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Comparison of Properties of Woodchuck Hepatitis Virus and Human Hepatitis B Virus Endogenous DNA Polymerases

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The principal properties of the DNA polymerases of woodchuck hepatitis virus and human hepatitis B virus were compared. The enzymes of both viruses exhibited optimal activities in the same range of pH, ionic strength, and $MgCl_2$ concentration. Like human hepatitis B virus DNA polymerase, the woodchuck hepatitis virus DNA polymerase was strongly inhibited by phosphonoformic acid but not by phosphonoacetic acid and aphidicolin. Similar inhibition patterns for both enzymes were observed with arabinofuranosyl nucleotides (9-β-D-arabinofuranosyladenine-5'-triphosphate, 1-β-D-arabinofuranosylcytosine-5'-triphosphate, 1-β-D-arabinofuranosylthymine-5'-triphosphate) and dideoxythymidine triphosphate, whereas no effect was obtained with corresponding nucleosides. The therapeutic significance of these results and the relevance of the woodchuck as an experimental animal model for the study of human hepatitis B virus infections are discussed.

A new class of virus, for which the tentative terms Icrons (2) and Hepa-DNA-virus (24) have been proposed, includes human hepatitis B virus (HBV), woodchuck hepatitis virus (WHV) (28), Beechey ground squirrel hepatitis virus (13), and Pekin duck hepatitis virus (14). These viruses share the following characteristics: (i) the presence in blood of excess viral coat protein as tubular and spherical forms, (ii) double-shelled particles containing a small circular partially single-stranded DNA and a DNA polymerase, and (iii) a high proportion of chronic persistent infections associated with inflammatory liver diseases and, as documented for humans and woodchucks, hepatocellular carcinoma (21, 26). Among these, the WHV of *Marmota monax* and the HBV of humans have a close phylogenetic relationship as illustrated by nucleic acid homology (7, 8) and antigenic cross-reactivity (16, 32). HBV is responsible for chronic infections for which treatment with antiviral agents such as vidarabine (3, 20), vidarabine-5'-monophosphate (3), and acyclovir (3) is actually under clinical evaluation. To assess the possibility of blocking viral replication, the HBV DNA polymerase has been well characterized by studies on the inhibition of this enzyme by intercalating agents (10), pyrophosphate analogs (18), and arabinofuranosyl nucleotides (9). Like HBV, WHV particles contain a DNA polymerase which fills in the large single-stranded region in the genome, generating a fully double-stranded DNA. Therefore, woodchucks may represent a potentially useful animal model for in vivo studies of antiviral agents against HBV. To further evaluate this possibility, we report here, in a comparative study, the major properties and the inhibition patterns by arabinofuranosyl nucleotides for HBV and WHV DNA polymerases.

MATERIALS AND METHODS

Reagents and compounds used. Unlabeled triphosphates (dATP, dCTP, dGTP, and dTTP), phosphonoacetic acid (PAA), and phosphonoformic acid (PFA) were obtained from Sigma Chemical Co., St. Louis, Mo. Thymidine, 1-β-D arabinofuranosylthymine (ara-T), and 1-β-D-arabinofuranosylcytosine (ara-C) were from Calbiochem-Behring, Paris, France, and 9-β-D arabinofuranosyladenine (ara-A) and 9-β-D-arabinofuranosyladenine-5'-monophosphate (ara AMP) were from Parke-Davis, Laboratoires Substantia, Paris, France. Dideoxythymidine (ddT), dideoxythymidine triphosphate (ddTTP), 9-β-D-arabinofuranosyladenine-5'-triphosphate (ara-ATP), and 1-β-D-arabinofuranosylcytosine-5'-triphosphate (ara-CTP) were purchased from P-L Biochemicals, Milwaukee, Wis. 1-β-D-Arabinofuranosylthymine-5'-triphosphate (ara-TTP) was a gift from G. A. Gentry, Mississippi Medical Center, Jackson, Miss., which was tritiated (0.33 Ci/mmol) by the Commissariat à l'Energie Atomique, Gif sur Yvette, France. [^{3}H]dTTP (41 Ci/mmol), [^{3}H]dATP(24 μCi/mmol), and [^{3}H]dGTP (9.5 Ci/mmol) were purchased from the Amersham Radiochemical Center, Versailles, France. Aphidicolin was a gift from A. H. Todd, Imperial Chemical Industries, Cheshire, England.

