

## Induction of Endogenous Virus and of Thymidine Kinase by Bromodeoxyuridine in Cell Cultures Transformed by Friend Virus

(erythroleukemia cells/differentiation/spleen focus-forming virus)

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**ABSTRACT** Thymidine kinase positive (TK<sup>+</sup>) N type cell lines that had been transformed by spleen focus-forming virus were established by transformation with NB tropic Friend virus complex. Thymidine kinase deficient (TK<sup>-</sup>) cell clones were isolated. Some of these cell clones release 1000- to 100,000-fold reduced amounts of Friend virus complex as compared to the TK<sup>+</sup> parental cell clone. TK<sup>-</sup> clones were grown in medium without BrdUrd. Some of these TK<sup>-</sup> clones can be induced to release endogenous helper virus and transforming spleen focus-forming virus on reexposure to 10<sup>-6</sup>-10<sup>-4</sup> M BrdUrd. The induced Friend virus complex is of N host range as expected with induced endogenous virus in N-type cells. Before the induction of the endogenous virus spleen focus-forming virus complex, an induction of thymidine kinase (ATP:thymidine 5'-phosphotransferase, EC 2.7.1.75) activity is observed. The latter is possibly a prerequisite for the induction of endogenous virus in TK<sup>-</sup> cells. Induction of thymidine kinase activity and of endogenous virus is transient and always correlated. The role of BrdUrd and another thymidine analogue, azidothymidine, in interfering with C-type virus release in virus positive cells is discussed. Azidothymidine is unable to induce endogenous virus. Induction of endogenous virus by BrdUrd and inhibition of virus release in virus positive cells is apparently not caused by the same mechanism.

BrdUrd inhibits the replication of C-type tumor viruses (1), whereas in some instances BrdUrd or IdUrd can induce endogenous C-type virus (2-4). In order to study the action of BrdUrd on virus replication and its role in the induction of endogenous virus we have used Friend virus (FV) infected and transformed DBA-2 mouse spleen cells in culture. The FV complex consists of two components, the lymphatic leukemia helper virus (LLV-F) and the replication-defective erythroid cell-transforming spleen focus-forming virus (SFFV-F) (5, 6). The RNA of the two virus types is presumably different in size and structure (7). We have established various cell lines transformed by the SFFV-F (8, 9) which, after 4 years of tissue culture, still liberate biologically active FV of NB host range. The SFFV component of tissue culture origin transforms erythroid mouse cells (10).

BrdUrd and IdUrd are thymidine analogues and can replace thymidine as precursors for DNA synthesis. Neither substance is incorporated into RNA. Azidothymidine,

another thymidine analogue, can be phosphorylated to azidothymidine triphosphate. However, the presence of an N<sub>3</sub> group at the 3' terminal of the deoxysugar interferes with esterification, so presumably azidothymidine can only be added terminally to the growing DNA strand. Both azidothymidine as well as BrdUrd can be used to decrease the virus titer of BrdUrd-sensitive erythroleukemic cells. We have isolated several BrdUrd-resistant clones by treatment of sensitive cells with high doses of BrdUrd (11) and have shown that some of these clones have either decreased or barely detectable thymidine kinase (ATP:thymidine 5'-phosphotransferase, EC 2.7.1.75) activity (11). Furthermore, they have a 10<sup>2</sup>- to 10<sup>4</sup>-fold decreased virus titer. The decrease in C-type virus release can also be shown by electron microscopy.

A temporary thymidine kinase activation is observed if BrdUrd is added to some virus negative lines. The same cells release large numbers of endogenous C-type and spleen focus-forming virus on exposure to BrdUrd but not to azidothymidine. The RNA tumor virus which is induced by BrdUrd has mainly N-type host range properties unlike the original NB type FV complex which has been used for the transformation of our erythroid cell lines. Thymidine kinase induction and virus induction are always correlated. We have independent evidence (Ostertag, Crozier, and Swetly, unpublished), that some of the thymidine phosphorylating enzymes are under viral control. These data constitute the first evidence for involvement of thymidine kinase in C-type virus replication and induction.

