Transient Inhibition of Avian Myeloblastosis Virus Reproduction by Amethopterin and Fluorodeoxyuridine

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Avian myeloblastosis virus cannot initiate its reproduction in the presence of amethopterin or fluorodeoxyuridine. This inhibition is reversed by thymidine. Addition of either inhibitor after virus production has started does not inhibit further virus synthesis. In presence of either inhibitor, deoxyribonucleic acid synthesis is inhibited by over 90%, but ribonucleic acid synthesis is not affected. Cells resume their normal growth rate 24 hr after removal of either inhibitor.

Based upon the effects of inhibitors of deoxyribonucleic acid (DNA) synthesis, the replication of Rous sarcoma virus (RSV), and of its helper Rous-associated virus (RAV), may be divided into two temporal stages: (i) the initiation of virus reproduction which is prevented by inhibitors of DNA synthesis (1, 2, 17) and (ii) stages subsequent to initiation which are not affected by the same inhibitors. Since this property may be unique among certain ribonucleic acid (RNA) viruses, particularly the avian leukosis viruses, it seemed desirable to determine whether early events in the replication of avian myeloblastosis virus (AMV) were affected by inhibitors of DNA synthesis. To this end, we determined whether this phenomenon occurs under rigorous conditions of DNA inhibition in which cells were not irreversibly damaged but were able to resume normal growth after removal of the inhibitors. Under these conditions, the initiation of AMV reproduction was found to be inhibited in the presence of inhibitors of DNA synthesis. Continuation of virus production, however, was not affected.

MATERIALS AND METHODS

Virus stocks. The BAI strain A of AMV used in this study is the same as that used by Vogt and Rubin (18).

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Bryan "high titer" RSV (RAV-1) was obtained from H. Rubin (9) and was propagated in secondary chick embryonic cells. The Newcastle disease virus (NDV) used is the L-Kan 1948 strain.

Cell cultures. All cells were obtained from chick embryonated eggs of the White Leghorn breed (Code No. K-137 Kimber Farms Inc., Pomona, Calif.). Fibroblast cultures were prepared either from decapitated whole 7- to 10-day-old embryos or from thigh muscles of 15-day embryos (4). Cell counts were obtained on samples of trypsinized dishes. Samples of cell cultures intended for virus infection were first subjected to the standard RIF test (14) in order to establish that they were free of leukosis virus. Cultures of yolk sac cells were prepared from 13- to 15-day embryos as reported by Baluda (3). Cultures of chick leukemic myeloblasts were prepared as previously described (13). All cultures were used within 24 to 48 hr after plating.

Media. The standard nutrient medium used is a modification of that used by Baluda and Goetz (4). For NDV infection, chicken serum was omitted and the amount of calf serum doubled. For DNA inhibition studies, a thymidine (TdR)-less medium was prepared with dialyzed sera and without tryptose phosphate. Tryptose phosphate was found to reverse the effect of the inhibitors used; no inhibition of DNA synthesis occurred if 10% tryptose phosphate was present in the test medium and only 40% inhibition occurred with only 1% tryptose phosphate.

Virus assays. AMV was titrated *in vitro* by cell conversion in secondary cultures of yolk sac cells by end-point dilution (4). Two cultures were used for each dilution step. To reverse the possible inhibitory effect of the drugs, 10^{-5} M TdR was added to test samples which had to be assayed for virus.

The titer of RSV (RAV-1) was assayed by the formation of Rous sarcoma foci on whole embryo fibroblast cultures using medium M199 as described by Rubin (14).

NDV was titrated *in vitro* by plaque formation on embryonic fibroblasts by a modification of the method of Franklin, Rubin, and Davis (8). The modification used an overlay medium consisting of equal volumes of 1.8% agar and a nutrient medium composed of 84% M199A, 10% tryptose phosphate, 5% calf serum, and 1% of a 3.6% solution of sodium bicarbonate.

DNA inhibition studies. Amethopterin was used at a concentration of 10^{-5} M; fluorodeoxyuridine (FUDR), 10^{-6} M, and TdR, 10^{-5} M. Uridine was added to a final concentration of 10^{-5} M.