Woodchucks. The animals used in this study belonged to a colony of woodchucks (*Marmota monax*) established in our laboratory and made up of animals imported from Dutchland Laboratories Inc., Denver, Pa. They were kept indoors (10 to 25°C), maintained in separate cages, and fed with rat chow, vegetables, and water ad libitum. The animal used in this study was a 3-year-old naturally infected male, and it was observed for 1 year with bimonthly specimens of blood.

Serological assays. Hepatitis B surface antigen (HBsAg), anti-HBs, and antibody to hepatitis B core antigen (anti-HBc) were determined by using commercially available radioimmunoassays (Abbott Laboratories, North Chicago, Ill.). Dane particle-associated hepatitis B core antigen was determined by a microtiter solid-phase radioimmunoassay as described by Purcell et al. (22). Woodchuck sera were tested by counter immunoelectrophoresis (28) for woodchuck hepatitis surface antigen (WHsAg) and anti-WHs by using reference reagents kindly provided by J. Summers and the same radioimmunoassays (Abbott Laboratories) used for HBsAg and anti-HBs, taking advantage of the cross-reactivity between WHsAg and HBsAg (17).

Preparation of (HBV). Serum was obtained from an immunosuppressed patient positive for HBsAg. Viral particles were pelleted from 250 ml of serum by three successive centrifugations through a discontinuous sucrose gradient (10 to 20%) as described by Summers et al. (28). The final pellet

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was resuspended in 3 ml of CsCl solution (density, 1.16 g/cm^3) containing 10 mM Tris-EDTA and layered on top of a CsCl gradient (density, 1.22 g/cm^3 to 1.30 g/cm^3) in 10 mM Tris-EDTA, pH 7.2. After centrifugation at 35,000 rpm for 22 h in a Beckman SW41 rotor, fractions (0.6 ml) were collected from the top of the tubes (Fig. 1A). Fractions (15, 16, and 17) containing heavy Dane particles and the peak DNA polymerase activity were pooled, dialyzed against 50 mM Tris-hydrochloride, pH 7.6–0.15M NaCl–0.02% NaN_3 and finally diluted to 20 ml of this buffer containing 0.02% bovine serum albumin. This solution was divided into portions (0.5 ml), stored at −80°C, and used as a standard HBV-associated DNA polymerase preparation. With 10 μl of this preparation, 2,000 cpm was obtained in the standard DNA polymerase assay.

Preparation of WHV. Blood was drawn from a WHsAg chronic-carrier woodchuck. Virions were purified from 40 ml of serum as described above by CsCl gradient centrifugation (Fig. 1B). The fractions (13, 14, 15, and 16) containing the peak DNA polymerase activity were pooled, dialyzed, diluted to 7 ml in buffer, divided into portions (0.5 ml), and stored at −80°C. Ten microliters of this standard WHV preparation was used for each DNA polymerase assay, with 1,800 cpm of [^{3}H]dTTP incorporated under standard conditions.

