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Tumorigenicity, Immunogenicity, and Virus Production in Mouse Melanoma Cells Treated with 5-Bromodeoxyuridine

(murine leukemia virus/helper virus/tumor suppression/antigens/electron microscopy)

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ABSTRACT **Bromodeoxyuridine (BrdU), whether administered in a 30-hr pulse of 30 μg/ml or continuously in low concentrations (1–3 μg/ml), significantly increased production of particles with the morphology of murine leukemia virus in a mouse melanoma (B16) cell line. Particles were very rare in control cells, detectable only by electron microscopy. By contrast, in many experiments with BrdU-treated cells the numbers of virus particles counted by electron microscopy increased over 100-fold, and other tests for murine leukemia virus (plaque assay and tests for group-specific antigens 1 and 3 and for Gross cell-surface antigens) became positive. All BrdU-treated cells, regardless of drug concentration or length of treatment, in addition to showing loss of both pigment and of piled-up morphology, were suppressed in tumorigenicity compared with the control cells. These effects were all reversible. A significant percentage of mice injected with BrdU-treated cells were protected against subsequent tumor formation when challenged with malignant control cells. The degree of protection conferred on the mice correlated well with the number of virus particles counted in the injected cells. There was also good correlation between the amount of cell-associated virus and the degree of suppression of malignancy for cells treated continuously with 1 μg of BrdU per ml, but not as good for cells treated for short periods with higher concentrations of BrdU.**

Reversible suppression of tumorigenic potential of mouse melanoma cells grown in culture for long periods in medium containing low concentrations of the thymidine analog 5-bromodeoxyuridine (BrdU) has been demonstrated (1, 2). Morphology of these cells changed dramatically during growth in the presence of BrdU, although the growth rate remained essentially normal when the concentration was 1 μg/ml. These effects required DNA synthesis, and BrdU was incorporated into the DNA (1, 2). Further, mice injected with a clonally derived strain grown continuously in the presence of 1 μg of BrdU per ml developed a degree of immunity to subsequent challenge with the 100% malignant melanoma cells in direct proportion to the number of prior injections with the BrdU-treated cells. Four inoculations at weekly intervals immunized 90% of the mice (2). We also observed large numbers of particles with the morphology of murine leukemia viruses in cells grown continuously in medium containing BrdU, as compared with untreated cells.

Recent reports indicated that a short pulse with high concentrations of BrdU (20–200 μg/ml), or of other halogenated pyrimidines, can "induce" previously unexpressed virus in various mouse (3, 4) and rat (5) cells, including embryonic mouse cell lines. In view of this, a series of experiments was undertaken to determine whether similar treatment of the melanoma cell line would (*a*) produce reversible suppression of malignancy, (*b*) alter the morphology, as did longer periods of growth with 1–3 μg of BrdU per ml and, (*c*) "induce" virus production and release. When positive results were obtained in all three of these parameters with a short pulse, correlations were sought between the number of viruses associated with cells treated with BrdU in various ways and for different lengths of time, and between suppression of malignancy and induction of immunogenicity.

Abbreviations: RM, normal culture medium not containing BrdU; MuLV, murine leukemia virus; gs, group specific.

MATERIALS AND METHODS

The pigmented clone $B_5$59 was derived (6, 7) from a melanoma (B16), which originated spontaneously in a C57BL/6 mouse (8). $B_5$59 clone was the parental line for all cells used in these experiments (Fig. 1). Methods of cell culture, cloning, and growth with or without BrdU were the same as reported (1, 7), except that very recently Falcon microtest plates II were used for cloning.

Cells were periodically monitored for mycoplasma by Hayflick's method (9), by autoradiography with [^{3}H]-thymidine (6.7 Ci/mmol), and by electron microscopy. All tests for contamination were negative.

Replicate pellets of washed and trypsinized cells were used for tumorigenicity tests and for electron microscopic studies. The standard test for tumorigenicity was by subcutaneous injection of 10^6 viable cells into 6- to 8-week old C57BL/6J mice. Tumors were scored as positive only if they continued to grow progressively and killed the mouse. Surviving animals were checked for tumors twice weekly for at least 80 days.

