

H-2b Antigen. The cytotoxicity titers (50% kill) of 2 experiments follow: (a) B59 (control) cells, 1/8; C471 (treated) cells, 1/80; and (b) B59 cells, 1/16; C471 cells, 1/16. Chart 2 shows the results of one of the experiments.

Table 2
Tumorigenicity of BUdR-grown cells in animals immunosuppressed with ATS

<table>
<thead>
<tr>
<th>No. of cells</th>
<th>Control cells (untreated)</th>
<th>4–5 days in BUdR</th>
<th>7–8 days in BUdR</th>
<th>13–16 days in BUdR</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^3</td>
<td>4/16</td>
<td>9/9</td>
<td>3/4</td>
<td>5/11</td>
</tr>
<tr>
<td>10^4</td>
<td>15/15</td>
<td>4/5</td>
<td>9/18</td>
<td>4/11</td>
</tr>
<tr>
<td>10^5</td>
<td>6/6</td>
<td>6/6</td>
<td>3/10</td>
<td>4/19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of ATS-treated mice with tumors/total no. of mice</th>
<th>MLP (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13/8 (130)</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>20/20 (36)</td>
<td>15 ± 2</td>
</tr>
</tbody>
</table>

* MLP, mean latent period, mean ± S.D.; p < 0.02.

* Numbers in parentheses, number of days surviving animals were observed.

Table 3
Tumorigenicity of BUdR-grown cells (3µg/ml) in neonatal mice

Newborn mice received i.p. injections of 10^2 to 10^4 trypsinized, washed cells, usually within 24 hr after birth.

<table>
<thead>
<tr>
<th>No. of cells</th>
<th>No. of mice with tumors/total no. of mice</th>
<th>MLP (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^3</td>
<td>4/16</td>
<td>9/9</td>
</tr>
<tr>
<td>10^4</td>
<td>15/15</td>
<td>4/5</td>
</tr>
<tr>
<td>10^5</td>
<td>6/6</td>
<td>6/6</td>
</tr>
</tbody>
</table>

* All animals with tumors died. Surviving animals were kept under observation for 96 to 117 days.

* Each mouse was given 1 or 2 × 10^5 cells.

* Each mouse was given 0.5 or 1 × 10^5 cells.
Antigenicity of Melanoma Cells Grown in BUdR

DISCUSSION

The ability of BUdR-treated melanoma cells to form tumors in immunosuppressed and neonatal mice suggests that a large component of the loss of malignant potential of these cells may be ascribed to a change in their antigenicity. This is particularly striking in the cells grown continuously in 1 μg of BUdR per ml; these grow as quickly and as well in animals with depressed immune competence as do the untreated melanoma cells in control animals. The plating efficiency and population doubling times of these cells are essentially unchanged from control cells (Table 1). In addition, uptake of BUdR-3H into their nuclei, as determined by autoradiography, is normal. As previously reported (9, 10) if allowed to grow in RM, C3471 cells gradually revert to piled growth, pigmentation, and 100% tumorigenicity.

Probably, both change in capacity to grow and increased immunogenicity combine to produce the effects noted in cells grown in 3 μg of BUdR per ml for 1 week or more. Such cells have decreased plating efficiency and increased doubling time (Table 1). These changes, however, cannot alone explain their inability to form tumors in normal animals. The number of untreated melanoma cells needed to produce tumors in 50% of control adult animals at the time of these experiments was about 5 × 10⁶ cells. Therefore, one would expect at least 50% of control mice inoculated with 10⁶ cells of 5% plating efficiency (3 μg/ml for 14 days) to develop tumors, but none did. The number of untreated cells needed to produce tumors in 50% of neonates is between 10² and 10³ cells (Table 3). When cells treated with 3 μg of BUdR per ml were inoculated into neonates and ATS-treated animals, a significant percentage of them (Tables 2 and 3) developed tumors. This would indicate that the cells were able to grow in vivo but one factor, at least, that prevented them from growing in the normal host was its immune response. The latent period was significantly longer than that for untreated cells, as might be expected from their decreased plating efficiency and increased doubling time. Thus, growth of these cells in vivo in immunologically compromised mice may be analogous to their ability to regain tumorigenic potential after growth in medium without BUdR (9). The presence of pigment in some of the tumors in neonates and ATS-treated mice further suggests that morphological reversal of BUdR-treated cells occurs in vivo.

Cells grown in 3 μg of BUdR per ml for 4 to 5 days also have lower tumorigenic potential and longer latent periods in neonates than have control cells (Table 3). As already reported (11), 1 million of these cells form tumors in only 30% of adult animals. Their plating efficiencies and doubling times are close to control levels and therefore other factors must explain this
A known antigen, GCSA, was demonstrated on BUdR-grown C3471 cells but was not detected on control B59 cells. GCSA is the cell surface antigen found on cells producing naturally occurring (Gross) leukemia virus and could be a factor in their increased antigenicity. Other antigenic differences are currently being sought, and the sera of mice immunized with C3471 cells are being tested against known antigens.

Results similar to ours were obtained by Barbieri et al. (2) who found the C-type particles, GCSA, and decreased tumorigenicity occurred in "spontaneously" transformed C57BL/6 lung cells after prolonged culture with methylcholanthrene. Untreated control cultures were negative for C-type particles and antigen and retained high tumor-producing capacity. Stephenson and Aaronson (12) also found an increase in cell surface antigens and immunogenicity associated with virus production by mouse sarcoma virus-transformed cells. Lieber and Todaro (4) have hypothesized that transformed cells that express endogenous C-type virus may be more antigenic than those that do not, thus providing a natural means of controlling neoplastic cells.

Other experiments (Chart 2) indicate a 10-fold increase in histocompatibility antigen H-2β on C3471 cells as compared with control melanoma cells. While it is difficult to relate this to the immunogenicity of these BUdR-grown cells in C57BL/6 mice, it is another example of a change in a cell surface antigen in these cells as compared with untreated cells.

Our working hypothesis is therefore that increased antigenicity of BUdR-grown mouse melanoma cells is an important component of their decreased tumorigenic potential. This allows the normal host to reject them before they can revert to the malignant state as they can in vitro. The antigenicity may be due to production of cell surface antigens related to the induction by BUdR (1, 5) of endogenous C-type virus similar to the naturally occurring leukemia virus. One such antigen may be GCSA, demonstrated on C3471 cells. The rejection of melanoma cells by mice previously given injections of C3471 cells could well be related to immunological sensitization to an antigen shared by both cell strains. That this antigen may be the GCSA demonstrable by current techniques in C3471 cells, but not in untreated melanoma cells, is plausible in view of the tremendous increase in virus production in these BUdR-grown cells as compared with their parental line, in which virus could be demonstrated only by electron microscopy. This hypothesis is supported by the earlier finding (10) of proportionality between the number of cell-associated virus particles produced by the BUdR-treated cells and the degree of protection they afford against challenge with malignant melanoma cells. If the antigenicity of human tumor cells could be increased by incubation in BUdR, application of this work to human cancer might be possible.

ACKNOWLEDGMENTS

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REFERENCES

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