5-Bromodeoxyuridine-Tolerant Melanoma Cells

In Vitro and In Vivo

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The thymidine analog 5-bromodeoxyuridine (BrUdR) incorporated into DNA has been successfully used as a selective agent (1, 2) or mutagen (3) on various animal cells. Some of its effects are understandable in terms of base substitution in the genetic material; others, however, such as the reversible suppression of cytodifferentiation (4–7), remain unexplained.

We have examined the effects of prolonged treatment with high BrUdR concentrations on the in vitro and in vivo characteristics of a murine malignant melanoma (B16) cell line and have already reported the isolation and altered phenotype of B16 cells tolerant to 45 μg BrUdR/ml (8). Moreover, these did not require continued exposure to the analog-containing environment (9). The data presented here review and extend our previous observations and support the hypothesis that B16 cells tolerant to high analog doses (1) are stable amelanotic variants and (2) do not retain their altered phenotype by virtue of residual BrU-DNA. We also demonstrate, by means of cesium chloride (CsCl) equilibrium-centrifugation analysis, that tolerant cells incorporate less analog into DNA than control cells. When cells chronically exposed to BrUdR were tested in vivo, tumorigenicity was reduced but not completely extinguished. On the other hand, cells withdrawn from the BrUdR-containing media eventually become as tumorigenic as control cells.

MATERIALS AND METHODS

Cells. B16 malignant melanoma developed spontaneously in a C57BL mouse at the Jackson Laboratory in 1954 (10). In 1964, two cell strains, HFH-14 and HFH-18, were established in vitro (11). A pigmented cell line, 440B, derived from one of these strains (HFH-18) was used almost exclusively in the present studies. Chart 1 gives a detailed history of this cell line. As a monolayer culture, it prolifer-

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ates equally well in Medium 199 or in Eagle's Minimal Essential Medium (MEM) supplemented with 10% fetal-calf serum and antibiotics. Media and serum were purchased from Grand Island Biological Co. (Grand Island, NY).

A detailed history of cells chronically treated with the thymidine analog 5-bromodeoxyuridine (BrUdR) (California Biochemicals) is given in Chart 2 (Group 1 cells). We began by exposing mass cultures of 440B growing in MEM to increasing concentrations of the analog in November, 1969, initiating the regimen with 3 \( \mu \text{g/ml} \), and raising it first to 6 \( \mu \text{g/ml} \) and then to 10 \( \mu \text{g/ml} \). Thereafter the cells were cultured in media containing BrUdR at 2 \( \mu \text{g/ml} \) increments up to 20 \( \mu \text{g/ml} \) and 5 \( \mu \text{g/ml} \) increments up to 45 \( \mu \text{g/ml} \). In March, 1972, we increased the dosage to even larger increments and presently maintain three sublines in 45 \( \mu \text{g/ml} \), 300 \( \mu \text{g/ml} \), and 500 \( \mu \text{g/ml} \) BrUdR, respectively.

Chart 3 outlines the history of cells propagated in MEM containing 10 \( \mu \text{g BrUdR/ml} \), recultured in analog-free MEM for 4 months, and then injected into mice. Cells from these tumors were subsequently reestablished in vitro in MEM for 8 months when an additional subline was started in Medium 199 (Group 2 cells).

Group 3 cells (Chart 4) are those that had been growing in MEM with a final concentration of 30 \( \mu \text{g BrUdR/ml} \) and were then transferred to analog-free MEM for 5 months. At that time, an additional line was established in Medium 199 which also lacked the analog. Both of these lines are called "released" cells.

NP, an amelanotic cell line, was established in culture from a nonpigmented tumor produced by the injection of cells of a nonpigmented HFH-18 subline (12).

Cytological examination. To inspect cellular morphology, we seeded cells onto a 22 \( \times \) 45-mm coverglass in a 60 \( \times \) 15-mm Falcon plastic culture dish or a two-chambered slide (Lab Tek, Miles Laboratory, Westmount, IL). When they had grown to a confluent sheet, the cells were rinsed in isotonic saline, fixed in methanol, and stained with May–Grunwald–Giemsa.

We continuously observed the morphology of all cells but include here only representative samples from different monitorings.

Tumor production. Tumors were established at several different times by subcutaneously injecting cells suspended in 0.2 ml phosphate-buffered saline into 6-week-old male C57BL/6 mice. The number of cells, as well as of mice tested, varied. Tables 1 and 2 and legends to Charts 2–4 give the figures for each test.