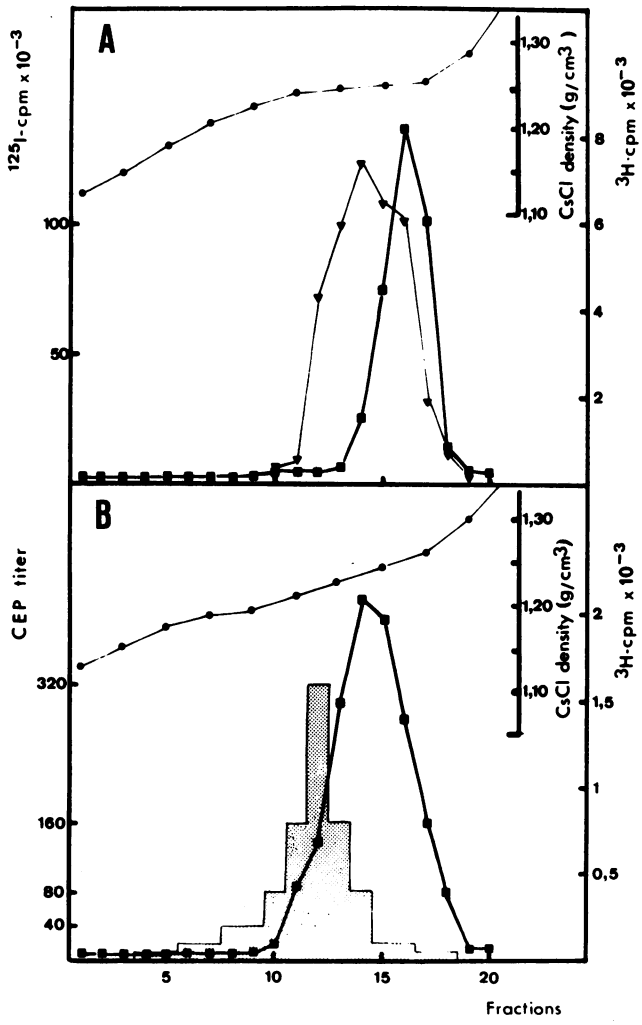

FIG. 1. (A) Density gradient centrifugation for purification of Dane particles. After centrifugation, fractions were collected from the top of the tubes and tested for DNA polymerase activity (5 μl) (■) and Dane particle-associated HBcAg (5 μl) (▼). CsCl density (●) is also shown. (B) Density gradient centrifugation for purification of WHV. Fractions were assayed for DNA polymerase activity (5 μl) as described in Materials and Methods (■) and for WHsAg by counterimmunoelectrophoresis (CEP; shaded area).

DNA polymerase assay. DNA polymerase was assayed as described by Kaplan et al. (12) with minor modifications. The reaction was carried out in a 50-μl final volume containing 50 mM Tris-hydrochloride, pH 7.5, 40 mM $MgCl_2$, 60 mM NH_4Cl, 100 mM dATP, dCTP, and dGTP, 0.4 μM [^{3}H]dTTP (41 Ci/mmol), 0.5% Nonidet P-40, 10 mM 2-mercaptoethanol, and inhibitor at indicated concentrations (see below). The reaction was started by addition of 15 μl of the standard Dane particle preparation. After 2 h at 37°C we added 50 μl of pronase E (E. Merck AG, Darmstadt, West Germany) at 0.5 mg/ml in 10 mM Tris-hydrochloride, pH 7.4–10 mM EDTA. After a further incubation for 1 h at 40°C, the reaction was stopped by addition of 1 ml of cold 15% trichloroacetic acid in distilled water containing 50 mM sodium PP_i. Under such conditions, the incorporation of [^{3}H]dTTP into the viral DNA was linear for 2 h. Acid-precipitable radioactive material was then collected on glass fiber filters (GF/C; Whatman, Inc., Clifton, N.J.). Filters were washed 12 times with 5 ml of cold 3% trichloroacetic acid and then once with 95% ethanol by using a Millipore manifold. Radioactivity on the dried filter was measured by liquid scintillation. All experiments were conducted under the above-mentioned conditions except in the case of the inhibition studies for ddT, ddTTP, and ara-TTP in which [^{3}H]dATP or [^{3}H]dGTP was used as labeled nucleotide. The same applied to other nonradioactive nucleotides used at different concentrations as indicated in the text.

Specificity of viral DNA polymerase assay. Standard HBV and WHV preparations were tested for the specificity of enzyme activity associated with HBV or WHV precipitation with anti-HBs or anti-WHs serum before detergent treatment. Twenty-five microliters of the HBV or WHV preparation was mixed with either 25 μl of rabbit anti-HBs serum or normal rabbit serum (HBV sample) or with 25 μl of woodchuck anti-WHs or normal woodchuck serum (WHV samples) and incubated for 1 day at 4°C. Goat anti-rabbit immunoglobulin G (IgG) (15 μl; for HBV samples) or rabbit anti-woodchuck IgG (15 μl; for WHV samples) was then added. After a further incubation for 1 day at 4°C, the mixtures were centrifuged at 3,000 × *g* for 15 min. DNA polymerase activity was then measured on 25 μl of the supernate as described above.

RESULTS

Specificity of the DNA polymerase activity. Heavy Dane particles and WHsAg particles containing DNA polymerase activity were isolated by CsCl density gradient centrifugation as described in Fig. 1. When immunoprecipitated by their homologous antibodies (anti-HBs and anti-WHs) the HBV and WHV particle preparations lost more than 90% of their DNA polymerase activity (Table 1). In the same manner, the reaction of anti-WHs with HBV particles and of anti-HBs with WHV particles resulted in a lower but significant decrease of the DNA polymerase activity. (Table 1).