For the measurements of DNA or RNA synthesis, ^3H -deoxycytidine (^3H -CdR), specific activity 2.4 c/mm; or ^3H -cytidine (^3H -CR), specific activity 2.4 c/mm (Schwarz Bio Research Inc., Orangeburg, N.Y.), was added to a final concentration of 0.02 to 0.5 $\mu\text{C}/\text{ml}$. For studies of RNA synthesis with labeled CR, unlabeled CdR was added to a final concentration of 10^{-5} M to minimize incorporation of label into DNA, and uridine was omitted from the medium.

Assay of radioactivity was performed by a modification of the method devised by Mans and Novelli (12). At the completion of exposure to radioactive substrate, the cultures were washed once with phosphate-buffered saline and trypsinized, and the detached cells were transferred to centrifuge tubes. After centrifugation, the volumes were reduced to 0.10 to 0.15 ml. The cells were resuspended and disrupted by freezing and thawing 3 to 5 times until nearly homogeneous suspensions were obtained. These suspensions were transferred to paper discs (Whatman no. 3 MM chromatography paper, 2.3 cm diameter); they were then numbered and mounted on straight pins, dried in an oven at 70 C, extracted three times with 5% trichloroacetic acid, and successively washed in 95% alcohol, 100% alcohol, and then in acetone. Each disc was placed in a scintillation counting vial to which was added 10 ml of scintillation phosphor in toluene. The radioactivity of the sample was measured in a model 1200 Ansitron scintillation counter. Background was determined from blank discs carried through the same extraction and washing procedure.

Autoradiography. The emulsion coating technique reviewed by Kopriwa and Leblond (10) was employed. Sterile cover slips were placed in 60-mm plastic tissue culture dishes and cells were cultured thereon. Fixation in Carnoy's solution was followed by coating and by lightly staining with methylene blue.

In some studies, deoxyribonuclease digestion was performed. Recrystallized deoxyribonuclease (Worthington, Code DPFF) free of ribonuclease activity was dissolved in phosphate buffer (pH 7.0) with gelatin (0.1%) and magnesium sulfate (0.003 M) to a final concentration of 8.0 mg/100 ml. Slides were dipped into the deoxyribonuclease solution and kept there at 20 C for 3 hr. Treated slides were stained by the Feulgen procedure, washed, dried, and coated with emulsion for autoradiography.

Isolation and purification of ^3H -labeled virus. AMV was labeled with ^3H -uridine as previously reported (13). The purification of ^3H -uridine-AMV from 10 ml of tissue culture medium by ammonium sulfate precipitation at 50% saturation and density gradient centrifugation in sucrose have also been described (13).

RESULTS

Effect of amethopterin and FUDR upon cell division, and DNA and RNA synthesis, in uninhibited cells. Experiments were carried out to demonstrate that there was no irreversible damage to cells under the conditions of inhibition of DNA synthesis used in this study. The general pattern of initiation of inhibition of DNA synthesis, and the continuation of RNA synthesis at a normal rate, in cells cultured in TdR-less medium containing either amethopterin (10^{-5} M) or FUDR (10^{-6} M) were determined. In addition, the reversion of inhibition of DNA synthesis by TdR, the recovery from inhibition, and the resumption of normal cell multiplication after removal of inhibitors were also investigated.

These phenomena were investigated with two types of cell cultures: (i) muscle fibroblasts which multiply well *in vitro* and produce AMV after infection but do not show morphological conversion, and (ii) yolk sac cultures which do not grow well *in vitro* but produce AMV and show morphological conversion after infection (3).

Muscle fibroblast culture. Growth of fibroblasts was inhibited by either amethopterin (10^{-5} M) or FUDR (10^{-6} M), but increase in cell number resumed at a normal logarithmic rate 24 hr after release from 24-hr inhibition (Fig. 1).

Incorporation of radioactively labeled DNA precursors into fibroblasts exposed to amethopterin was inhibited within 30 min, and maximal inhibition, to a level of less than 10% of normal, was attained within 1 hr (Table 1). After a 24-hr exposure to inhibitors, DNA synthesis resumed at a normal rate within 30 min after removal of inhibitors (Table 1). This inhibition was manifest with either ^3H -CdR or ^3H -deoxyuridine as a radioactive precursor, but the former was used in these experiments so that TdR reversal of inhibition may be accomplished without dilution of incorporation of the radioactive precursor.