### MATERIALS AND METHODS

**Tissue Culture.** Establishment, origin (DBA-2 mice, NB tropic Friend virus complex), and maintenance of the parental erythroleukemic cell lines FSD-1 and of clone FSD1/F4 (= F4) as well as of the BrdUrd-resistant cell clones B8 and B16 have been described (8, 9, 11). B8 is a cell clone with barely detectable and B16 with intermediate thymidine kinase activity (11). B8 and B16 have been now grown in the absence of BrdUrd for more than 2 years. Subclones of B8 have been isolated (B8/1-B8/22) using agar cloning techniques. All subclones of B8 and the parental population are resistant to high concentrations (1 mg/ml) of BrdUrd and are TK<sup>-</sup>.

**Virus Assay.** The presence of RNA tumor viruses was checked by electron microscopy and by the XC assay (14). The A31 and XC cells were provided by Contract E-73-2001-N01 within the Special Virus-Cancer Program, NIH, PHS,

Abbreviations: FV, Friend virus; SFFV-F, spleen focus-forming virus of FV; SFFV, spleen focus-forming virus; SFFU, spleen focus-forming units; LLV-F, lymphatic leukemia virus of FV; Me<sub>2</sub>SO, dimethylsulfoxide; TK<sup>+</sup>, thymidine kinase positive cells; TK<sup>-</sup>, thymidine kinase deficient cells.

through the courtesy of Dr. A. Hackett. For all virus assays, cells were grown to a density of  $1$  to  $5 \times 10^6$  cells per ml. Medium was exchanged 24 hr before removing the tissue culture supernatant from which the cells were pelleted. The continued *in vivo* activity of the LLV-F and of the transforming SFFV was determined by injecting tissue culture supernatant into the lateral tail veins of 2- to 6-month old N or B-type mice (12, 13). Spleen foci of SFFV transformed cells were counted after fixation of the spleen in 70% ethanol.

In another experiment, 0.2-ml aliquots of tissue culture supernatant from various cell lines were injected intraperitoneally into mice. Three weeks later the mice were killed, the spleen examined, and the weight recorded (15).

**Electron Microscopy.** Cell pellets were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 followed by postfixation with 1% osmium tetroxide in isotonic veronal-acetate buffer, pH 7.2, and with 0.5% uranylacetate in 0.1 M acetate buffer, pH 3.9. After dehydration in ethanol, small portions of the cell pellets were embedded in EPO. Sections were cut and stained with 3% aqueous uranyl-acetate and 0.6% lead citrate. The number of C-type viruses were counted in samples of at least 50 BrdUrd-treated cells and 75 untreated control cells.

**Thymidine Kinase Activity.** The thymidine kinase assay was performed on the cells as described (11).

**Azidothymidine.** 3'-Azido-2',3'-dideoxythymidine (3), first prepared by a four step method (16), was synthesized by a shorter procedure via 2,3'-anhydrothymidine (2). This intermediate was prepared as described by Kowolik *et al.* (17) with 70% yield.

One millimole of 2 was refluxed with 5 mmol of lithium azide in 50 ml of di-methyl-formamide for 2 hr. The mixture was evaporated, dissolved in chloroform-methanol (8:2), filtered and purified over a silica gel column with chloroform-methanol (98:2). The main fraction was evaporated and yielded 70% of 3. The physical constants were as described earlier (16).

## RESULTS

**Inhibition of Virus Replication by BrdUrd and Azidothymidine.** The BrdUrd-sensitive cell clone F4 is resistant to BrdUrd concentrations below  $10^{-5}$  M. The virus titers were determined at  $8 \times 10^{-6}$  M BrdUrd or  $2.5 \times 10^{-4}$  M azidothymidine. It can be seen that both inhibitors (Fig. 1a and b) decrease the transforming activity of the SFFV to less than 5% of that of untreated cells. After more than 3 weeks of growth in  $8 \times 10^{-6}$  M BrdUrd or  $2.5 \times 10^{-4}$  M azidothymidine, the cells return to somewhat higher levels of transforming units (Table 1). Changes of growth rate have not been found if cells are treated with  $8 \times 10^{-6}$  M BrdUrd (Fig. 1a) or are only minimal after treatment with  $2.5 \times 10^{-4}$  M azidothymidine (Fig. 1b). The decrease in virus release seems to be a direct effect on C-type virus release.

