3'-Azido-3'-Deoxythymidine Inhibits the Replication of Avian Leukosis Virus

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We tested the ability of the thymidine analog 3'-azido-3'-deoxythymidine (BWA509U) to inhibit the replication of the retrovirus avian leukosis virus. Inhibition was measured with two different assays: (i) inhibition of a single round of virus replication and (ii) inhibition of virus spread through a cell culture. With both assays, we detected inhibition of virus growth, although inhibition of a single round of virus replication required a 40-fold higher drug concentration than did inhibition of virus spread. We also detected variations in the concentrations of drug needed to inhibit virus replication in different cell types. Higher concentrations of drug were needed to inhibit virus replication in chicken embryo fibroblasts than in the continuous quail cell line QT6. Viral DNA synthesis in infected cells was shown to be inhibited in the presence of the drug. The triphosphate form of the analog acted as a competitive inhibitor of purified viral reverse transcriptase, with a K_i of $0.09 \pm 0.003 \mu\text{M}$, and was incorporated as a chain terminator during reverse transcription of the natural viral RNA substrate *in vitro*.

Retroviruses are widely distributed among vertebrates. These viruses share a common life cycle that includes the reverse transcription of virion RNA into double-stranded DNA (23). A virus-encoded DNA polymerase (reverse transcriptase) present in the virion is responsible for the synthesis of the double-stranded DNA product (1, 21), a process that takes place in the cytoplasm of the infected cell (22). The use of a biochemically distinct polymerase and the cytoplasmic localization set viral DNA synthesis apart from the synthesis of cellular chromosomal DNA.

Retrovirus polymerases do not have an associated 3' exonuclease activity and therefore are incapable of repairing misincorporated bases (3). This observation suggests that retrovirus polymerases may be more sensitive to inhibition by chain-terminating nucleotide analogs than are polymerases that can repair misincorporated bases. Recently, the thymidine analog 3'-azido-3'-deoxythymidine was shown to be a potent inhibitor of human immunodeficiency virus and other mammalian retroviruses (13, 16). For human immunodeficiency virus, both virus replication and virus-induced cell killing were inhibited by the analog. This inhibition was obtained at a concentration of azidothymidine that did not inhibit cell growth. We have extended these studies by examining the inhibitory effect of azidothymidine on the replication of avian leukosis virus (ALV). Azidothymidine was shown to be a potent inhibitor of viral DNA synthesis in infected cells and, in its triphosphate form, to inhibit purified viral reverse transcriptase with both synthetic and native template primers. We also observed differences in cell sensitivity to azidothymidine. At concentrations that greatly inhibited virus replication, the growth rate of QT6 cells (14), a quail cell line, was not affected. In contrast, the growth of chicken embryo fibroblasts (CEF) was much more sensitive to the toxic effects of the drug.

MATERIALS AND METHODS

Cells and viruses. Fertile chicken eggs (C/O, virus negative) were obtained from H and N Farms, Redmond, Wash. CEF and QT6 cells (14) were maintained as described previously (15).

Virus stocks were obtained following Polybrene-mediated transfection of CEF with infectious plasmid clones of viral DNA (11). Avian neo retrovirus (ANV) was obtained from pANV-A. The construction of pANV-A will be described elsewhere (J. Olsen, W. Osheroff, and R. Swanstrom, manuscript in preparation). This plasmid contains the neomycin resistance (*neo*) gene and simian virus 40 promoter cloned downstream of the viral *env* gene. The resulting plasmid contains the replicative genes of the Schmidt-Ruppin A strain of Rous sarcoma virus derived from the SRA-2 DNA clone (5), with the *v-src* gene being replaced by the *neo* gene. Expression of the *neo* gene in animal cells confers resistance to cell killing by G418 (19). ALV was obtained from pALV-Cla. The construction of pALV-Cla will be described elsewhere (Olsen et al., in preparation). This plasmid contains the replicative genes of the Schmidt-Ruppin A strain of Rous sarcoma virus and lacks all *v-src* sequences.

The infectivity of ALV was titrated by placing dilutions of virus-containing medium on fresh QT6 cells and subsequently assaying for the presence of reverse transcriptase activity as described below. The infectious titer (in infectious units per milliliter) was determined from the dilution endpoint 30 days after infection.