Incorporation of radioactivity into DNA, with ^3H -CdR used as precursor, was not inhibited if TdR was present at a concentration of 10^{-5} M along with 10^{-5} M amethopterin or 10^{-6} M FUDR (Table 2).

Incorporation of ^3H -CR into RNA was not inhibited by amethopterin or FUDR (Table 2). Total incorporation appeared partially inhibited in fibroblasts because of lack of increase in cell number, but incorporation per cell was usually normal or increased in amount, perhaps due to cell enlargement.

Examination of autoradiographs show that inhibited cells had fewer than 10% as many grains (all less than 10 grains per cell) as did control cells (all more than 100 grains per cell). All these

FIG. 1. Inhibition of fibroblast growth by amethopterin (Am) and fluorodeoxyuridine (FUDR). Secondary cultures of fibroblasts, 0.8×10^6 cells per dish, were washed three times with phosphate-buffered saline and incubated in 5 ml of medium with or without inhibitors for 24 hr, at which time the medium was replaced by standard medium. Am was used at a concentration of 10^{-5} M and FUDR at 10^{-6} M. The control contained 10^{-5} M thymidine. Duplicate cultures in each medium were trypsinized and counted at intervals of 24 hr. Each point represents the average cell count.

grains were removed by deoxyribonuclease treatment. The reduction in incorporation of ^3H -CdR into DNA in the presence of inhibitors was therefore uniform. All the cells showed a decrease of approximately 90% in their DNA synthesis.

Yolk sac cell culture. These did not show a significant increase in cell number in 2 days under normal growth conditions. Microcinematography, performed by Jesse Siskin, revealed no mitosis in 60 hr. However, incorporation of radioactively labeled precursors into DNA did occur, although at a lower rate per cell than that observed in fibroblasts. Incorporation of precursors into RNA was also much lower than in fibroblasts. Measurements of incorporation of tritiated precursors revealed marked inhibition of DNA synthesis but not of RNA synthesis in the presence of amethopterin (10^{-5} M) or FUDR (10^{-6} M). Results are presented in Table 2.

These results demonstrate that, while DNA synthesis was inhibited by 90%, RNA synthesis was not affected and TdR completely reversed the inhibitory effect of the drugs. The cells resumed normal DNA synthesis within 30 min, and normal multiplication within 24 hr after removal of inhibitors. The cells, therefore, ap-

TABLE 1. Inhibition of DNA synthesis by amethopterin (10^{-5} M)

Step	Radioactivity incorporated	
	Counts/min ^a	Per cent
Initiation of inhibition^b		
Control without amethopterin	24,621	89.0
TdR control	27,668	100
0 hr of preincubation	4,843	17.5
0.5 hr of preincubation	3,742	13.6
1 hr of preincubation	2,366	8.6
2 hr of preincubation	2,280	8.3
4 hr of preincubation	2,223	8.1
Recovery from inhibition^c		
TdR control	1,005	100
Amethopterin control	97	9.7
0 hr	937	93
1 hr	1,104	110
2 hr	998	99

^a Amount of ^3H -radioactivity incorporated into DNA per culture was determined as described in Materials and Methods. Background counts have been deducted.

^b Secondary cultures of fibroblasts, 2.0×10^6 cells per dish, were washed three times with phosphate-buffered saline and incubated for the times indicated in medium with amethopterin (10^{-5} M) before replacement by identical medium to which $0.5 \mu\text{C}/\text{ml}$ of ^3H -deoxycytidine had been added. Cells were harvested 20 hr after the addition of radioactivity. The control medium contained TdR (10^{-5} M).

^c Secondary cultures of fibroblasts, 1.2×10^6 cells per dish, were washed three times with phosphate-buffered saline and incubated for 24 hr in medium containing amethopterin (10^{-5} M), at which time the medium was replaced with standard medium. At the intervals noted, this medium was replaced by medium containing $0.5 \mu\text{C}/\text{ml}$ of ^3H -deoxycytidine. One control was initially incubated with medium containing TdR (10^{-5} M) instead of amethopterin. Another control was incubated for 24 hr in medium with amethopterin (10^{-5} M) and ^3H deoxycytidine was added in inhibitory medium. All radioactive cultures were harvested after 0.5 hr of exposure to ^3H -deoxycytidine.

peared not to be irreversibly damaged under our conditions of inhibition of DNA synthesis.

