Inhibition of Hepatitis B Virus Deoxyribonucleic Acid Polymerase by the 5'-Triphosphates of 9-β-D-Arabino furanosyladenine and 1-β-D-Arabino furanosylcytosine

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9-β-D-Arabino furanosyladenine (ara-A), 1-β-D-arabino furanosylcytosine (ara-C), and their 5'-triphosphates (ara-ATP and ara-CTP) were tested for ability to inhibit the hepatitis B virus (HBV)-associated deoxyribonucleic acid (DNA) polymerase. Ara-C did not inhibit the HBV DNA polymerase at the concentrations tested, ara-A did so by 50% at a concentration of 30 mM, with the inhibition noncompetitive with respect to deoxyadenosine 5-triphosphate (dATP). Ara-ATP and ara-CTP inhibited the DNA polymerase test competitively with respect to dATP and dCTP, respectively. Both compounds were also active after initiation of the DNA polymerase reaction. The inhibition caused by ara-ATP and ara-CTP was shown to be reversible, with no evidence that ara-ATP or ara-CTP was incorporated into the HBV DNA.

Three different morphological forms exhibiting hepatitis B surface antigen (HBsAg) determinants can be distinguished in sera of individuals with acute or chronic type B hepatitis: a 22-nm spherical particle, a filamentous particle 22 nm in diameter and variable in length, and a spherical 42-nm Dane particle. The Dane particle is the only HBsAg form to consist of an inner core (hepatitis B core antigen [HBcAg]) and an outer shell (HBsAg) (4). A subpopulation of Dane particles contains a circular double-stranded deoxyribonucleic acid (DNA) and a DNA polymerase (7, 8, 20). This population of Dane particles is believed to represent the complete hepatitis B virus (HBV). The DNA polymerase was found to be located within the HBcAg (7). During the reaction, the DNA polymerase fills single-stranded regions of the HBV DNA, which comprise between 15 and 45% of its total length, in the presence of triphosphates and a detergent (6, 9).

Recent studies indicate that 9-β-D-arabinofurano- syladenine (ara-A) has antiviral activity when administered to individuals suffering from chronic type B hepatitis (2, 10, 18). It was observed that this therapy led to a significant reduction of serum Dane particles as indicated by a decrease of Dane particle-associated HBcAg and HBV-specific DNA polymerase activity. In addition, a marked decrease in HBsAg and hepatitis B e antigen (HBeAg) concentration was noted (2, 10, 18). These observations led us to study the effect of ara-A and its triphosphate (ara-ATP) on the Dane particle-associated DNA polymerase. 1-β-D-Arabinofurano- sylcytosine (ara-C) and its triphosphate (ara- CTP), which are known to exert intolerable toxicity when administered to humans (11), were used for comparison.

MATERIALS AND METHODS

Materials. Ara-A, ara-C, ara-ATP, and ara-CTP were purchased from Sigma Chemical Co., St. Louis, Mo. [3H]dTTP (deoxythymidine triphosphate; specific activity, 50 Ci/mmol) was obtained from New England Nuclear Corp. dATP, dCTP, and dGTP (deoxyguanosine triphosphate) were purchased from P-L Biochemica ls.

Assays. HBsAg was determined by using a commercially available radioimmunoassay (AUSR A II, Abbott Laboratories, Chicago, Ill.). Dane particle-associated HBcAg was determined by microtiter solid-phase radioimmunoassay as described by Purcell et al. (19). DNA-dependent HBV-specific DNA polymerase activity was measured as described by Kaplan et al. (7).

Preparation of Dane particles. Sera of HBsAg carriers were screened for HBV-specific DNA polymerase activity. Those containing large quantities of complete Dane particles as judged by high levels of DNA polymerase activity were selected for Dane particle isolation. Dane particles in 240 ml of serum were pelleted in an SW27 rotor at 25,000 rpm at 4°C for 16 h. The resulting pellet was suspended in 2 ml of 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 0.1% (wt/vol) bovine serum albumin (BSA). Six milliliters of each Dane particle concentrate was layered on 6 ml of 20% (wt/wt) sucrose containing 0.1% BSA and pelleted in an SW41 rotor at 40,000 rpm at 4°C for 4 h. The resulting pellet was again dissolved in 200 μl of PBS-BSA. This Dane particle concentrate was diluted to 2 ml with PBS-BSA and layered on top of a cesium chloride gradient consisting of 1.5 ml of
1.32 g/ml CsCl, 2.5 ml of 0.25 g/ml CsCl, and 6 ml of 0.20 g/ml CsCl. After centrifugation in an SW41 rotor at 32,000 rpm at 4 °C for 16 h, fractions were taken by bottom puncture and assayed for DNA polymerase activity (10 μl) and Dane particle-associated HBcAg (10 μl) (Fig. 1). Fractions 6 to 8 containing heavy Dane particles as indicated by HBcAg activity and a peak of DNA polymerase activity were pooled and dialyzed against several changes of PBS-BSA and finally diluted to 30 ml, using PBS-BSA as the diluent. Twenty microliters of this Dane particle preparation contained approximately 2,000 cpm in a DNA polymerase test, whereas a test lacking Dane particles revealed approximately 40 cpm after a 3-h DNA polymerase reaction. This Dane particle preparation served as Dane particle source in the DNA polymerase inhibition test described below.