Density gradient centrifugation studies. Approximately 10\(^6\) cells were subcultured into 250-ml flasks (Falcon Plastics). Twenty-four hours later, 45 \( \mu \text{g/ml} \) BrUdR were substituted for the MEM in control cultures of "acute-45" cells, whereas "chronic-45," "chronic-500," control, and "released" cells were fed 45 \( \mu \text{g/ml} \) BrUdR, 500 \( \mu \text{g/ml} \) BrUdR, and MEM, respectively. The released cells used were transferred from 30 \( \mu \text{g BrUdR/ml} \) into MEM and proliferated in the analog-deficient medium for 2 years. After feeding, 1 \( \mu \text{Ci/ml} \) \(^3\text{H-deoxyadenosine (G)} \) (11.1 Ci/mmole) or 0.05 \( \mu \text{Ci/ml} \), \(^1\text{C-deoxyadenosine-8} \) (38.8 mCi/mmole) (both from New England Nuclear, Boston, MA) were added. Twenty-four to 28 hr later, the cells were trypsinized, centrifuged, and washed three times with Hanks' balanced salt solution.

DNA was extracted from the different classes of cells, generally 3–5 \( \times \) 10\(^6\) cells per class, by a modification of the procedure of Penman (13). Each cell group

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**CHART 1. In vitro and in vivo history of 440B and P 51 cells.**
In vitro and in vivo history of 440B cells continuously cultured in BrUdR (Group 1 cells). $a = 69$ and $b = 45$ amelanotic tumors.
Chart 3. In vitro and in vivo history of cells transferred from 10 µg BrUdR/ml into analog-free media (Group 2 cells). $a = 10$, $c = 20$, $d = 100$, $e = 20$, $f = 49$, $g = 50$, $h = 58$, and $i = 107$ amelanotic tumors. $b = 10$ amelanotic tumors with pigmented nodules.
TABLE 1  
TUMORIGENICITY OF BrUdR-TREATED AND UNTREATED B16 CELLS

| Cells                      | No. cells injected | Days postinjection tumors were | Tumor weight (g)  
|----------------------------|--------------------|-------------------------------|------------------|
|                            |                    | Palpable | Visible       | Palpable | Visible       |
| Untreated 440B             | $10^6$             | 14 (10/10)$^b$ | 18 (10/10) | 2.5 ± 0.37 (20d)$^c$ 
| Untreated NP               | $5 \times 10^6$   | 10–12 (5/5) | 14–15 (5/5) | 4.0 ± 0.43 (20d) 
| 440B Chronically treated with 45 µg BrUdR/ml | $10^4$ (8/22/72) | 79 (5/5) | 87 (1/5) | 0.6 ± 0.10 (101d) 
|                            | $10^4$ (8/30/72) | 41 (10/10) | 112 (4/5) | 3.4 ± 1.0 (55d) 
|                            | $3 \times 10^5$ (4/25/72) | 23 (5/5) | 40 (5/5) | 5.0 ± 0.70 (51d) 
|                            | $5 \times 10^5$ (6/29/72) | 21 (10/10) | 35 (10/10) | 6.0 ± 0.90 (51d) 

$^a$ = mean ± SD; $^b$ = number of tumors/number of mice injected; $^c$ = day postinjection.

TABLE 2  
TUMORIGENICITY OF CELLS CHRONICALLY TREATED WITH BrUdR AND TRANSFERRED TO MEM

| BrUdR concentration | Time in MEM | Number of cells | Days postinjection tumors were | Tumor weight (g)  
|----------------------|-------------|----------------|-------------------------------|------------------|
| 30 µg/ml (10 mos)    | 5 months    | $5 \times 10^6$ | 12 (10/10)$^b$ | 16 (10/10) | Not determined 
| 45 µg/ml (2 yrs 7 mos) | 7 days | $10^4$ | 42 (7/9) | 54 (9/9) | 2.9 ± 1.1 (54)$^c$ 
|                      | 14 days | $10^4$ | 41 (9/10) | 51 (10/10) | 3.9 ± 0.83 (56) 
|                      | 21 days | $10^4$ | 38 (7/8) | 46 (8/8) | 4.0 ± 0.96 (53) 

$^a$ = mean ± SD; $^b$ = number of tumors/number of mice injected; $^c$ = day postinjection.

was pooled as a frozen pellet, with 2–5 vol of unlabeled BCS-1 (Green monkey kidney) cells for carrier. The extraction was performed on whole cells rather than separated nuclei, and the treatment with deoxyribonuclease was omitted. The aqueous phase from the last chloroform–isoamyl alcohol extraction was made 1 M with respect to NaCl, the RNA precipitate was centrifuged to a pellet, and DNA was spooned from the supernatant under 2 vol of ethanol. This spooled DNA was redissolved in 0.1 × SSC (1.5 × 10⁻² M sodium chloride + 1.5 × 10⁻² trisodium citrate, pH 7.0) and used without further treatment.