Effect of inhibitors upon AMV infection. This was investigated in three stages: (i) initiation of virus production; (ii) continuation of virus production; and (iii) determination of virus production by isolation of purified virions labeled with ^3H -uridine.

Initiation of virus production was carried out as follows: Secondary fibroblast or yolk sac cultures were preincubated for 2 hr in inhibitor media, and

TABLE 2. *Effects of inhibitors on DNA and RNA synthesis^a*

Medium	Radioactivity incorporated (counts/min) ^b			
	Expt 1 ^c		Expt 2 ^d	
	³ H-CdR	³ H-CR	³ H-CdR	³ H-CR
Standard.....	2,544	3,038	2,791	264
TdR-less.....	2,360	—	2,693	271
TdR-less + TdR 10 ⁻⁶ M.....	2,407	—	—	—
TdR-less + TdR 10 ⁻⁶ M + Am 10 ⁻⁵ M.....	2,225	3,148	2,832	283
TdR-less + TdR 10 ⁻⁶ M + FUdR 10 ⁻⁶ M.....	2,351	2,972	2,705	236
TdR-less + Am 10 ⁻⁵ M.....	225	2,419	320	259
TdR-less + FUdR 10 ⁻⁶ M.....	181	2,360	236	228

^a Secondary cultures of fibroblasts or yolk sac cells were washed with phosphate-buffered saline and preincubated for 2 hr in designated medium, which was then replaced by fresh medium with added radioactive precursor. At 24 hr, the cells were trypsinized and radioactivity assayed as before. Abbreviations: ³H-CdR = ³H-deoxycytidine; ³H-CR = ³H-cytidine; TdR-less = thymidine-less; Am = amethopterin; FUdR = fluorodeoxyuridine.

^b Amount of radioactivity incorporated per culture. Background counts have been deducted.

^c Fibroblasts, 2.4×10^6 per dish; ³H-CdR, 0.1 μ Ci in 3 ml; ³H-CR, 0.1 μ Ci in 3 ml.

^d Yolk sac cells, 1.2×10^6 per dish; ³H-CdR, 1.0 μ Ci in 3 ml; ³H-CR, 1.0 μ Ci in 3 ml.

TABLE 3. *Inhibition of AMV infection in presence of inhibitors^a*

Medium	AMV produced (CU $\times 10^{-4}$) ^b		Cell number ($\times 10^{-4}$) ^c		³ H incorporated (counts/min) ^d	
	Fibroblasts	Yolk sac cells	48 hr	96 hr	³ H-CdR	³ H-CR
TdR-less + TdR 10 ⁻⁶ M.....	2.8	1.3	10.2	—	2,351	2,049
TdR-less + TdR 10 ⁻⁶ M + Am 10 ⁻⁵ M.....	1.9	1.3	9.8	—	—	—
TdR-less + TdR 10 ⁻⁶ M + FUdR 10 ⁻⁶ M.....	1.3	0.6	11.3	—	—	—
TdR-less + Am 10 ⁻⁵ M.....	<0.01	0.05	1.9	5.0	225	1,587
TdR-less + FUdR 10 ⁻⁶ M.....	<0.01	0.01	1.7	5.0	181	1,381

^a Secondary cultures of fibroblasts or yolk sac cells were washed three times with phosphate-buffered saline, preincubated for 2 hr in experimental medium, then infected with AMV in a volume of 0.2 ml. After 2 hr, 5 ml of experimental medium was added. At 24 hr, experimental medium was removed, the cells were washed three times with phosphate-buffered saline (with 10% chicken serum in the case of yolk sac cells, which detach from the dishes in the absence of serum), and standard medium was added. Virus was harvested 48 hr later, i.e., at 72 hr postinfection.

^b Infectivity titer, in converting units (CU) of AMV released in the supernatant fraction of: 2.4×10^6 fibroblasts infected with a multiplicity of infection of 0.013; and 1.5×10^6 yolk sac cells infected with a multiplicity of infection of 0.05. Each titration was done in duplicate on replicate samples.