DNA polymerase inhibition test. Stock solutions of ara-A, ara-C, ara-ATP, and ara-CTP were made in distilled water. The DNA polymerase inhibition test contained the following reactants (final concentration given in parentheses) in a total volume of 120 μl: NH₄Cl (100 mM), tris(hydroxymethyl)amino- methane-hydrochloride, pH 7.4 (130 mM), MgCl₂ (30 mM), dATP, dCTP, and dGTP (each 400 μM), [3H]dTTP (20 μM), 0.5% (vol/vol) Nonidet P-40, and various amounts of the compounds tested. The reaction was started by the addition of 20 μl of the standard Dane particle preparation. After a 3-h reaction at 37°C, the total volume was spotted on a Whatman 3MM filter paper disk, washed intensively in trichloro- acetic acid, and counted in a liquid scintillation counter (7). The specific counts per minute obtained with the compound were related to those without a compound and expressed as percent activity.

RESULTS

Experiments with fewer than four triphosphates revealed that the HBV DNA polymerase required all four triphosphates for optimal activity. The standard DNA polymerase test contained all triphosphates in vast excess. When the individual triphosphates (dATP, dCTP, and dGTP) were titrated, each could be reduced to a concentration of 0.2 μM without affecting the incorporation of [3H]dTTP into the HBV DNA.

Ara-A, a compound shown to exhibit antiviral activity in different systems, was tested for its ability to inhibit the DNA polymerase test. A 50% inhibition of the DNA polymerase assay was obtained at an ara-A concentration of 30 μM (Fig. 2B). This inhibition was not competitive with respect to dATP. Ara-ATP inhibited the DNA polymerase assay by 50% at a concentration of 15 μM.

Fig. 1. Dane particles were concentrated from a serum of an HBsAg carrier by repeated pelleting and then subjected to cesium chloride density gradient centrifugation in an SW41 rotor as described in the text. The fractions obtained were analyzed for Dane particle-associated HBcAg (10 μl) and DNA polymerase activity (10 μl). Two populations of Dane particles were resolved: (i) a heavy Dane particle population indicated by the coincidence of HBcAg activity and a peak of DNA polymerase activity, and (ii) a light Dane particle population which was associated with a peak of HBcAg and thus represented the predominant Dane particle population. Heavy Dane particles which contain a circular double-stranded DNA and a DNA polymerase were used to test the effect of ara-A, ara-C, ara-ATP, and ara-CTP on the HBV DNA polymerase.
is, however, limited by the fact that asynchrony was found to be competitive with respect to dATP but not to dCTP and dGTP (Fig. 3B).

Similar experiments were performed with ara-C and ara-CTP. Ara-C did not inhibit the DNA polymerase test up to a concentration of 60 μM. Reduction of the dCTP content in the reaction mixture did not result in an inhibition of the DNA polymerase test by ara-C (Fig. 2A). In contrast, a 50% inhibition of the DNA polymerase assay was noted at an ara-CTP concentration of 0.4 μM. This inhibition was found to be competitive with respect to dCTP, but not to dATP and dGTP (Fig. 3A).

The HBV-associated DNA polymerase exhibited optimal activity in the presence of MgCl₂ concentrations between 30 and 300 mM. Experiments carried out with 30 and 300 mM MgCl₂, respectively, revealed that the inhibition caused by ara-ATP and ara-CTP was not dependent on the MgCl₂ concentration (data not shown).

To determine whether ara-ATP and ara-CTP were active also after initiation of the DNA polymerase reaction, 2 mM ara-ATP or ara-CTP was added to the DNA polymerase test at different times during the reaction. Ara-ATP and ara-CTP inhibited the incorporation of triphosphates into the HBV DNA also when they were added 15, 30, 45, and 60 min after initiation of the reaction (Fig. 4), indicating that ara-ATP and ara-CTP were also active during the elongation step of the DNA polymerase reaction.