Principal properties of the WHV and HBV DNA polymerases. Optimal activity of the HBV and WHV DNA polymerase was observed between pH 7.2 and 8.0 and at high salt concentration (0.15 M NaCl or 0.2 M KCl). Both enzymes required high $MgCl_2$ concentration (20 to 120 mM) for

TABLE 1. DNA polymerase activity in HBV and WHV particle preparations after immunoprecipitation by anti-HBs and anti-WHs

Antibody	% Endogenous DNA polymerase after immunoprecipitation	
	HBV	WHV
Negative control	100	100
Anti-HBs	6	22
Anti-WHs	52	7

optimal activity. No significant enzyme activity was observed with $MnCl_2$, $CaCl_2$, or $BaCl_2$ at concentrations of 1 to 50 mM. A low incorporation (about 15% of the normal value) of [^{3}H]dTTP was found with $ZnCl_2$ at a 30 mM concentration. WHV and HBV DNA polymerase showed similar sensitivity to *N*-ethylmaleimide and *N*-methylmaleimide and ethanol. A concentration of 100 mM *N*-ethylmaleimide or *N*-methylmaleimide was required to obtain total inhibition of both enzymes, whereas a 10 mM concentration had little effect (5% inhibition). The addition of 10 or 20% (vol/vol) ethanol decreased both DNA polymerase activities by 30 and 70%, respectively. No inhibition was obtained with aphidicolin at a 300 μM concentration, which specifically inhibits the cellular DNA polymerase-α (data not shown).

Effect of PAA and PFA. The same sensitivity to PAA and PFA was observed for the HBV and WHV DNA polymerases. The two enzymes were highly resistant to PAA (no inhibition at 1 mM of PAA) but were inhibited by low concentrations of PFA (50% inhibition at 10 μM) (Table 2).

Effect of triphosphate analogs. HBV and WHV DNA polymerases were inhibited by ara-TTP and ddTTP. The inhibition of these two analogs was studied in four successive assays, each containing one of the four natural triphosphates at low concentration (0.2 μM), the other two nonradioactive natural triphosphates in excess (100 μM), and [^{3}H]dATP and [^{3}H]dGTP as labeled nucleotides. In this experiment (data not shown), increased inhibition of ara-TTP and ddTTP occurred at a low concentration of dTTP but did not change for low levels of dATP, dGTP, or dCTP. At 0.2 μM dTTP, the ara-TTP/dTTP ratio giving a 50% inhibition of the DNA polymerase was about 0.25 for both HBV and WHV DNA polymerases, whereas ddTTP/dTTP ratios differed slightly (1 for HBV, 0.4 for WHV). Furthermore (Table 2), similar ratios of ara-TTP to dTTP (1.5) and ddTTP to dTTP (1.0) were obtained for both enzymes at 100 μM of dTTP. When the DNA polymerase assays were carried out with [^{3}H]ara-TTP (specific activity, 0.33 Ci/mmol) instead of [^{3}H]dTTP (specific activity, 4.1 Ci/mmol), no incorporation of [^{3}H]ara-TTP in HBV or WHV DNA was observed, with concentrated preparations showing about 10,000 cpm of [^{3}H]dTTP incorporation after 4 h of reaction. Ara-CTP and ara-ATP had also the same inhibitory effect on the HBV and WHV DNA polymerases at 100 μM concentrations of the corresponding natural triphosphate dCTP and dATP (Table 2).

Effect of nucleoside analogs. No significant inhibition was observed with either ara-A, ara-C, ara-T, or ddT (Table 2). Only very high concentrations of ara-AMP inhibited the DNA polymerases (18 and 30%) inhibition of HBV and WHV DNA polymerases at a 20 mM concentration, respectively.

DISCUSSION

The close structural and clinical-pathological similarities observed between WHV and HBV suggest that the woodchuck may represent a very promising model for studying persistent hepatitis virus infections and their relation to the development of primary liver cancer (2, 21, 26). DNA polymerases enclosed in the HBV and WHV virus particles can be immunoprecipitated by their homologous antibodies (anti-HBs and anti-WHs). Immunoprecipitation of the particle-associated DNA polymerase activity also occurred in a heterologous system, i.e., anti-WHs with HBV particles and anti-HBs with WHV particles, thus confirming the existence of common antigenic determinants on the HBs and WHs antigens (32). Comparison of the properties of these two virus-associated DNA polymerases show them to have remarkably similar behavior. Many properties of these enzymes are similar to those described for the cellular DNA polymerase-β (15, 29): high salt and $MgCl_2$ concentrations for optimal activity, insensitivity to PAA, sulfhydryl group blocking agents, and aphidicolin (11). However, WHV and HBV DNA polymerases differ from DNA β-polymerases in optimal pH and sensitivity to ethanol, ddTTP, and PFA (23, 29). Since the DNA β-polymerases of human and woodchuck liver have not been studied, our results do not permit us to form any conclusions about the viral or host origin of these two enzymes.