^c Cell counts at 48 and 96 hr in fibroblast cultures.

^d Counts/min (less background) per fibroblast culture incorporated during period 2 to 24 hr, after 2 hr of preincubation in experimental medium using 0.1 μ Ci of ³H-deoxycytidine or 0.1 μ Ci of ³H-cytidine in 3 ml of medium.

then infected with AMV at input multiplicities of 0.1 to 1.0. Free virus was removed by washing prior to the replacement of inhibitor medium by standard medium at 24 hr postinfection. Newly produced virus was harvested 24 or 48 hr after standard medium was added. The results (Table 3) indicate that amethopterin or FUdR inhibits the initiation of virus production under conditions where cellular DNA synthesis is inhibited but RNA synthesis is not. This inhibition of virus

infection is not attributable to cell death, because cell growth resumes at a normal rate as it does in uninfected cells after removal of inhibitors.

Similar results were obtained with yolk sac cultures (Table 3).

Continuation of virus production was carried out as follows: Cultures already producing virus were similarly treated with inhibitors. The cells used were converted yolk sac cells (myeloblasts). Cells were incubated for 24 hr in medium contain-

TABLE 4. Continuing AMV production in presence of inhibitors^a

Medium	AMV produced (CU $\times 10^{-3}$) ^b				Cell number ($\times 10^{-6}$) ^c		DNA synthesized (counts/min) ^d
	0 hr	24 hr	48 hr	72 hr	24 hr	72 hr	
Standard	47	31	70	150	—	28	3,029
TdR-less		31	—	104	—	25	3,261
TdR-less + TdR 10^{-5} M		14	—	224	8.7	27	3,253
TdR-less + Am 10^{-5} M		31	70	150	4.2	19	302
TdR-less + FUDR 10^{-6} M		14	—	104	4.1	18	297

^a Converted AMV-producing cells (myeloblasts) were washed three times with phosphate-buffered saline and resuspended in experimental medium at 4.2×10^6 cells per 5 ml. After 24 hr, the cells were resuspended in standard medium. The medium was changed again at 48 hr (24 hr after removal from inhibition). Medium was harvested for virus assay at 24, 48, and 72 hr, intervals of 0, 24, and 48 hr after inhibition. Abbreviations: TdR-less = thymidine-less; Am = amethopterin; FUDR = fluorodeoxyuridine.

^b Conversion units of AMV produced. 0 hr = virus produced by 4.3×10^6 cells during 24 hr preceding experiment; 24 hr, 48 hr, 72 hr = virus harvested at end of 24, 48, or 72 hr, respectively, of incubation in experimental medium. Two titrations of each sample were carried out using duplicate assay plates.

^c Cell counts at 24 and 72 hr.

^d Counts/min (less background) incorporated per culture during period 2 to 24 hr, in experimental medium using $0.1 \mu\text{C}$ of ^3H -deoxycytidine in 5 ml of medium.

FIG. 2. Continued virus production in presence of inhibitors determined by incorporation of ^3H -uridine into free virions. After three washings with phosphate-buffered saline, 10^7 leukemic myeloblasts in 10 ml of thymidine-less (TdR-less) medium were incubated for 2 hr in presence of either amethopterin (10^{-5} M) or fluorodeoxyuridine (10^{-6} M). A control culture was incubated in TdR-less medium alone. ^3H -uridine ($2.5 \mu\text{C}$ per dish) was then added and the supernatant fractions were harvested 9 hr later. The supernatant fractions were cleared of cell debris by centrifugation in the Sorvall SS34 rotor at 10,000 rev/min for 10 min. ^3H -uridine-labeled AMV was precipitated from the supernatant fractions with ammonium sulfate at 50% of saturation, layered (in a volume of 0.7 ml) over a 4.5-ml linear sucrose gradient (15 to 60%) containing tris(hydroxymethyl)aminomethane-HCl, pH 7.4 (10^{-2} M), and ethylenediaminetetraacetic acid (10^{-3} M), and centrifuged in the Spinco SW39 rotor at 36,000 rev/min for 3 hr at 2 to 4 C. Fractions were collected from the bottom of the tube and a 100- μliter sample of each was placed in Bray's scintillation fluid and assayed for ^3H radioactivity.

ing amethopterin (10^{-5} M) or FUDR (10^{-6} M), then resuspended in standard medium, which was changed at 48 hr. Supernatant fluids which were harvested at 24, 48, or 72 hr were assayed for virus. Under these conditions, virus production was not inhibited, although inhibition of cell multiplication and of DNA synthesis occurred (Table 4).