The presence of virus was ascertained by electron microscopy. Cells were always changed to fresh medium 16–24 hr before harvest by trypsinization. Cell pellets [1200 rpm (326 × *g*) for 5 min] were fixed in 2% glutaraldehyde (0.1 M cacodylate buffer, pH 7.2) for 90 min, rinsed in buffer, post-fixed in 1% osmium tetroxide for 60 min, and left overnight in 0.5% aqueous uranyl acetate (pH 4.7) at 4°. After dehydration in ethanol and in propylene oxide, small fragments of the cell pellets were embedded in Epon. Sectioning was by a Porter–Blum MT-2 ultramicrotome, equipped with a diamond knife. Thin sections were stained with 5% uranyl acetate (in absolute methanol) and lead citrate, and examined with a Siemens Elmiskop 1A electron microscope operated at 80 kV. To estimate the number of cell-associated viruses in

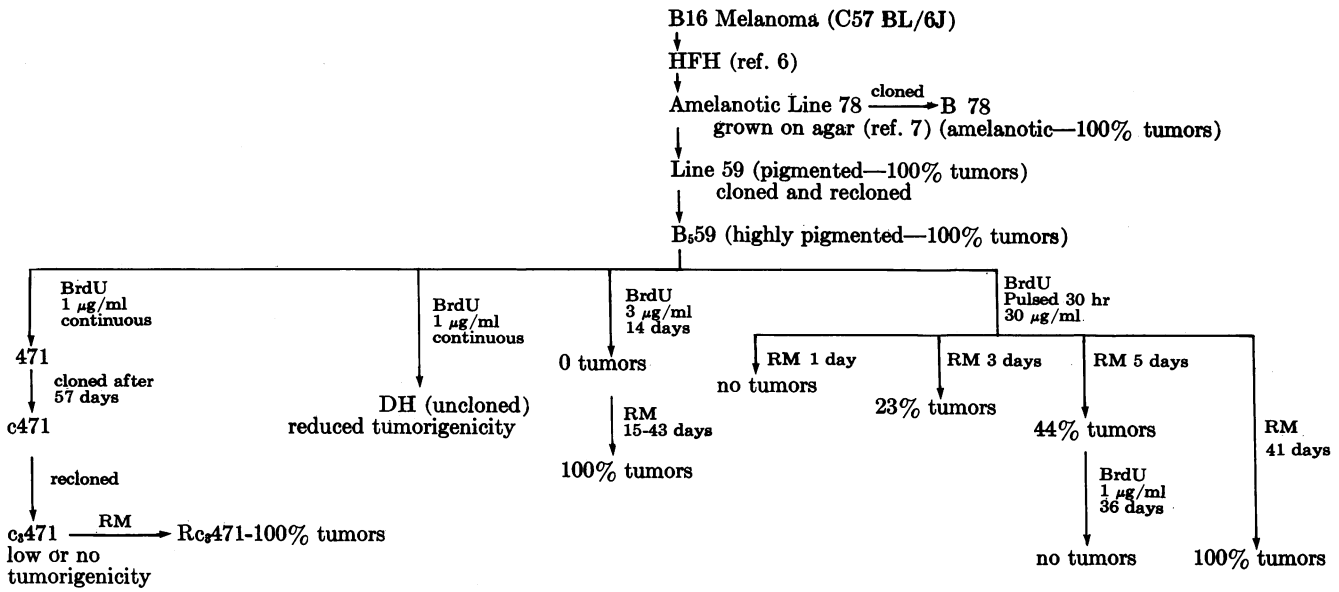

FIG. 1. Lineage and treatment of melanoma cell strains.

control and BrdU-treated cells, budding and extracellular viruses were counted in samples containing at least 60 nucleated cell sections for each BrdU-treated sample, and 120 cell sections in controls. Sections on the grid were screened to assure that each cell section was scored only once. The mean and standard deviation of the mean number of virus particles per cell section were calculated and compared.

Assay and typing of the virus by the methods of Rowe *et al.* (10) and Pincus *et al.* (11) were performed by Dr. W. P. Rowe. Dr. William Hardy tested for group-specific antigens (*gs*-1 and *gs*-3) of murine leukemia virus (MuLV) by indirect immunofluorescent technique (12, 13). Gross cell-surface antigen was assayed by the indirect absorption method, with E ♂ G2* spleen cells as antigen and with a specific antiserum, both supplied by Dr. E. A. Boyse (14).

BrdU was obtained from Nutritional Biochemicals Co., Cleveland, Ohio, and solutions made about once weekly were kept in the dark at 4°.