**BrdUrd-Resistant Cell Clones with Low Levels of Virus Release.** High levels of BrdUrd ( $10^{-3}$  M) were added to cells adapted for growth in  $10^{-5}$  M BrdUrd to obtain resistant cells. We first observe an initial increase in the titer of transforming units and then a steep decline. The resistant cells were cloned and checked for viral release. The virus titer is very low in some of the BrdUrd-resistant clones. With clone

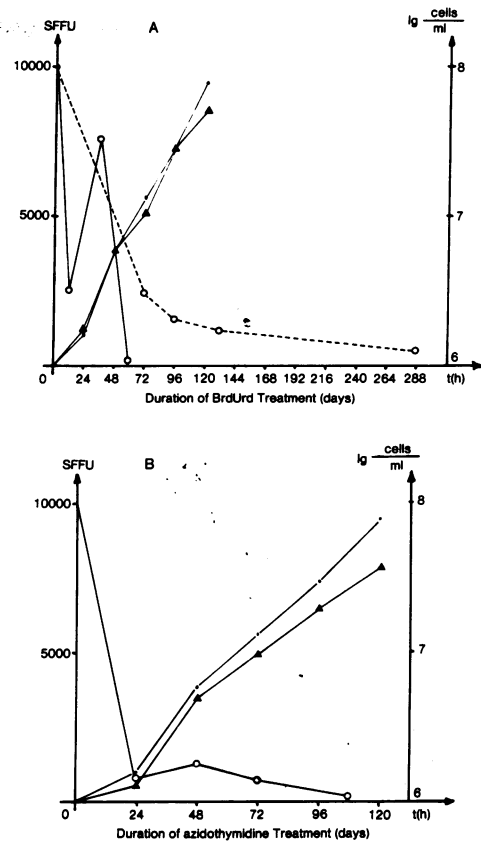


Fig. 1. Inhibition of virus release (SFFV) by the thymidine analogues BrdUrd (A) and azidothymidine (B) in TK<sup>+</sup> cell line F4. Growth curves of cells treated with  $8 \times 10^{-6}$  M BrdUrd or  $2.5 \times 10^{-4}$  M azidothymidine. (A) Virus release: ○—○, BrdUrd (200  $\mu$ g/ml); ○---○, BrdUrd (2.5  $\mu$ g/ml). Growth: ●—●, control; ▲—▲, BrdUrd (2.5  $\mu$ g/ml). (B) ○—○, virus release, azidothymidine ( $2.5 \times 10^{-4}$  M); Growth: ●—●, control; ▲—▲, with azidothymidine ( $2.5 \times 10^{-4}$  M).

B8 we obtained, for example, less than five spleen focus-forming units per  $10^6$  cells as compared to the parental cell line F4 with 1000 to 10,000 SFFV per  $10^6$  cells. A similar difference is found in the XC assay (Table 1). BrdUrd-resistant cell clone B16 released intermediate levels of virus. A similar difference is observed by electron microscopy (Table 1). Intraperitoneal injection of supernatant of B8 cells in Balb/c mice results in only a slight increase in spleen weight (data not shown).

**Effect of Dimethylsulfoxide ( $Me_2SO$ ) on BrdUrd-Resistant Cell Clones B8.** Both F4 and B8 cells differentiate to hemoglobin producing normoblasts after exposure to  $Me_2SO$  (9, 11). We also observe a 20- to 100-fold increase in the yield of C-type tumor viruses as estimated by the spleen focus-forming assay if 1-1.5%  $Me_2SO$  is added to BrdUrd-sensitive cells (F4) or to the partially resistant cell line B16 (10). In contrast, cell clones B8, B8/3, and B8/22 with very low basic levels of virus release do not show an increase in SFFU if  $Me_2SO$  is added (Fig. 3, Table 1). Supernatant of  $Me_2SO$ -treated virus negative B8 cells does not induce an increase in spleen weight. No increased amounts of extracellular C-type particles are observed by electron microscopy. (Table 1).