Virus production by isolation of purified virions labeled with ^3H -uridine was determined as follows: The continued production of infectious virus in presence of inhibitors (Table 4) might not reflect the de novo production of AMV, but rather the delayed release of virus synthesized before the addition of inhibitors. To ascertain that AMV is produced in the presence of inhibitors, the following experiment was carried out. Virus-producing cells, i.e., leukemic myeloblasts, were incubated for 2 hr in the presence of amethopterin (10^{-5} M) or FUDR (10^{-6} M) to arrest DNA synthesis, then ^3H -uridine was added to the inhibitor medium. After 9 hr of incubation, the supernatant fluids were tested for the presence of free ^3H -uridine-labeled AMV. The supernatant fraction of leukemic cells, incubated in TdR-less medium without inhibitor, was used as a control. The amount of ^3H -uridine-labeled AMV produced was determined by the distribution of ^3H after purification of the virus by density gradient centrifugation as previously described (13). The results in Fig. 2 show that AMV was synthesized at a normal rate by virus-producing cells in the presence of amethopterin or FUDR.

On the other hand, (Fig. 3), there was no significant amount of ^3H -uridine-labeled AMV pro-

duced if inhibitors of DNA synthesis were present at the time infection was initiated.

Effect of inhibitors upon NDV infection. To determine whether other cell functions needed for

synthesis of RNA viruses might be impaired by amethopterin or FUDr, the cells were tested for their capacity to produce NDV in presence of inhibitors. NDV was chosen because it has some properties which are similar to those of AMV, e.g., large RNA genome (7), chemical composition, structure, and mode of maturation of the virions (11).

Since the latent period of NDV (about 4 hr) is much shorter than that of AMV (14 to 24 hr), the cells might sustain more damage before AMV production actually begins than they do before NDV production begins. A comparison was therefore made of the amount of virus produced by cells preincubated in amethopterin or FUDr for 2 or 14 hr before infection with NDV. Virus was adsorbed for 2 hr, and the supernatant medium was harvested 20 hr later. There was no inhibition of NDV infection and the length of the preincubation time in inhibitors did not affect the results (Table 5).

Effect of inhibitors upon RSV (RAV-1) infection. In the present study, cells maintained in TdR-free media were exposed to relatively low concentrations of inhibitors, amethopterin (10^{-5} M) or FUDr (10^{-6} M). These inhibitory conditions were also tested for their ability to inhibit the initiation of RSV (RAV-1) reproduction, as had been reported to be the case with higher concentrations of inhibitors (1, 2, 17). If chick embryonic fibroblasts were infected with RSV (RAV-1) after 2 hr of exposure to either drug, and cultured in presence of either drug for 24 hr, they produced less than 3% of the amount of virus produced by uninhibited cells. During that time, DNA synthesis was inhibited by over 90%, but RNA synthesis was not affected. In addition, RSV (RAV-1) production was still only 3% of that in untreated

FIG. 3. Inhibition of initiation of virus production in presence of inhibitors determined by incorporation of ^3H -uridine into free virions. Three cultures of chick embryonic fibroblasts, previously found to be free of leukosis virus by the RIF test, were washed three times with phosphate-buffered saline and incubated for 2 hr in presence of either amethopterin (10^{-5} M) or fluoro-deoxyuridine (FUDr) (10^{-6} M) in thymidine-less medium. A control culture was incubated in thymidine-less medium alone. Each culture was then infected with AMV at a multiplicity of infection of about 1.0. After 2 hr, the virus inoculum was removed, each culture was washed twice with 10 ml of phosphate-buffered saline, and thymidine-less medium was added with either amethopterin or FUDr or no inhibitor, as before. ^3H -uridine (25 μC per culture) was then added. The cultures were incubated for 25 hr, at which time the supernatant fractions were harvested and assayed for ^3H -labeled AMV as in Fig. 2.