RESULTS

When 100% tumorigenic clone $B_5$59 was grown with BrdU at any of the concentrations or time periods tested, tumorigenicity decreased (Tables 1, 2 and refs. 1 and 2). Continuous growth in 1 μg of BrdU per ml gradually lowered tumorigenicity, but it was necessary to clone the cells to obtain strains of zero or very low tumorigenic potential, indicating that there was heterogeneity in the cell population grown with BrdU in the medium. Clones selected for lowest tumorigenicity began to produce more tumors after 7–8 subcultures and had to be recloned to derive new strains of equally low tumorigenic potential (Table 1). For example, clones *b* and *c* of line 471 each produced a tumor in only 1 of 10 animals tested, whereas clone *a* produced tumors in 7 out of 10 mice (1). Clone *c* continued to be almost nontumorigenic until subculture 11, when it produced tumors in 5 out of 10 mice. On recloning, subclone c_1 was found to be highly tumorigenic, but subclone c_3 was not tumorigenic up to the 8th passage, after which tumorigenicity began to increase (Table 1). All clones and subclones derived from cells growing in 1 μg of BrdU per ml had approximately the same generation time and cloning efficiency as the parental melanoma cell line (2). When returned to growth in normal medium without BrdU (RM), all clones became 100% tumorigenic (2).

When melanoma cells were grown in 3 μg of BrdU per ml, tumorigenicity started to decrease at 48 hr and always reached zero by 14 days (1, 2). Cells at first grew at the normal rate in culture, but growth slowed during the second week. Cloning efficiency was also lowered at this time (2). When returned to normal medium, cells gradually returned to 100% tumorigenicity in most experiments (2).

After a 30-hr pulse with 30 μg of BrdU per ml, a procedure similar to that followed by Lowy *et al.* (3) and Aaronson *et al.* (4), melanoma cells were tested for tumorigenicity at various times after return to RM. After 24 hr in RM, tumorigenicity was zero but increased to 44% by day 5. The cells reversed to 100% tumorigenicity by day 41 (Table 2). When, after 5 days in RM, the cells were grown in medium with 1 μg of BrdU/ml, their tumorigenic potential was zero by day 41 after pulsing. Pulsed cells exhibited morphological changes comparable to those occurring during the first days of continuous treatment with low concentrations of BrdU (1). Cells flattened and changed to a more fibroblastic type; their pigmentation decreased. The remaining pigment granules were located in the juxtanuclear area. Those cells, which continued to grow in 1 μg of BrdU per ml, became completely unpigmented and fibroblast-like. When returned to normal medium, cells gradually reverted to the usual piled-up, pigmented form.

To investigate a possible correlation between reduction of tumorigenicity and the number of cell-associated viruses, an electron microscopic study was made of cells continuously grown in BrdU (1–3 μg/ml) or pulsed as described and then reversed in normal medium. Particles (budding, enveloped

* E ♂ G2 is a transplanted leukemia that was induced in a C57BL/6 mouse by Gross virus (14).

TABLE 1. *Tumorigenicity of melanoma cells grown continuously on BrdU*

Cell line or clone	Passage no.	BrdU* No. of days	No. with tumors/No. of mice injected
$B_5$59 (control)	All passages	—	100/100
471 (uncloned)	5	29	20/26
DH (uncloned)	15	92	2/5
	39	210	6/7
*c*471 (cloned once)	5–7	130–143†	1/15
		73–86‡	
	11–17	154–190†	6/25
		97–133‡	
$c_3$471 (recloned)	2–8	197–235†	0/40
		65–103‡	
	12–16	263–290†	2/10
		131–158‡	
	22–45	300–450†	17/25
		168–318‡	

* All cell lines except controls were incubated in medium with 1 μg of BrdU per ml.

† No. of days since cells began growth in BrdU-containing medium.

‡ No. of days since cells were last cloned in BrdU-containing medium.

A and type C) (15) were counted as described in *Methods*. Table 3 and Fig. 2 give the results of these studies and Fig. 3 shows a representative electron micrograph.

Fig. 2 shows that the number of viruses in all BrdU-treated cells was significantly greater than in control cells. There are variations in the number of budding and extracellular viruses depending on mode and length of treatment.