Selection for G418-resistant colonies. On the day prior to infection, QT6 cells (5.0×10^5) were seeded in 60-mm tissue culture plates in medium containing various concentrations of azidothymidine. Twenty-four hours later, the medium was removed and replaced with medium containing ANV (4×10^3 CFU/ml). After 2 h at 37°C, the virus was removed and replaced with medium containing 225 μg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml. The medium was changed every 2 to 3 days. Azidothymidine was present in

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the medium throughout. The number of G418-resistant colonies was determined after 10 to 12 days.

Enzyme assays. Reverse transcriptase assays were used to monitor viral infection and were performed as described previously (15). For the kinetics study (see Fig. 4), the reaction volumes were increased to 150 μ l and contained either filtered culture medium from infected cells (0.45- μ m-pore filters) or 0.1 U of purified avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.).

The incorporation of [3 H]TMP into DNA was assayed by the DEAE filter-binding technique (12) and liquid scintillation spectrophotometry. Kinetic constants were determined by fitting the data to a hyperbola by using the method of Wilkinson (25) and the computer program of Cleland (4). Enzyme inhibition was analyzed by the method of Spector and Hajian (20).

The levels of deoxythymidine- and azidothymidine-phosphorylating activities in cell extracts were measured as described previously (6). Products were trapped on DEAE paper disks.

Viral DNA synthesis in vitro. Viral genomic 70S RNA was isolated from the Prague C strain of Rous sarcoma virus as described previously (15). Synthesis of viral DNA in vitro was carried out in 10- μ l reaction mixtures containing 50 mM Tris hydrochloride (pH 8.0), 8 mM $MgCl_2$, 40 mM NaCl, 2 mM dithiothreitol, 50 μ g of 70S RNA per ml, 50 μ M dATP, 50 μ M dCTP, 50 μ M dGTP, 50 μ M TTP, 650 nM [α - 32 P]dTTP (800 Ci/mmol; New England Nuclear Corp., Boston, Mass.), 100 μ g of actinomycin D (Sigma Chemical Co., St. Louis, Mo.) per ml, and 16 U of avian myeloblastosis virus reverse transcriptase. When needed, dideoxyribonucleoside triphosphates were added at the following concentrations: ddATP, 44 μ M; ddCTP, 20 μ M; ddGTP, 10 μ M; and ddTTP, 40 μ M. The reaction mixtures were incubated at 37°C for 30 min. Each reaction was terminated by the addition of a 2- μ l solution of 0.1 M EDTA and 1.5 N NaOH, and the viral RNA was allowed to hydrolyze for 18 h at 22°C. The solution was neutralized, and the DNA products were concentrated by ethanol precipitation and fractionated by electrophoresis through a 12% acrylamide sequencing gel (17).

Analysis of unintegrated viral DNA. Unintegrated viral DNA was isolated from whole cells by the method of Hirt (8) as described previously (15), except that the DNA was not extracted with acid-phenol. Unintegrated DNA was fractionated by electrophoresis in 1.0% agarose (SeaKem LE; FMC Corp., Marine Colloids Div., Rockland, Maine). The DNA in the gel was nicked by exposure to ethidium bromide and UV light, transferred to nitrocellulose paper (BA85; Schleicher & Schuell, Inc., Keene, N.H.), and hybridized at 42°C for 24 h in 50% formamide–10% dextran sulfate as described previously (2). The radioactive probe was prepared by nick translation (12) of pANV-A to a specific activity of 1.5×10^8 dpm/ μ g with [α - 32 P]dTTP (600 Ci/mmol; New England Nuclear) as the radioactive precursor. The concentration of the radioactive probe in the hybridization solution was 10 ng/ml. Hybridization was detected by autoradiography with Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.). For quantitation of the amount of hybridization, radioactive bands were excised from the nitrocellulose filter by using the exposed autoradiogram as a template and counted in Omnifluor (New England Nuclear). It was found that under the above-described hybridization conditions, the amount of hybridized DNA probe was proportional to the amount of DNA loaded on the gel over the concentration range of DNAs obtained from the

Hirt extracts (data not shown). We and others (15) have found that circular DNA is relatively easy to detect in QT6 cells. In various experiments, circular DNA represented 50 to >90% of the total unintegrated viral DNA detected.