Finally, we tested whether the inhibition caused by ara-ATP was also competitive after initiation of the DNA polymerase reaction. At an ara-ATP concentration of 40 μM, the DNA polymerase test was not inhibited at a dATP concentration of 400 μM but completely inhibited at a dATP concentration of 0.2 μM (Fig. 3B). Therefore, we ran a DNA polymerase test that contained a dATP concentration of 0.2 μM and an ara-CTP concentration of 40 μM. At different times (0, 15, 30, 45, 60, 90, 120, 135, 150, 165, and 180 min) after the DNA polymerase reaction was started, the dATP concentration was adjusted to 400 μM. The data obtained after a 3-h reaction were compared with those obtained in a standard test lacking ara-ATP and were expressed as percent activity. The results (Fig. 5B) were plotted in relation to the time at which the dATP concentration was adjusted to 400 μM. It is evident from the data that the inhibition of the HBV DNA polymerase caused by ara-ATP was reversible even after initiation of the DNA polymerase assay, which strongly argues against incorporation of ara-ATP into the HBV DNA. An experiment carried out with ara-CTP revealed results similar to those obtained with ara-ATP (Fig. 5A).

DISCUSSION

Ara-A and ara-C have been shown to be promising antiviral and carcinostatic agents (3, 11, 21-23). After entering the cell, both compounds are readily converted by the cell into their corresponding mono-, di-, or triphosphates (1, 12, 15-17). The triphosphates of these compounds (ara-ATP and ara-CTP) were shown to be the active components and to inhibit ribonucleic acid-directed DNA polymerases, DNA-directed DNA polymerases, and mammalian DNA polymerases alpha and beta (11). Several viruses which are known to induce DNA polymerases in the infected cell do not contain a DNA polymerase in the virion (24). HBV (Dane particle) is
among the few viruses to contain a DNA polymerase within the virus particle (24). The DNA polymerase of the Dane particle was found to be DNA dependent and was localized within the core (HBcAg) of the Dane particle (7). It is well established that this enzyme incorporates triphosphates into single-stranded regions of the circular HBV DNA. The function of this HBV DNA polymerase in the infected hepatocyte is still not known. This is largely due to the fact that HBV infection is restricted to humans and to chimpanzees, where HBV replication takes place in the liver. In addition, HBV has so far resisted propagation in tissue culture.

We have studied the effects of ara-A, ara-C, ara-ATP, and ara-CTP on the DNA polymerase reaction. Ara-C did not affect the DNA polymerase reaction, and ara-A did so only at high concentrations. Chromosome breaks have been observed after incubation of leukocytes with

![Graph A](Image1)

**Fig. 3.** Inhibitory activity of ara-CTP (A) and ara-ATP (B) on the HBV-associated DNA polymerase was tested as in Fig. 2.
high concentrations of ara-A (14), and a similar mechanism might be responsible for the inhibition of the HBV DNA polymerase by ara-A. As reported previously with other DNA polymerases (5, 11), ara-ATP and ara-CTP inhibited the DNA polymerase reaction competitively with their respective triphosphates. Ara-ATP and ara-CTP were found to be active after initiation of the DNA polymerase reaction; this statement has not been excluded and the timing of events in the replication reaction is not established. In addition, our data strongly argue against incorporation of ara-ATP and ara-CTP into HBV DNA.

Pollard et al. (18) and Chadwick et al. (2) described reduction of Dane particles, HBeAg, and HBsAg in the serum of patients with chronic type B hepatitis who received ara-A therapy. Since the enzymes involved in HBV replication
Laboratory polymerase DNA polymerase. Very uncertain whether the dCTP concentration was 0.2 μM (A). At different times (0, 15, 30, 45, 60, 90, 120, 150, 180 min) after the DNA polymerase reaction was started, the dCTP concentration was adjusted to 400 μM. A similar experiment was carried out with ara-ATP (B). The ara-ATP concentration was 40 μM; dATP concentrations were 0.2 and 400 μM, respectively.

in the infected hepatocyte are still not known, it remains uncertain whether the above effect is due to inhibition of a virus-specific or a host DNA polymerase. Very recent studies in this laboratory revealed that the HBV-associated DNA polymerase meets many properties that have been described for the cellular DNA polymerase beta (G. Hess, W. Arnold, and K.-H. Meyer zum Büschenfelde, manuscript in preparation). If additional studies can prove that the HBV-associated DNA polymerase does represent a host-coded enzyme, reduction of Dane particles under ara-A therapy is probably due to inhibition of host enzymes (most likely DNA polymerase alpha). If so, attempts to terminate an HBsAg carrier state might benefit from stimulation of host defense mechanisms to destroy virus-infected cells while viral replication is reduced or arrested under ara-A therapy.
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