When cells that were grown in 1 μg of BrdU per ml for different lengths of time (cloned and uncloned) were compared with each other and with control and reversed cells (Fig. 2*A* and *B*), there was an inverse correlation between the number of virus particles and degree of tumorigenicity. This was less evident with other modes of treatment (Fig. 2*C* and *D*). Cells pulsed for 30 hr showed very few viruses after 5 days in RM and produced tumors in only 44% of the inoculated mice. When the same cells were then allowed to grow in 1 μg of BrdU/ml, the number of viruses increased and tumorigenicity decreased to zero. If the same pulsed cells grew in RM for a total of 41 days, virus counts increased to a lesser extent, but tumorigenicity returned to 100%. On the other hand, cells grown for 14 days in medium containing 3 μg of BrdU/ml were completely nontumorigenic, but very few virus particles were observed.

TABLE 2. *Tumorigenicity of melanoma cells after pulse of BrdU (30 μg/ml) for 30 hr and return to RM*

No. of days in RM	% tumorigenicity
1	0 (0/19)
3	23 (3/13)
5	44 (7/16)
41	100 (9/9)

TABLE 3. *Number of viral particles and immunogenicity of control and treated cell lines*

Cells	No. of days on BrdU*	Percent animals protected with 3 prior injections	Viral particles†
$B_5$59 (control)	0	0 (0/20)	0.10 ± 0.09
$B_5$59 (treated)	14 (3)	17 (2/12)	0.43 ± 0.35
DH-39			
(uncloned)	205 (1)	60 (3/5)	7.18 ± 1.54
$c_3$471-8 (cloned)	229 (1)‡	70 (12/20)	18.87 ± 3.6

* Number in parentheses represents concentration of BrdU (μg/ml).

† Mean number of extracellular and budding particles per ultrathin cell section ± standard deviation.

‡ 80 days after recloning.

Different passages of subclone c_3 revealed various numbers of viruses (Fig. 2*B*). Two subcultures of c_3 of zero tumorigenicity had the highest counts of virus of all cultures studied. The virus count in subculture 36 was markedly reduced and tumors grew in 80% of the inoculated mice. When c_3 cells

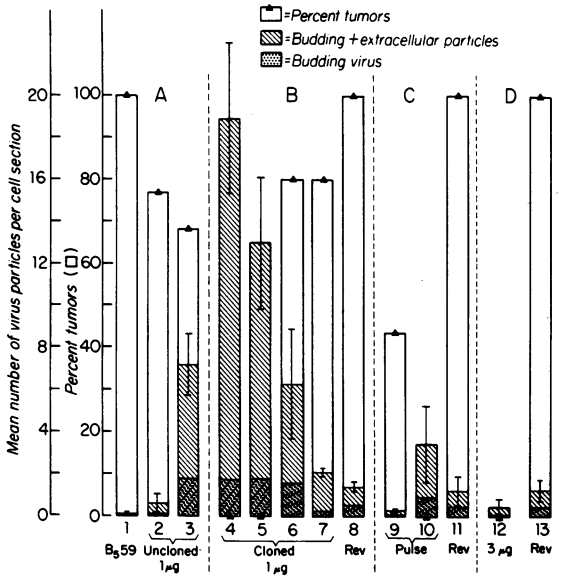

FIG. 2. Relationship between electron microscopy counts of extracellular and budding viral particles (cell-associated) and percentage of tumorigenicity of control and BdrU-treated cells. (*A*) $B_5$59 controls and uncloned BrdU (1 μg/ml)-treated cells. **1.** $B_5$59 control cells. **2.** Cells treated for 29 days. **3.** Cells treated for 208 days. (*B*) Cloned BrdU (1 μg/ml)-treated cells of different passages, and the same clone reversed by growth in RM. Number of days in BrdU-containing medium is the same as in Table 1. **4.** $c_3$471-8. **5.** $c_3$471-12. **6.** $c_3$471-22. **7.** $c_3$471-36. **8.** Reversed $c_3$471 (201 days on BrdU followed by 220 days in RM). (*C*) Cells pulsed for 30 hr in 30 μg of BrdU per ml and subsequently treated as follows: **9.** Into RM for 5 days. **10.** Into RM for 5 days and then into BrdU (1 μg/ml) for 36 days. **11.** Into RM for 41 days for reversal. (*D*) **12.** Cells treated in BrdU (3 μg/ml) and **13.** Cells treated in BrdU (3 μg/ml) and then reversed by growth in RM for 35 days.