Samples of the labeled DNA were mixed with a stock cesium chloride solution in 0.1 × SSC in combinations shown in Fig. 9a–c to give a volume of 3 ml and a density of approximately 1.72. These were centrifuged under oil for 60–70 hr, at 30,000 rpm in a SW 39 rotor at 20°C. Fractions were collected from below, densities determined pycnometrically with a calibrated micropipet, and aliquots of each fraction assayed in a scintillation counter. Double-label separation and normalized plotting were done by computer.

RESULTS

In vitro studies. The morphology of the two untreated cultures, 440B and NP, is illustrated in Figs. 1 and 2, respectively. The former are lightly pigmented in vitro whereas the latter are amelanotic but both are spindle-shaped, lack contact

CHART 4. In vitro and in vivo history of cells transferred from 30 µg BrUdR/ml into analog-free media (Group 3 cells). a = 10, b = 15, c = 20, d = 46e = 120, and f = 54 amelanotic tumors.
inhibition, and pile high on each other. At doses as low as 3 μg/ml BrUdR, the cellular morphology of 440B altered, and continued to do so, as we increased exposure to the analog. Cells maintained in medium containing BrUdR were more contact inhibited than control cells but, to some extent, still formed multilayered areas. Moreover, they were greatly enlarged, unpigmented, and flattened. These morphological changes persisted as the analog concentration was increased and are apparent in cultures currently maintained in 45 μg/ml (Fig. 3) or 500 μg/ml BrUdR (Fig. 4).

With one exception, all cultures transferred into MEM (Fig. 5) or Medium 199 (Fig. 6) from 10 μg BrUdR/ml were morphologically similar to group 1 and 2 cultures. In February 1971, we observed small colonies of small, spindle-shaped,
pigmented cells among the enlarged, flattened amelanotic cells. Only one bottle of this subline survived at that time and in it only the larger, amelanotic cells proliferated. Although continuously maintained in analog-free medium since then, the smaller, pigmented cells have not been detected again.

Fig. 5. Cells grown in BrUdR (final concentration: 10 µg/ml) for 7 months and transferred to analog-free MEM for 4 months. After 1 passage in vivo, they were reestablished in culture in analog-free MEM for 1 year 10 months. See Chart 3.

Fig. 6. Cells grown in BrUdR (final concentration: 10 µg/ml) for 7 months and transferred to analog-free MEM for 4 months. After one passage in vivo they were reestablished in culture in analog-free MEM for 8 months and then transferred to analog-free Medium 199 for 1 year 4 months. See Chart 3.

Fig. 7. Cells grown in BrUdR (final concentration: 30 µg/ml) for 10 months and transferred to analog-free MEM for 8 months. They then underwent two passages in vivo before they were reestablished in culture. Then, after 2 additional months in analog-free medium, they underwent two more passages in vivo and were reestablished in vitro in analog-free MEM for 13 more months. See Chart 4.

Fig. 8. Cells grown in BrUdR (final concentration: 30 µg/ml) for 10 months and transferred to analog-free MEM for 5 months. They were frozen in liquid nitrogen and after 4 months thawed, reestablished in MEM for 1 month, and transferred to Medium 199 for 1 year 4 months. See Chart 4.
Fig. 9. Paired cultures labeled with $^{14}$C or $^3$H deoxyadenosine for 24–28 hr. Abscissa: fraction number; ordinate (left) counts normalized to percentage of total; ordinate (right) g/cc. a. Chronic-45 ($^{14}$C) $\triangle$ and control ($^3$H) $\bullet$. b. Acute-45 ($^{14}$C) and control ($^3$H) $\bigcirc$. c. Released ($^{14}$C) $\bigtriangleup$ and control ($^3$H) $\bigcirc$. d. Chronic-500 ($^{14}$C) $\bigcirc$ and control ($^3$H) $\bigcirc$.

After repeated subcultures in BrUdR-free media for more than 2 years, group 3 cells retained their enlarged, somewhat flattened, unpigmented phenotype. These cells are similar (Figs. 7 and 8) to those of group 1 (Figs. 3 and 4) and group 2 (Figs. 5 and 6). No morphological differences were detected between cells grown in MEM or Medium 199.