High-pressure liquid chromatography analysis. Neutralized perchloric acid extracts of cells treated for 24 h with 50 μ M [3 H]azidothymidine were analyzed by high-pressure liquid chromatography as described previously (6). Azidothymidine nucleotides were separated by elution with a linear gradient of 0.015 to 1 M KH_2PO_4 (pH 3.5) developed over 110 min at a flow rate of 0.5 ml/min. Unlabeled nucleotides were analyzed in a similar manner.

RESULTS

Inhibition of ANV replication in quail cells by azidothymidine. We constructed a replication-competent avian retrovirus (ANV) that carries and expresses the *neo* gene from Tn5 (Olsen et al., in preparation). The ANV genome was constructed by using a modified form of the ALV genome (10). Since *neo* provides a marker for dominant selection against cell killing by the compound G418 (19), cell transformation to G418 resistance after virus infection provides a quantitative measure of a single round of virus replication. Clonal cell lines that grow in the presence of G418 have a single copy of the ANV genome, indicating that the resistant colonies arise as the result of infection by a single virus particle (data not shown). We used this assay to determine if azidothymidine could affect the ability of ANV to transform the continuous quail cell line QT6 (14) to G418 resistance. Increasing the amount of azidothymidine in the culture medium decreased virus replication, as measured by the decrease in G418-resistant colonies (Fig. 1). The amount of azidothymidine required to inhibit colony formation 70% was approximately 40 μ M. The amount required to inhibit colony formation 90% was 75 μ M.

Lack of inhibition of QT6 cell growth by azidothymidine at concentrations that inhibit virus replication. To determine the effects of azidothymidine on QT6 cells, we studied parameters of cell growth. In the first experiment, we examined the effect of azidothymidine on the plating efficiency of QT6 cells previously infected with ANV and selected for G418 resistance. In this experiment, 10^3 ANV-transformed QT6 cells were seeded into 60-mm dishes containing medium with various concentrations of azidothymidine. These cells had not previously been exposed to the drug. Twelve days later, the number of colonies on duplicate plates was determined (Fig. 1). Concentrations of azidothymidine up to 80 μ M had no effect on the efficiency of colony formation (Fig. 1); however, at azidothymidine concentrations greater than 120 μ M both the colony formation (Fig. 1) and the number of cells per colony (data not shown) significantly declined. Similar results were obtained for uninfected cells (data not shown).

In the second experiment, we examined the effect of azidothymidine on the growth rate of QT6 cells. In this experiment, 5×10^4 QT6 cells were seeded into wells of tissue culture plates (2 cm²). After 3.5 h, the medium was removed and replaced with medium containing azidothymidine. At various times, the cells were removed by trypsin treatment and counted. Figure 1 shows the results obtained from counting cells 4 days after seeding. At this time, cells without drug were rapidly growing. At 80 μ M azidothymidine, by which virus infection was inhibited more than 90%, there was no decrease in cell growth. At 200 μ M

FIG. 1. Effect of azidothymidine (N_3dThd) on the replication of ANV and on the growth of QT6 cells. Solid circles represent colony formation following infection of QT6 cells by ANV. QT6 cells (5.0×10^5) were infected and selected for G418 resistance as described in Materials and Methods. Azidothymidine was added to the cells 24 h prior to infection. Each point is the average of duplicate measurements. The 100% value in the absence of azidothymidine corresponds to 347 colonies. Open circles represent colony formation of ANV-infected QT6 cells plated at 10^3 cells per 60-mm dish. Each point is the average of duplicate measurements. The 100% value corresponds to 150 colonies. Open triangles represent the number of QT6 cells present after 4 days of culturing in the presence of azidothymidine. The 100% value is 3.8×10^5 cells.

azidothymidine, there was a slight (12%) decrease in cell growth. At higher concentrations (500 μM), the drug was cytotoxic. These data suggest that azidothymidine concentrations up to 80 μM can inhibit the replication of ANV without affecting the ability of QT6 cells either to plate at a low density or to grow at a normal rate.