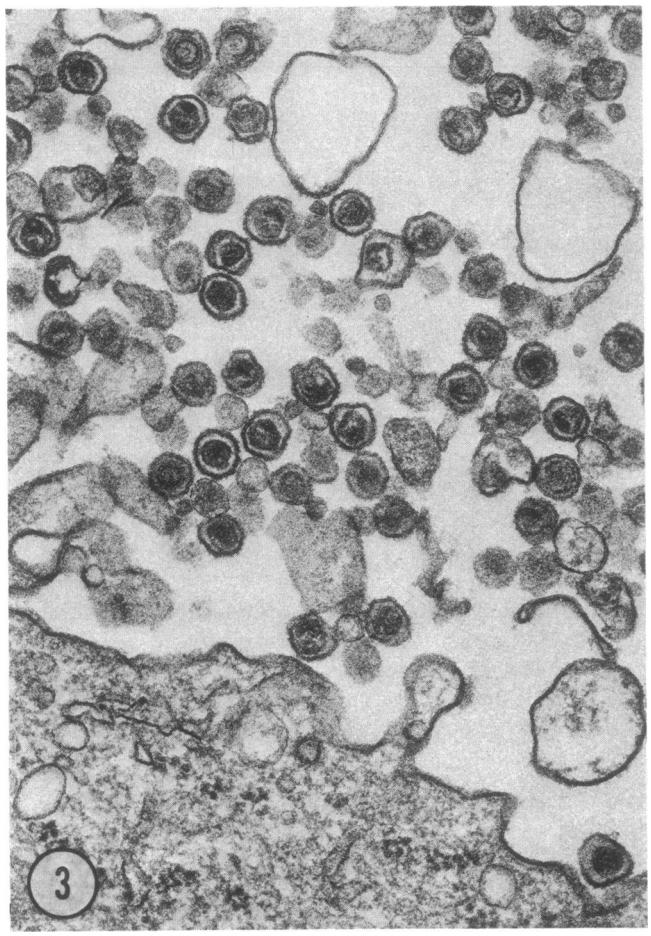

FIG. 3. Typical accumulation of virus particles having the morphology of murine leukemia virus (enveloped A and type C) located in close contact with the surface of a $c_3$471 cell treated with 1 μg BrdU per ml for 248 days. Magnification $\times$50,240.

were completely reversed in normal medium to 100% tumorigenicity, the mean virus count per cell section was lower than that for any of the subcultures growing in BrdU. All reversed populations continued to release similar small numbers of virus particles (Fig. 2).

A closer correlation exists between number of cell-associated viruses and degree of immunogenicity (Table 3). The greatest protection against challenge with 10^6 cells of the 100% tumorigenic parental line was offered by subclone $c_3$471-8, which had the highest virus counts, while passages with lower virus counts offered less protection. Melanoma cells gave no protection when subtumorigenic numbers were injected 3 times, at weekly intervals, before challenge.

The ultrastructure of the observed particles resembled that of murine leukemia type C and enveloped A viruses (Fig. 3).

Cells grown in medium with BrdU ($c_3$471-15) and supernatant fluid were assayed by Dr. W. Rowe by the UV-XC method (10). The results indicated the presence of a murine leukemia virus with a titer of $10^{1.9}$ plaque-forming units per 0.1 ml of medium. No virus was discernible by this method in the control cells. $c_3$471 cells were also strongly positive for MuLV *gs*-1, as well as *gs*-3 antigens, whereas the $B_5$59 cells were negative, as assayed by the indirect immunofluorescence technique by Dr. W. Hardy (12, 13). Preliminary results in this laboratory indicate that the $c_3$471 cells possess the Gross cell-surface antigen, which was not detected in the control cells.

DISCUSSION

When mouse melanoma cells are grown in a medium containing BrdU several changes occur. Malignancy and pigmentation are suppressed and cells no longer grow in piled-up colonies, but form "density-dependent" monolayers of flattened cells. All of these changes are reversible and apparently dependent on continued DNA synthesis (1, 2). This report shows that the reversible changes in malignancy and morphology occur whether the BrdU is present in low concentration for a continuous period ranging from days to months, or in high concentration (30 μg/ml) for about a single cell cycle (30 hr). No virus (titrated by the UV-XC test, ref. 10), *gs*-1 or *gs*-3 antigens (assayed by indirect immunofluorescence), or Gross antigen (assayed by indirect absorption) were detected in the malignant melanoma cells. However, when cell sections were examined by electron microscopy, occasional virus particles were detected. By contrast, when the same cells were grown in the presence of BrdU, *gs*-1 and *gs*-3 antigens were found, as was Gross antigen, the UV-XC test became positive, and electron microscopic counts of particles with the morphology of MuLV were significantly increased, frequently more than 100-fold. This is analogous to the induction of MuLV by BrdU and IdU (5-iododeoxyuridine) in mouse embryo cells from various strains (3, 4).