**Induction of Thymidine Kinase Activity in BrdUrd-Resistant Cell Clones.** Thymidine kinase-deficient B8 and B16 cells

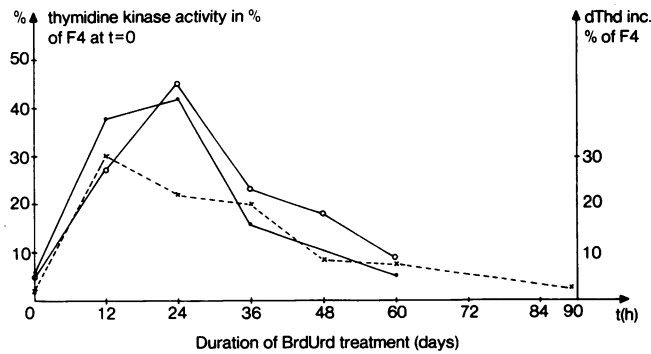


FIG. 2. Induction of thymidine kinase activity and thymidine incorporation in the TK<sup>-</sup> B8 and B8/3 cells by treatment of cells with 200  $\mu$ g/ml of BrdUrd. Details of the treatment are those described for virus induction with BrdUrd. Thymidine kinase activity in B8 (○—○), in B8/3 (●—●); dThd incorporation in B8/3 (×—×—×).

have been grown in absence of BrdUrd for 3 years. The activity in B8 cells was monitored routinely every 4–8 weeks and an increase in activity has not been obtained in 3 years' growth of clone B8, but a marked increase could be found with B16. The thymidine kinase activity of B8 is about 5% of that of TK<sup>+</sup> control cells. The incorporation of BrdUrd or Thd

TABLE 1. Virus release in various cell clones, and after treatment with (A) Me<sub>2</sub>SO, (B) BrdUrd, (C) azidothymidine or (D) dThd

N-type mice*	B-type cells†	Electron microscopy‡	Treatment	Days
<i>Cell clone F4A</i>				
1 × 10 <sup>4</sup>	2.5 × 10 <sup>6</sup>	11.3	—	—
6 × 10 <sup>5</sup>	n.t.	(Not counted) many	(A) 1%	2
5 × 10 <sup>2</sup>	n.t.	n.t.	(B) 8 × 10 <sup>-6</sup> M	12
8 × 10 <sup>3</sup>	n.t.	n.t.	(B) 8 × 10 <sup>-6</sup> M	150
<i>Cell clone F4A, BrdUrd-resistant</i>				
8 × 10 <sup>2</sup>	n.t.	n.t.	(B) 6 × 10 <sup>-4</sup> M	180
<i>Cell clone B8</i>				
1–7	5 × 10 <sup>2</sup>	0.03–0.45	—	—
6	n.t.	n.t.	(A) 1.5%	1.3
2.2	n.t.	0.1	(A) 1.5%	2.5
6 × 10 <sup>3</sup>	5 × 10 <sup>3</sup> to 1.2 × 10 <sup>4</sup>	4.2	(B) 6 × 10 <sup>-4</sup> M	2
5	n.t.	0.5	(C) 5 × 10 <sup>-4</sup> M	1.4
5	n.t.	n.t.	(C) 5 × 10 <sup>-4</sup> M	2
5	n.t.	n.t.	(C) 5 × 10 <sup>-4</sup> M	4
10	n.t.	0.4	(D) 2.5 × 10 <sup>-4</sup> M	2
<i>Cell clone B16</i>				
3.7 × 10 <sup>2</sup>	n.t.	6.2	—	—
1.8 × 10 <sup>4</sup>	n.t.	n.t.	(A) 1.5%	2.5
1.6 × 10 <sup>3</sup>	n.t.	1.9	(B) 6 × 10 <sup>-4</sup> M	1.5
1.3 × 10 <sup>2</sup>	n.t.	n.t.	(B) 6 × 10 <sup>-4</sup> M	10
1.2 × 10 <sup>1</sup>	n.t.	n.t.	(B) 6 × 10 <sup>-4</sup> M	Permanent

n.t., not tested.

\* SFFU/10<sup>6</sup> cells.