In vivo studies. All of the 114 tumors derived from cells chronically treated with BrUdR were completely amelanotic (Chart 2). Furthermore, tumor detection was much more delayed than in completely pigmented tumors produced by the
injection of untreated 440B cells or in totally unpigmented tumors resulting from untreated NP cells (Table 1). Both of the untreated cell lines, whether pigmented or nonpigmented, generated tumors palpable by Day 14 and visible by Day 18 after subcutaneous injection of 10⁶ cells. By contrast, when tested in March, 1972, tumors from cells chronically treated with 45 μg BrUdR/ml were delayed as long as 112 days and at the time of termination weighed considerably less than those resulting from control cell inocula. However, 5 months later the same cell number produced palpable tumors by Day 41 and visible tumors by Day 55 after injection. Even when mice were inoculated with 3 × 10⁶ or 5 × 10⁶ BrUdR-tolerant cells, tumors were not visible until 40 and 35 days, respectively.

Of the 424 tumors produced by cells transferred to analog-free media from medium containing 10 μg BrUdR/ml (Chart 3), only 10 had small pigmented nodules embedded in nonpigmented tumor tissue. We also observe a mixture of small melanotic and large amelanotic cells growing simultaneously in our cultures (see Results, In Vitro Studies).

When cells withdrawn from 30 μg BrUdR/ml and subsequently subcultured in MEM were inoculated into C57BL/6 mice (Chart 4), 265 totally nonpigmented tumors resulted.

In a limited study of the time required for cells tolerant to BrUdR and reduced in tumorigenic potential to reach the same level of tumorigenicity as control cells (Table 2), we found that cells proliferating in BrUdR for 10 months with a final concentration of 30 μg/ml and transferred to MEM were as tumorigenic as controls after 5 months (Table 1). Recently we have subcultured cells from 45 μg/ml BrUdR into MEM and tested them in vivo 1, 2, and 3 weeks later (Table 2). Clearly, this time span is not sufficient to completely reverse the suppression of tumorigenicity to that of untreated 440B cells; however, all tumors in these tests were completely amelanotic.

Density gradient centrifugation studies. Figure 9 a–e shows the buoyant density profiles of DNA isolated from 440B, chronic and acute-45, chronic-500, and released cells tested in paired cultures. Cells treated with BrUdR for 2 years 9
than untreated cells ($\rho = 1.697$) but not as much as control cells exposed for only months (1 year 2 months in 45$\mu$g/ml) incorporated more analog ($\rho = 1.714$) one generation time ($\rho = 1.749$) to the same drug dose. Significantly, both released cell and control cell DNA had the same buoyant density. Although the BrUdR exposure to chronic-500 cells was more than 10 times that to chronic-45 cells, the DNA buoyant density of the former ($\rho = 1.719$) is somewhat larger than that of the latter ($\rho = 1.713$) but not as great as that of DNA from acute-45 cells ($\rho = 1.749$).

The bands of DNA from both chronic-45 and chronic-500 cells were broader than the band of normal DNA, and showed a prominent shoulder or small peak on the light side. This broadening of the main peak suggests a heterogeneity in density, and the light peak may be related to satellite DNA. This would be a large peak for satellite, but it should be remembered that we measured the uptake of exogenous deoxyadenosine, not the optical absorbance (mass) of the DNA. These are not necessarily the same. Further experiments will be required to analyze the broad, asymmetric distribution of DNA from BrUdR-tolerant cells.

**DISCUSSION**

Our results suggest that changes in the in vitro phenotype of 440B cells cultivated in and tolerant to high BrUdR concentrations are irreversible and independent of continued analog exposure. Three independent lines of evidence support this hypothesis. First, the altered morphological characteristics and pigment loss persisted when tolerant cells were continuously subcultured in BrUdR-free medium for 2 years. We believe that the putative revertant cells that were mixed with the enlarged, amelanotic cells originated by contamination with non-BrUdR-treated control cells. Since we have not detected such revertants either in vitro or in vivo since February, 1971, this explanation seems justified.

Second, we depleted the tolerant cell BrU-DNA by injecting cells into host animals. In our experience, even though untreated 440B cells are lightly or seemingly nonpigmented in vitro, they always generate darkly pigmented tumors in vivo. Of the 803 tumors produced by injecting mice with cells previously exposed to the analog, 793 were completely amelanotic. The 10 exceptional tumors developed from the contaminated cultures described above.

Third, density-gradient centrifugation data (Fig. 9c) confirm that there is no residual BrU-DNA in cells retaining the altered phenotype.