Effect of azidothymidine on virus spread. The effect of azidothymidine on virus spread was tested with another assay. In this experiment, the ALV parent of ANV was used to infect QT6 cells in the presence of different concentrations of the thymidine analog. Virus replication was monitored by noting the appearance of reverse transcriptase-containing virus particles in the culture medium. A low level of virus appeared in the control culture 2 days after infection, and the cultures were monitored for another 2 weeks. After 5 days of infection, virus in the medium increased linearly. The approximate biological titer of virus at day 14 in the absence of drug was 10^5 infectious units per ml (data not shown). The increase in virus from days 5 to 14 was inhibited 70% in the presence of 1 μM azidothymidine (Fig. 2). The level of inhibition was 85% at 3 μM and 95% at 10 μM . No virus was detected at higher concentrations of azidothymidine (30, 80, and 200 μM).

We next tested the effect of azidothymidine on the replication of ALV in CEF. Again, the course of infection was monitored by noting the appearance of virus-associated reverse transcriptase activity in the medium. The amount of virus produced by cultures of CEF grown in the presence of different concentrations of azidothymidine is shown in Fig. 2. Virus production was inhibited 40% at 1 μM azidothymidine and 80% at 10 μM azidothymidine. Overall, the amount of virus produced at different concentrations of azidothymidine was approximately twice that produced in QT6

cells. However, in contrast to QT6 cells, the inhibitory effect on the growth of CEF was pronounced. Over a 3-day period, cell growth was inhibited 50% at 5 μM azidothymidine and 60% at 10 μM azidothymidine (Fig. 2).

Inhibition by azidothymidine of viral DNA synthesis in infected cells. The anticipated target of azidothymidine is the viral DNA polymerase reverse transcriptase. To test this hypothesis, we examined viral DNA synthesis in infected cells. QT6 cells that had been preincubated with various concentrations of azidothymidine for 24 h were infected with ALV. At 24 h after infection, unintegrated DNA was isolated by the method of Hirt (8), and the presence of viral DNA was detected by agarose gel electrophoresis and Southern blot analysis (Fig. 3A). In this experiment, form I viral DNA was more prominent than form III viral DNA. There was no detectable inhibition of viral DNA at an azidothymidine concentration of 1 μM (Fig. 3B). Approximately 65% inhibition was apparent at 30 μM azidothymidine. At 80 μM azidothymidine, the amount of viral DNA was estimated to be reduced 90%, and at 200 μM azidothymidine, viral DNA was barely detectable above the uninfected control. These results show that with increasing concentrations of azidothymidine there was a decrease in the amount of viral DNA synthesized in infected cells. Also, the level of inhibition of viral DNA at each concentration of the analog closely paralleled the level of inhibition of a single round of virus replication, as measured by the G418 resistance assay (Fig. 1).

Effect of azidothymidine on virus release in productively infected cells. To determine the effect of azidothymidine on the release of virus from productively infected cells, we passaged QT6 cells fully infected with ALV in medium containing 30 μM azidothymidine and maintained them for

FIG. 2. Effect of azidothymidine (N_3dThd) on the spread of ALV through cultures of QT6 cells and CEF. QT6 cells were seeded in medium containing various concentrations of azidothymidine and infected 24 h later with ALV at a multiplicity of infection of 10^{-2} infectious units per cell. At various times after infection, the medium was assayed for reverse transcriptase activity. The 11-day values were replotted as a function of azidothymidine concentration (inset). Also shown in the inset are data for a similar experiment with CEF, and included are the results of experiments measuring the effect of azidothymidine on the growth of CEF and QT6 cells.

30 days. Virus production was monitored by measuring the reverse transcriptase activity in the culture medium. In the culture that was exposed to azidothymidine, there was only a slight (10 to 20%) decrease in reverse transcriptase released into the medium over the course of the experiment (data not shown).

The infectivity of the virus produced in the presence of azidothymidine was also tested. In this experiment, QT6 cells were infected with 4×10^2 CFU of ANV in the presence of 30 μM azidothymidine. The resulting G418-resistant colonies were pooled. Azidothymidine was maintained in the medium continuously. The virus produced by the pooled G418-resistant colonies was found to have the same colony-forming ability, when normalized to reverse transcriptase activity, as the parental virus stock (data not shown). From these experiments, we conclude that azidothymidine has only a slight effect on the release of virus particles from productively infected cells and has no effect on the infectivity of the virus produced.