† XC, plaques/10<sup>6</sup> cells.

‡ C-type particles/cell cross-section.

into cellular DNA is below 5% of that observed in control cells (11). BrdUrd (200  $\mu$ g/ml) was added to B8 cells. We observe a transient induction or activation of thymidine kinase (Fig. 2). After 30 days of BrdUrd exposure, cells were grown for another 10 days without BrdUrd. BrdUrd was then added again. This second exposure at a short interval in BrdUrd-free medium does not lead to induction of thymidine kinase. Cells revert to inducibility if cells treated with BrdUrd for 30 days are kept in medium without BrdUrd for longer periods. This reversion to inducibility is a stable trait of only a few cells in the population (unpublished observations). We isolated 21 subclones of uncloned BrdUrd-resistant cells after growth for 2 years in nonselective BrdUrd-free medium to exclude the argument that induction of thymidine kinase activity was restricted to TK<sup>+</sup> revertants. All of the 21 subclones were shown to be TK<sup>-</sup> and most of these clones were inducible (ref. 11, and unpublished results).

To further exclude the argument that induction of thymidine kinase activity and endogenous virus activation (see below) was due to TK<sup>+</sup> revertants, B8 cells which were shown to be stable with respect to thymidine kinase activity were subcloned (clones B8/1–22) and immediately after subcloning (not more than 3 weeks later) were exposed to BrdUrd so that thymidine kinase activity (Fig. 2, Table 2) before and after exposure to BrdU could be measured. All of the eight subclones tested for thymidine kinase activity were TK<sup>-</sup> and five of these were inducible for virus release (Table 2). One of these (B8/3) was checked for thymidine kinase inducibility and it is inducible at a high level (Fig. 2).

Reversion rates to TK<sup>+</sup> cells of 6 × 10<sup>-9</sup> (18) or 4 × 10<sup>-7</sup> (19, 20) per cell generation have been reported for other cell lines. For the purpose of calculating the TK<sup>+</sup> revertants in our cell clone B8/3, we assume a reversion rate of 10<sup>-6</sup> per cell generation. This estimate is too high for B8 and B8/3 but too low for cell clone B16 (unpublished results). The proportion of TK<sup>+</sup> cells appearing in 3 weeks could thus be maximally 30 × 10<sup>-6</sup> if we use the generation time for B8 cells which is about 0.8 days. That this estimate is realistic and that no pronounced selection for TK<sup>+</sup> cells could have occurred is shown by the fact that thymidine kinase activity during 3 years of growth of B8 without BrdUrd did not increase within the reliability of our method of calculating the activity. The induction of thymidine kinase activity in B8/3 cells is maximally 40% that of TK<sup>+</sup> F4 cells (Fig. 2). This is sufficient to account for 30% of normal incorporation of [<sup>3</sup>H]dThd into cellular DNA (Fig. 2) after induction. This cannot be due to the calculated 3 × 10<sup>-5</sup> TK<sup>+</sup> revertants after growth of B8/3 for 3 weeks. After cloning, the 30% and perhaps all cells of the TK<sup>-</sup> cell clone B8/3 are able to incorporate BrdUrd or dThd after induction. Thymidine kinase induction and 30% normal dThd incorporation of previously TK<sup>-</sup> cells is observed in less than 12 hr after exposure to BrdUrd. Cellular selection does not occur. By autoradiography, we could show that at least 70–80% of all cells in an inducible TK<sup>-</sup> cell population are able to incorporate dThd after induction (Crozier, M.A. thesis, 1973).

*Induction of Endogenous N-Type Virus and SFFV by BrdUrd.* BrdUrd at 200  $\mu$ g/ml, if added to sensitive cell clone F4A, decreases overall virus yield (Fig. 1a). During the first to second day of treatment, possibly a transient increase in virus yield is obtained (Fig. 1a). This would confirm electron microscope observations of other authors (21). In