TABLE 5. NDV infection in presence of inhibitors^a

Medium	NDV produced (PFU $\times 10^{-6}$) ^b		Cell number ($\times 10^{-6}$) ^c	DNA synthesized (counts/min) ^d
	Expt 1	Expt 2		
TdR-less	2.5	3.2	2.1	1,381
TdR-less + Am 10^{-5} M + TdR 10^{-5} M	2.3	3.6	2.4	1,646
TdR-less + FUDr 10^{-6} M + TdR 10^{-5} M	2.3	2.8	1.9	1,142
TdR-less + Am 10^{-5} M	2.9	2.9	0.5	96
TdR-less + FUDr 10^{-6} M	2.3	3.7	0.5	102

^a Secondary fibroblast cultures were washed three times with phosphate-buffered saline and experimental medium was added. After 2 hr of preincubation at 37 C, Newcastle disease virus (NDV) was adsorbed to cells for 2 hr. Inoculum was removed and cells were washed with phosphate-buffered saline (final dilution 10^7 -fold). Virus was harvested 20 hr after the inoculum was removed. Replicate cultures were used for each medium.

^b Experiment 1: 7.0×10^5 cells infected with input multiplicity of 0.8 after 2 hr of preincubation. Experiment 2: 7.0×10^5 cells infected with input multiplicity of 0.8 after 14 hr of preincubation.

^c Cell counts at time of harvest in experiment 2.

^d Counts/min (less background) per dish, incorporated during 14 to 36 hr, using 0.25 μC of ^3H -deoxycytidine in 3 ml of medium.

cells, 72 hr after amethopterin or FUdR had been removed when the cells were multiplying exponentially. Inhibition of RSV (RAV-1) infection was also reversed by TdR (10^{-5} M). Exposure of chick embryonic cells that were producing RSV (RAV-1) to amethopterin or FUdR for 24 hr did not arrest further virus production. Thus, both AMV and RSV (RAV) behaved similarly in presence of amethopterin or FUdR.

DISCUSSION

The present study shows that the reproduction cycle of AMV, like that of RSV (RAV) plus RAV (17) and of RAV alone (1), may be divided into an early phase sensitive to repression by inhibitors of DNA synthesis and into a later phase that is not repressible. Bader has shown (2) that with a mixture of RSV (RAV) plus RAV, the sensitive phase lasts for approximately 12 hr after infection.

In the present experiments, failure of infection by AMV in the presence of amethopterin or FUdR is not attributable to cell death or irreversible cell damage, and in fact infection by NDV is supported, as is continuing infection with either AMV or RSV. DNA synthesis, but not RNA synthesis, is strongly inhibited by amethopterin and FUdR, as is consistent with the known mechanism of inhibition of dihydrofolate reductase by amethopterin (5) and of thymidylate synthetase by FUdR (6). Amethopterin and FUdR in effect inhibit the same reaction, thymidylate synthesis, since amethopterin blocks the regeneration of tetrahydrofolic acid from dihydrofolic acid. Inhibition of DNA synthesis by a different mechanism, i.e., blocking deoxycytidine synthesis, is accomplished by cytosine arabinoside a known inhibitor of RSV infection (2). That inhibition of DNA synthesis is responsible for the repression is further supported by the reversal of the effects of amethopterin and FUdR by TdR and of cytosine arabinoside by CdR. This apparent dependence upon DNA synthesis in the early phase of infection seems to be a characteristic property of avian leukosis viruses. The replication of another unrelated virus, Visna virus of sheep, which has some characteristics similar to those of avian leukosis viruses, also appears to be inhibited in a transient fashion by inhibitors of DNA synthesis (16).

However, the phenomenon described in this study does not imply that DNA is directly implicated in the replication of viral RNA; an enzyme or cellular function associated with DNA synthesis might be involved. For example, Temin (15) has presented evidence that interference with mitosis after exposure to RSV prevents normal

virus production. The significance of this phenomenon with respect to virus reproduction, oncogenesis, or compatibility of virus synthesis with cellular proliferation remains to be determined.

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