Inhibition of reverse transcriptase in vitro by azidothymidine triphosphate. To test directly the hypothesis that the triphosphate of azidothymidine ($N_3dThdTP$) inhibits viral DNA synthesis, we measured the ability of $N_3dThdTP$ to inhibit the incorporation of dTMP by the viral reverse transcriptase by using the synthetic template primer poly(rA) · oligo(dT)₁₂₋₁₈. We used purified reverse transcriptase from a closely related virus, avian myeloblastosis virus, and also virus-containing medium from CEF infected with ALV as a source of enzyme. This latter source allowed us to assay the same virus stock that was used in the infectivity experiments. The results with the purified reverse transcriptase (Fig. 4) show that $N_3dThdTP$ competitively inhibited the incorporation of dTMP catalyzed by reverse transcriptase. The apparent K_m for dTTP was $80 \pm 0.7 \mu M$, and the K_i for $N_3dThdTP$ was $0.09 \pm 0.003 \mu M$. These results indicate that $N_3dThdTP$ has a much greater affinity for the

enzyme than does the natural substrate dTTP. Similar results were obtained in experiments with disrupted virus from the culture medium as the source of enzyme. In these experiments we determined an apparent K_m of $40 \pm 7 \mu M$ for dTTP and a K_i of $0.07 \pm 0.014 \mu M$ for $N_3dThdTP$ (data not shown). These apparent K_m values are in close agreement with values reported previously (9).

Incorporation of $N_3dThdMP$ during reverse transcription of viral RNA. We next investigated the effect of $N_3dThdTP$ on DNA elongation by using purified reverse transcriptase with viral genomic RNA as the template primer (23). Figure 5 shows an analysis of DNA products synthesized in the absence and presence of $N_3dThdTP$. As expected, in the absence of $N_3dThdTP$, the predominant DNA product was strong-stop DNA, the 101-nucleotide-long transcript that is initiated on the endogenous tRNA^{Trp} primer and extends to the 5' end of the viral RNA template (23). In the presence of $N_3dThdTP$, the 101-base DNA transcript was again observed; however, a number of new, shorter DNA products now appeared. In each case, the new DNA product terminated at a thymidine residue, when compared with the truncated DNA product obtained from DNA synthesis in the presence of chain-terminating dideoxyribonucleoside triphosphates (17, 18). These results suggest that $N_3dThdMP$ is incorporated as a thymidine analog during reverse transcription of viral RNA and results in DNA chain termination.

Phosphorylation of azidothymidine in avian cells. High-pressure liquid chromatography analysis of extracts prepared from CEF and QT6 cells following incubation with 50 μM azidothymidine revealed the presence of mono-, di-, and triphosphate derivatives of azidothymidine in extracts of CEF and QT6 cells. In both cell types, the level of azidothymidine monophosphate was higher than the levels of azidothymidine diphosphate and azidothymidine triphosphate (Table 1). Azidothymidine monophosphate levels in both cell types were substantially lower than the levels

FIG. 3. Effect of azidothymidine (N_3dThd) on ALV DNA synthesis in QT6 cells. (A) QT6 cells were seeded in medium containing various concentrations of azidothymidine and infected 24 h later with ALV at a multiplicity of infection of 0.9 infectious units per cell. Twenty-four hours after infection, unintegrated DNA was fractionated by the Hirt procedure (8), subjected to electrophoresis in 1% agarose, and transferred to nitrocellulose paper. Viral DNA was detected by hybridization with a ^{32}P -labeled viral DNA probe and autoradiography. The mock-infected culture was grown in medium without azidothymidine. After autoradiography, regions corresponding to circular and linear forms of viral DNA were excised from the nitrocellulose filter, and the amount of radioactivity was determined by liquid scintillation spectrophotometry. Regions from the lane containing the mock-infected sample served as background controls. (B) The values of the two DNA forms were summed and plotted as a function of azidothymidine concentration. The 100% value corresponds to 629 cpm above the background level. No hybridizing material was seen in the lower-molecular-weight range of the gel in panel A (not shown).

detected in human, mouse, or monkey cells (7). However, the levels of the diphosphate and triphosphate derivatives of azidothymidine were only about 2- to 10-fold lower than those detected previously in mammalian cells (7). The levels of the triphosphate form of azidothymidine in CEF and QT6 cells (0.3 μM) were equal to or greater than the K_i for the viral reverse transcriptase.