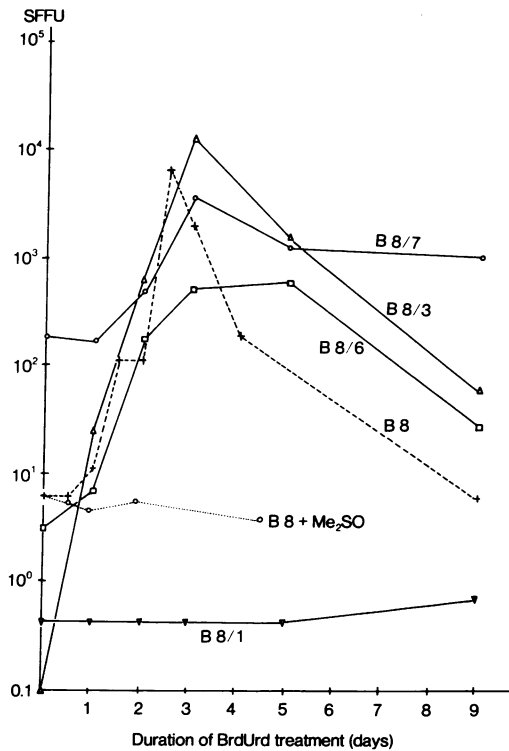


Fig. 3. Induction of endogenous and spleen focus-forming virus with  $6 \times 10^{-4}$  M BrdUrd in cell clone B8 and subclones of B8 from DBA2 mice. There is lack of induction of the FV complex with 1.5%  $\text{Me}_2\text{SO}$ . Most of the subclones show low or no detectable SFFV release in absence of BrdUrd; one subclone (B8/7) shows a relatively high level of SFFV release. No induction is detectable in B8/1, B8/4, or B8/22.

partially resistant cell clone B16 an initial increase and then a decrease of virus yield is found (Table 1).

B8, which is refractory to the  $\text{Me}_2\text{SO}$  induced increase of virus release, can be induced by BrdUrd ( $8 \times 10^{-6}$  to  $2 \times 10^{-3}$  M) to release C-type viruses (Table 1, Fig. 3). This increase of virus release can be detected (a) by electron microscopy (Table 1), (b) by increased spleen weight after injection of cellular supernatants in mice (data not shown), and (c) by formation of spleen foci in DBA-2 and 10- to 100-fold less in Balb/c mice (Fig. 4). The increase is much less if we use the XC assay with B-type indicator cells (Fig. 4). The induction of C-type virus has also been shown for several subclones of B8 which have been exposed to BrdUrd not later than 3 weeks after subcloning (see above) to exclude the argument that  $\text{TK}^+$  revertants are releasing the inducible endogenous virus (Fig. 3). Three out of eight subclones of B8 were not inducible (Fig. 3, Table 2). One of these three clones, B8/22, was checked for thymidine kinase induction and none was obtained (Table 2).

The virus release in individual cells in the induced and uninduced state was estimated by electron microscopy (unpublished results). In a B8 uninduced control population the virus release, i.e., the number of cells with virions adjacent to them or with budding viruses, fits the Poisson distribution. About 19% of the nontreated B8 cells are virus positive as compared to 40% in BrdUrd-induced B8 cells. The number of virions per cell in the induced B8 cells does not fit the Poisson distribution. A fraction of the B8 cells is not inducible. This agrees with our data of Table 2.