Triphosphate analogs such as ara-ATP, ara-CTP, ara-TTP, and ddTTP are known to be specific inhibitors of cellular and viral DNA polymerase (5, 6, 15). Very similar inhibitions by these four compounds were obtained for the HBV and WHV DNA polymerases. Enzyme inhibition by ara-TTP and dTTP was found dependent on the concentration of dTTP, and the rate of inhibition was the same for the both viruses.

Hess et al. (9) have reported an inhibitory effect of 50 μM ara-A on the HBV DNA polymerase. In our experiments, however, we were unable to dissolve ara-A at this concentration, and 20 mM ara-A did not inhibit the DNA polymerase activity. By contrst, ara-AMP exhibited only an inhibitory effect at very high concentration (20 mM). The mechanism of this inhibition by ara-AMP remains to be

TABLE 2. Comparison of inhibition of HBV and WHV DNA polymerases

Inhibitor	HBV DNA polymerase		WHV DNA polymerase	
	Maximum inhibition (% of control)[a]	I/S_{50}[b]	Maximum inhibition (% of control)[a]	I/S_{50}[b]
Aphidicolin	0		0	
PFA	95		95	
PAA	0		0	
ara-A	0		0	
ara-AMP	18		30	
ara-ATP	98	2.5	96	2.5
ara-C	0		0	
ara-CTP	98	2	98	2
ara-T	0		0	
ara-TTP	96	1.5	0	1.5
ddT	0		0	
ddTTP	98	1	98	1

[a] Inhibition noted at the following highest concentrations of inhibitors tested: aphidicolin, 0.3 mM; PFA and PAA, 1 mM; all others, 20 mM.

[b] Results are expressed as I/S_{50} ratios where I is the concentration of inhibitor giving a 50% inhibition of the DNA polymerase activity and S is the concentration of the corresponding natural triphosphate (dATP for ara-ATP, dCTP for ara-CTP, dTTP for ddTTP and ara-TTP).

explained. We cannot exclude that this inhibition was due to contaminants present in the ara-AMP preparation.

Although speculative, it is tempting to assign critical functions to the DNA polymerase contained in the viral particles in the replication of the virus; the enzyme seems to be necessary for the structural stability of the HBV genome (27) and may play a role in its replication and integration (25). In this view, the inhibition by ara-ATP, ara-CTP, and ara-TTP of the WHV and HBV DNA polymerases is of major importance. However, the effect of nucleoside triphosphates observed in vitro does not allow us to extrapolate on the efficiency of antiviral nucleoside analogs such as ara-A, ara-C, ara-T, or acyclovir (4) on the replication of the virus in the infected cells in vivo. The inhibition of viral replication by these compounds is generally due to a phosphorylation into their triphosphate analogs by virus-induced deoxypyrimidine kinase and consequent inhibition of viral DNA polymerase (1, 4, 17, 19). However, no deoxypyrimidine kinase has so far been reported in HBV or WHV particles, and neither of these two viruses has been reported to grow in culture. The woodchuck model may compensate for this crucial lack of a cell culture system for the hepatitis B virus. Our results confirm the striking relationship between HBV and WHV and therefore further reinforce the validity of woodchuck as a relevant animal model for in vivo studies of hepatitis DNA virus replication. The histopathological changes associated with chronic WHV infection in woodchuck are reminiscent of those associated with HBV infection in humans, including a very high incidence of hepatocellular carcinoma (21, 26), and occur in a shorter time frame. If one takes into account factors such as age, sex, hibernation, and consequent metabolic changes, the results obtained in woodchucks after administration of antiviral agents may provide basic information for antiviral therapy of HBV infection in humans. The woodchuck model also appears to be the fastest way to investigate whether the inhibition of viral replication can reduce the risk of development of hepatocellular carcinoma.

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