The level of intracellular TTP in QT6 cells was reduced when azidothymidine was present in the culture medium. After treatment of QT6 cells for 24 h with 50 μM

FIG. 4. Inhibition of purified avian myeloblastosis virus reverse transcriptase by N_3dThdTP . The inhibition constant (K_i [slope]) for N_3dThdTP was determined by replotting the slope versus the N_3dThdTP concentration (inset). The units for $1/v$ are pM^{-1} , and the units for $1/[\text{TTP}]$ are μM^{-1} .

azidothymidine, the intracellular levels of TTP dropped from the control level of 190 pmol/ 10^6 cells (130 μM) to 60 pmol/ 10^6 cells (40 μM).

Phosphorylation of deoxythymidine and azidothymidine by cell extracts. Cytosol extracts of QT6 cells catalyzed the phosphorylation of deoxythymidine and azidothymidine at 35 and 20 pmol/min per mg of protein, respectively, at 37°C. The ratio of these activities for the different substrates (1.7) was very similar to that observed previously with extracts of human cells or with purified human thymidine kinase (1.5 and 1.7, respectively). However, the absolute rates of phosphorylation were 31- to 37-fold lower with the QT6 cell extracts than with the human cell extracts (7). These results suggest that the low level of N_3dThdMP seen in QT6 cells is due, in part, to a low level of thymidine kinase activity.

DISCUSSION

Inhibition of ALV replication by azidothymidine. Retrovirus DNA polymerase is an attractive target for an inhibitor of virus replication since it is virus encoded and required for viral DNA synthesis (20). These studies show that the thymidine analog azidothymidine inhibits the replication of ALV in infected cells (Fig. 1 and 2) and, in its triphosphate form, is a potent inhibitor of purified avian myeloblastosis virus DNA polymerase activity (Fig. 4 and 5). There is a direct correlation between the level of inhibition of a single round of virus replication and the reduction of the amount of viral DNA synthesized in infected cells in the presence of the drug (Fig. 1 and 3). Since the inhibition of virus replication in QT6 cells occurs at levels of azidothymidine that do not affect the growth rate of the cells, it is likely that the action of the analog is specific for the virus rather than an indirect effect of cellular toxicity. Our results extend those of previous studies which showed that azidothymidine can inhibit human immunodeficiency virus and other mammalian retro-

FIG. 5. Chain termination by $N_3dThdTP$ during reverse transcription of viral RNA in vitro. In reactions containing $N_3dThdTP$, the concentration is indicated at the top of the figure. Reactions containing ddATP (lane A), ddCTP (lane C), ddGTP (lane G), or ddTTP (lane T) are also indicated. The DNA sequence on the left matches the known complementary sequence near the 5' terminus of the Prague C strain of Rous sarcoma virus RNA (18). Positions of ambiguity (where bands appear at the same position in all lanes) are in parentheses. The nucleotide length, in bases from the $tRNA^{Trp}$ primer, is indicated on the right.

viruses (13, 16). The fact that this analog inhibits an avian retrovirus and mammalian retroviruses whose polymerases are only distantly related at the sequence level (24) suggests that azidothymidine will be broadly inhibitory against retroviruses.

Assay-dependent inhibition. Our results show that there can be significant variability in the apparent level of inhibition depending on the type of assay and the type of cell used to measure virus growth. Using the same virus and cells in two different assays, we found a 40-fold difference in the concentration of azidothymidine needed to inhibit virus replication 70%. An assay designed to measure inhibition of a single round of replication was much less sensitive to inhibition than was an assay designed to measure an increase in the amount of virus produced by a culture after virus spread (Fig. 1 and 2). This difference can be understood by the fact that virus spread is the result of several rounds of virus replication, each of which can be inhibited. However, measuring the inhibition of a single round of virus replication provides a more stringent assessment of the inhibitory effect of this or any compound.