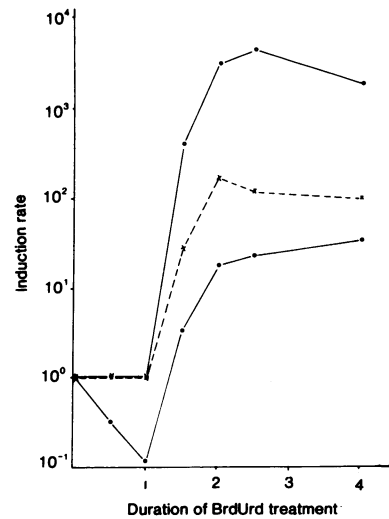


Fig. 4. Difference in apparent induction rate of endogenous virus (and SFFV) if different host range cells or mice are used. Cell clone B8/3 was treated with  $6 \times 10^{-4}$  M BrdUrd for the indicated time. Aliquots of the same tissue culture supernatant of control and treated cell cultures were used for the spleen focus-forming assay in N-type DBA-2 mice (O—O), in B-type Balb/c mice (X—X) and for the XC plaque assay with A31 B-type recipient fibroblasts (●—●). The induction rate is expressed in multiples of the control level (=1). No difference is obtained for the controls if Balb/c or DBA-2 mice are used. Supernatants of  $\text{TK}^+$  F4 cells yield equal number of spleen focus-forming units in N-type DBA-2 and B-type Balb/c mice. The number of spleen foci in N-type DBA-2 mice is  $10^4$  foci per supernatant of  $10^6$  BrdUrd induced B8/3 cells and 2 to  $3 \times 10^2$  in B-type Balb/c mice. The base level in A31 cells in the XC assay is  $5 \times 10^2$  for B8 and somewhat higher for B8/3.

The 20- to 100-fold lower number of spleen foci in B-type mice (Balb/c) and the higher number of foci in N-type DBA-2 mice (Fig. 4) during induction with BrdUrd, after injecting an aliquot of the same supernatant, proves that the major fraction of induced endogenous viruses is N tropic. The parental  $\text{TK}^+$  and virus<sup>+</sup> cell line F4 releases FV complex of NB host range. The major fraction of the inducible virus in B8 and B8/3 cells is, therefore, not LLV-F (NB tropic), but endoge-

TABLE 2. Thymidine kinase activity of F4, B16, B8, and B8 subclones

B8 subclones	dThd-nucleotides/dThd $\times 100$	Thymidine kinase inducibility	Induced virus release
1	1.3	n.t.	—
2	0.9	n.t.	++
3	1.4	+++	+++
4	0.8	n.t.	—
5	2.2	n.t.	++
6	2.3	n.t.	++
7	0.8	n.t.	++
22	0.7	—	—
B8	1.2	+++	+++
B16	10	+++	+++
F4	25	++	+

n.t., not tested

nous N tropic. The observed but low increase in the number of foci in B-type mice is a reflection of the incomplete resistance of B-type cells to N tropic virus (6).

BrdUrd-resistant B8 or B16 cells which were used once for induction of endogenous virus cannot be used a second time for induction. Neither thymidine kinase activation or induction nor virus release is obtained. BrdUrd-treated cells can revert to inducibility over longer periods of time. The calculated reversion rate to TK<sup>+</sup> and virus inducibility is very high, approximately 10<sup>-3</sup> per cell generation. This is higher than any known mutation rate in cell cultures (unpublished results).

No induction of virus can be obtained in inducible lines if high levels of azidothymidine are used (Table 1).

#### DISCUSSION AND SUMMARY

Some SFFV transformed erythroleukemic cell lines, e.g., F4, are permanently capable of releasing biologically active spleen focus-forming virus (SFFV) and LLV-F as a helper. BrdUrd or azidothymidine interfere initially with the release of C-type viruses and possibly release of LLV-F helper activity. LLV-F is either lost as in cell clone B8 or mutates to BrdUrd resistance in other cell clones if cells are exposed to BrdUrd for longer times. The virus titer in the latter case returns to high levels even in presence of BrdUrd (unpublished results). The virus titer in B8 and other cell clones was reduced permanently. Cell clone B8 is BrdUrd-resistant and deficient in thymidine kinase activity. B8 and some subclones of B8 have 10<sup>2</sup>- to 10<sup>6</sup>-fold reduced levels of LLV-F but must have retained the SFFV information since spleen focus forming activity can be induced in these cells. The FV complex of BrdUrd-sensitive cells (F4) responds with a 10- to 200-fold increase (10) if Me<sub>2</sub>SO is added. Me<sub>2</sub>SO, however, does not stimulate virus release in B8, although Me<sub>2</sub>SO-induced differentiation can be obtained (11). On treatment of the BrdUrd-resistant cell clones B8 with BrdUrd, C-type viruses that contain helper activity as well as genetic information for the SFFV are released. The BrdUrd-induced virus is N tropic and differs from the NB helper complex which initially was used to establish the parental cell line F4. The highest level of helper and transforming activity is obtained 2-3 days after BrdUrd exposure. The 10<sup>2</sup>- to 10<sup>6</sup>-fold increase of the SFFV activity could either be explained by rescue of already present SFFV genetic material by an endogenous virus or by rescue and induction of SFFV by BrdUrd.