MuLV-type viruses tend to adhere to the cells from which they are being released (15). We took advantage of this adhesiveness by counting the viruses in a *low-speed cell* pellet (see *Methods*). Similar methods of quantitation have been successfully applied in a study of the effects of BrdU on Friend leukemia cells (16). We believe this to be at least as valid a method as those that involve counting viruses released into a given volume of supernatant fluid. This belief is strengthened by our finding that electron microscopic counting detected rare virus particles in controls. These would have been classified as "nonproducer" cells by all other available methods. Further, we considered the counting of virus particles in the low-speed cell pellet biologically significant, since it was this material that was inoculated into the mice. Budding particles from control cells were too few to permit statistical analysis. Therefore, total cell-associated virus was counted and compared (Fig. 2). Wherever extracellular virus was numerous, budding also was extensive.

The most significant finding is the demonstration that in every case in which BrdU enhanced virus production, the cell tumorigenicity was reduced and, wherever measured, immunogenicity was increased. The degree of protection against challenge with the 100% malignant melanoma cells appeared proportional to the number of virus particles counted. The working hypothesis is that BrdU enhances MuLV production, conferring new properties on the cells, including a measurable level of antigenicity. Antigenicity is probably due to an increase to measurable levels of already existing antigens. These may be related to the rare particles found by electron microscopy in control cells, which would explain the ability of animals immunized with BrdU-treated cells to recognize and reject untreated melanoma cells.

Experiments to be reported elsewhere by Silagi, Weksler, and Newcomb show that cells grown for 14 days in medium with 3 μg of BrdU per ml that never form tumors in young

adult mice form tumors in some immunosuppressed adults and in some neonates, indicating that increased antigenicity is one important factor in BrdU-induced suppression of tumorigenicity. Virus production is probably not the only factor leading to decreased tumorigenicity, as indicated by the poor correlation between number of viral particles and suppression of tumorigenicity in the experiments with higher concentrations of BrdU for relatively short periods. It is possible that these conditions are somewhat toxic, as reflected in lowered plating efficiency (2). Short periods of treatment also may not give cells enough time to become producers of large amounts of virus.

BrdU-treated cells, which had lost tumorigenicity and then regained it after growth in RM, had more virus than controls. This may reflect the fact that not every cell in this population reverses. It should be emphasized that in the most extensively studied clone, $c_3$471, all passages growing in medium with BrdU that were studied by electron microscopy had higher counts than reversed cells.

The etiological role of the virus is completely unknown. Although the virus possesses serological and ultrastructural properties of MuLV, mice that were repeatedly injected with cells high in virus production did not develop leukemia, even when kept for 1 year (2). Some retained the ability to reject a challenge throughout this period. Preliminary studies with cell-free preparations of the BrdU-induced viruses indicate that they produce polykaryocytes in various mouse cell lines, unlike known MuLV strains.

Aaronson has described induction of focus-forming virus in nonproducer strains of murine sarcoma-transformed cells by treatment with BrdU, IdU, and IdC (5-iododeoxycytidine). These induced viruses had the host range and serological properties of the endogenous murine leukemia helper viruses (17). It is, therefore, of interest that the virus induced in the C57BL/6 melanoma cells was "B-tropic", growing better in BALB/c embryo cells than in NIH/Swiss embryo cells (11). C57BL/6 mice belong to the same host group as do BALB/c. Since Gross cell-surface antigen is always found in cells producing wild-type MuLV, it is not surprising that preliminary results indicate that the melanoma cells in which virus was induced were positive for this antigen.

Our findings of decreased tumorigenicity and increased antigenicity in BrdU-treated cells parallel the findings by Stephenson and Aaronson (18) in producer murine sarcoma-transformed cells as compared with nonproducer cells. Further, the presence of a virus in BrdU-treated cells that has the antigenic characteristics of an endemic MuLV, plus cytopathic characteristics unlike any described for known leukemia viruses, is consistent with the hypothesis that BrdU induced the endemic Gross strain of MuLV, which, in turn, acting as helper, rescued a possible murine melanoma virus. In any event, the increased antigenicity and decreased tumorigenicity of these cells after BrdU induction of virus may indicate new approaches to human tumor therapy.

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