Variability was also apparent when the results obtained with two different cell types, QT6 cells and CEF, were

TABLE 1. Anabolism of azidothymidine in CEF and QT6 cells^a

Cells	Concn (pmol/10 ⁶ cells) of indicated derivative of azidothymidine		
	Monophosphate	Diphosphate	Triphosphate
CEF	1.4 (0.9)	0.7 (0.5)	0.4 (0.3)
QT6	2.5 (1.7)	0.5 (0.4)	0.4 (0.3)

^a CEF and QT6 cells were incubated with 50 μM [3H]azidothymidine (424,000 dpm/pmol) for 24 h. Neutralized perchloric acid extracts of the treated cells were analyzed by high-pressure liquid chromatography. Concentrations were calculated based on a single-cell volume of 1.5 pl. Cell volume was determined by placing 100 μl of a cell suspension of known concentration in a capillary, pelleting the cells, and measuring the column height of the cell pellet. Values in parentheses represent micromolar concentrations.

compared (Fig. 2). Virus growth in QT6 cells, as measured by virus spread, was more sensitive to the analog than was virus growth in CEF, even though CEF showed much greater sensitivity to azidothymidine and the sensitivity was over the same range of concentrations that inhibited virus replication. Thus, at least some of the inhibition of virus spread in CEF was probably a result of a toxic effect on the host cell. The differential sensitivity of CEF and QT6 cells was not due to different levels of phosphorylated azidothymidine present in the cells, since these levels were approximately the same in each cell type (Table 1). It is not clear why virus replication was less sensitive to inhibition in CEF at equivalent levels of drug. One possible explanation may be in differences in the rate of virus spread through cultures. If virus spread in CEF is significantly more efficient than that in QT6 cells, it could obscure differences in the level of inhibition of DNA synthesis during a single round of infection. Virus sensitivity to azidothymidine, cellular toxicity from the analog, and ease of virus spread may all contribute to the final level of virus that is produced in a certain cell type or, potentially, in a certain tissue type.

Phosphorylation of azidothymidine in avian cells and interaction of its triphosphate with viral DNA polymerase. Extracts from QT6 cells catalyzed the phosphorylation of azidothymidine. The ratio of the rates of phosphorylation of thymidine and azidothymidine was very similar to that seen previously with human cells (7); it is likely that cytosolic thymidine kinase phosphorylates azidothymidine to its monophosphate in QT6 cells. The low level of thymidine kinase observed in the QT6 extracts correlates with the observation (Table 1) that the level of $N_3dThdMP$ was much lower than that seen previously in human cells (7).

At present, the mechanism(s) by which azidothymidine inhibits retrovirus DNA replication is not completely understood. $N_3dThdTP$ could inhibit ALV replication by chain termination of DNA synthesis (Fig. 5), by competitive inhibition of reverse transcriptase (Fig. 4), or by a combination of the two. The fact that a full-length DNA product is synthesized in infected cells treated with azidothymidine in the apparent absence of premature termination products (Fig. 3) suggests that competitive inhibition rather than chain termination may be the predominant mechanism of inhibition in infected cells. $N_3dThdTP$ must be able to compete with TTP to act as an inhibitor for the reverse transcriptase. With a K_i of 0.09 μM (Fig. 4) and an intracellular concentration of 0.3 μM in the presence of 50 μM azidothymidine (Table 1), $N_3dThdTP$ should be able to compete favorably with TTP, which has an apparent K_m of 40 to 80 μM and an intracellular concentration of 40 μM in the presence of 50 μM azidothymidine. These nucleotide concentrations should result in 60 to 70% inhibition. This level of inhibition is very

close to the 65% inhibition of viral DNA synthesized in infected cells in the presence of 30 μ M azidothymidine (Fig. 3) and similar to the level of inhibition of a single round of virus replication at an equivalent drug concentration (Fig. 1). However, a direct comparison between results obtained in vitro and in infected cells is difficult to make since we do not know the K_i of N_3 dThdTP for reverse transcriptase with its natural RNA and DNA templates.

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