In all other published cases of induction of endogenous virus by thymidine analogues, BrdUrd-sensitive cells have been used (2-4). These are killed after 3-5 days of treatment with BrdUrd. In the present case, the TK<sup>-</sup> cells used can be induced by BrdUrd for both thymidine kinase and release of endogenous virus. No pronounced loss of viability is observed on treatment with BrdUrd. The release of BrdUrd-induced virus and incorporation of BrdUrd is only transient. If after induction, BrdUrd is omitted and then added again, only very low levels of inducing activity and no inducibility of thymidine kinase is obtained (unpublished observations). These observations indicate (a) that the induced virus cannot reinfect the B8 cells to produce B8 cell lines which maintain the endogenous virus, and (b) that the induction of endogenous virus might involve excision and possibly partial loss of amplified provirus genetic information.

All transformed fibroblasts and melanoma cells show higher BrdUrd-inducible virus titers than nontransformed cells (22-

24). The yield of endogenous virus in B8, after induction with BrdUrd, also is much higher than in nontransformed cells (2-4).

Preceding the release of endogenous RNA tumor virus after application of BrdUrd, we observe an increase in thymidine kinase activity. This induction is a property of more than 50% of the TK<sup>-</sup> cells of clone B8. Probably all of the TK<sup>-</sup> cells in inducible subclones such as B8/3 are inducible. The induction of thymidine kinase activity in TK<sup>-</sup> cell clone B8 makes it possible for the cell to incorporate BrdUrd into the cellular DNA. This incorporation is possibly a prerequisite for the activation of N tropic endogenous virus and possibly also of transforming SFFV. This is supported by the absolute correlation between thymidine kinase induction and inducibility of endogenous virus. Constitutive TK<sup>-</sup> subclones of B8 cannot be induced to release endogenous virus. Both thymidine kinase activity and endogenous virus release are transient in TK<sup>-</sup> inducible cell clones. The reversion rate to TK<sup>-</sup> cells is very high, probably higher than 10<sup>-3</sup> per cell generation, whereas, reversion to constitutive TK<sup>+</sup> cells is much lower.

The thymidine kinase activity could possibly be induced by the endogenous virus preceding the release, or alternatively BrdUrd induces thymidine kinase activity independent of RNA tumor viruses release. Involvement of thymidine kinase activity during infection of cells with DNA viruses and DNA tumor viruses (25-27) and during induction with IdUrd of the Epstein-Barr virus in Burkitt lymphoblastoid cells (28, 29) has previously been shown.

A hybrid TK<sup>+</sup> cell line (NB8) of TK<sup>-</sup> uninducible clone (B8/22) with a TK<sup>+</sup> human cell line was established. The hybrid although constitutively TK<sup>+</sup> and able to incorporate BrdUrd cannot be induced to release SFFV. This dominance of virus noninducibility in the hybrid (Ostertag, Kiang, and Krooth, unpublished) shows that the presence of thymidine kinase activity is not sufficient to allow release and induction of endogenous virus.

The inhibition of LLV-F virus replication by both azidothymidine and BrdUrd seems to be an indication that the mechanism of inhibition is similar. Azidothymidine is phosphorylated to azidothymidine triphosphate but it presumably cannot be used for chain elongation during DNA synthesis. It, therefore, cannot be incorporated internally into the growing DNA strand and is likely to interfere with elongation. Cellular repair enzymes possibly remove azidothymidine from the ends of the growing chain but are not involved in viral repair. The low toxicity of azidothymidine for the cell but high toxicity for the virus would support such a speculation. In some instances azidothymidine might favorably replace BrdUrd for medical treatment of diseases caused by DNA viruses.

Azidothymidine, unlike BrdUrd, does not induce C-type viruses. We would, therefore, like to conclude that the induction of C-type viruses by BrdUrd does require integration of BrdUrd in the DNA as has been shown by others (30). Interference with virus replication is probably not due to integration of BrdUrd in cellular DNA but more likely due to a direct effect of BrdUrd on viral replication.

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