Other results from CsCl density-gradient centrifugation experiments reveal that tolerant cells incorporate less BrUdR into their DNA than do acutely exposed cells. The amount of BrU substituted for T in the DNA of various cell classes can be estimated. When completely substituted, E. coli DNA, which contains 25% T, gives a buoyant density increase of 0.1 g/cc (14). Mouse DNA containing 28% T (15) gives a shift of 0.112 g/cc when each T is replaced by BrU. In control vs chronic-45 DNA, the shift is 0.017 (Fig. 9a) and the degree of substitution approximately 15% of the total Ts. Similarly, the replacement in acute-45 and chronic-500 DNA is 46% and 19%, respectively (Fig 9b,d). When the buoyant densities of chronic-45 and chronic-500 DNA's spun together (Fig 9e) are compared, only a 6% increase in substitution was observed. This, added to the 15% replacement estimate for chronic-45 DNA, gives a value of 21% for chronic-500 DNA, in good agreement with the 19% calculated directly from density comparison of chronic-500 to control cell DNA. In summary, the maximum analog incorporation in cells chronically treated with BrUdR is only 21% of the total T com-
pared with 46% calculated for DNA from control cells exposed for 24–28 hr to 45 μg/ml BrUdR. This confirms our autoradiographic data (8) and supports the hypothesis that BrUdR-tolerant cells utilize much less analog than control cells.

How tolerance to as much substituted T as we calculated above is built up and maintained remains a vexing problem. Mouse heterochromatin, usually considered genetically inert, is satellite DNA, rich in AT sequences (16). Although heterochromatic regions were localized only in the centromeric regions of nonmalignant mouse cells, an SV 40-transformed mouse cell line possessed interstitial heterochromatin as well (18). We have also observed interstitial heterochromatic regions in control and tolerant 440B chromosomes (19). Whether the increase in modal chromosome number in tolerant cells (8) is sufficient to account for a significant rise in the amount of heterochromatin and thus the availability of extra Ts that could be replaced by BrU is not known. Since only 10% of the total normal mouse DNA is heterochromatin, it alone would not be enough to incorporate all the BrU estimated from our data.

Although BrUdR may have acted on nonnuclear entities, e.g., the cell membrane, to produce our amelanotic cells, it is difficult to explain how these modifications could be perpetuated for so many cell generations.

It is important to realize that few cells survived the rigorous selection conditions initially imposed on our cultures to obtain tolerant cells. Because the modified cell type was observed early in analog treatment, it probably originated either from the selection of a variant in the original cell population or from a mutation(s) induced by the analog. On the basis of morphological differences and chromosomal features (19), it appears to be an entirely different variant from NP.

Tolerant cells were not as tumorigenic in vivo as control cells but this characteristic varied. Since BrUdR incorporation at low levels has been shown to modify malignancy in amelanotic melanoma cells (7), the depressed tumorigenic potential in tolerant cells may result from the small quantities of the analog incorporated into the cell. This likelihood is supported by the fact that after 5 months in analog-free medium tolerant cells regain a tumorigenicity level comparable to that of untreated control cells.

Although the morphology of BrUdR-tolerant and released cells is similar, the former appear slightly larger and more flattened. This analog effect, which has been noted on various cell types (7, 20, 21), suggests a general control mechanism in the manufacture of cell-specific molecules. We suggest, as have others, that in suppressing a special molecular species (21) (in this instance, one required for tumorigenicity), BrUdR interferes with the manufacture of a common surface molecule, or vice versa.

SUMMARY

Malignant melanotic melanoma (B16) cells propagated for almost 3 yr in high BrUdR concentrations are tolerant to 500 μg/ml BrUdR. Tolerant cells—enlarged, flattened, and nonpigmented in vitro—produce only amelanotic tumors in vivo. These modifications in the B16 phenotype did not require continued exposure to the analog but persisted when these cells were transferred to an analog-free medium. The analog-containing environment was also eliminated by the injection of cells into host animals. In more than 800 tumors, some representing 27 animal passages, only 10 possessed pigmented nodules. All 10 originated from one culture which probably had been contaminated with pigmented cells from control cultures.

Data from cesium chloride density-gradient centrifugation show that the altered
morphology of cells withdrawn from BrUdR does not derive from residual BrU-DNA. Furthermore, cells chronically treated with BrUdR incorporate much less analog into DNA than control cells exposed to 45 μg/ml BrUdR for one generation time.

Although tumorigenicity is less in tolerant cells than in control cells, it is not completely destroyed; the reduction can be reversed by prolonged growth in medium lacking the analog.

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