Properties of Hepatitis B Virus Associated DNA Polymerase

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The nature of hepatitis B virus (HBV) particle associated DNA polymerase was studied in relation to various enzyme inhibitors including antiviral agents. HBV DNA polymerase required high concentration of MgCl₂ (≥30 mM) and neutral pH for its full activity. p-chloromercuribenzoate was a strong inhibitor (85% inhibition at 1 mM) but N-ethylmaleimide had much less inhibitory effect (20% inhibition at 10 mM). Phosphonoformic acid showed the greatest inhibitory effect on HBV-DNA polymerase (almost complete inhibition at 10 μM) among phosphonocompounds tested. Adenine arabinoside triphosphate (ara-ATP) and cytosine arabinoside triphosphate (ara-CTP) were competitive inhibitors with respect to their respective deoxyribonucleoside triphosphate (dATP and dCTP). Ara-CTP was a stronger inhibitor of HBV-DNA polymerase compared to ara-ATP. Ki values for ara-ATP and ara-CTP were 15.0 μM and 11.7 μM, respectively. HBV-DNA polymerase is characteristic in its ionic requirements and susceptibilities to certain inhibitors.

Key Words: HBV-DNA polymerase, PFA, ara-ATP, ara-CTP

Hepatitis B virus (HBV) DNA polymerase is a DNA dependent DNA polymerase (Kaplan et al., 1976; Robinson, 1977) and has a repair function that closes a single stranded gap of the HBV-DNA which comprises between 15% to 45% of its total length (Kaplan et al., 1973; Summers et al., 1975). It has been demonstrated that DNA polymerase activity found in the sera of patients infected with HBV was associated with the Dane particle, a 42 nm virion (Robinson and Greemman, 1974; Hirschman et al., 1975; Robinson, 1975). Alter and colleagues (1976) have observed that when a patient is HBe antigen and DNA polymerase positive, there is a phase of high level viral replication, followed by a phase of chronic HBs antigenemia. HBV-DNA polymerase and HBe Ag in the sera of patients infected with HBV have been evaluated by Albert et al. (1979). They have indicated that the persistence of both markers could be prognostic of chronic HBV infection. Although the period of HBe antigenemia is indicative for the infectivity of HBV, the clearance of HBe Ag occurs rather slowly and infrequently (Realdi et al., 1980; Viola et al., 1981). Craxi and colleagues (1983) have demonstrated that rapid changes in viral replication by an antiviral therapy were reflected by changes in HBV-DNA polymerase but not by changes in HBe antigen concentration. Therefore, HBV-DNA polymerase has been used as a serological marker for assessing viremia related to infectivity and for viral replication as well as for the selection of patients requiring antiviral therapy. In an attempt for the treatment of chronic HBs Ag positive liver disease, adenine arabinoside, a potential DNA polymerase inhibitor, has appeared to be effective in lowering of HBV-DNA polymerase activity and viral replication (Pollard et al., 1978; Bassendine et al., 1981; Weller et al., 1982). Phosphonoformic acid (PFA) has been reported to inhibit HBV-DNA polymerase more selectively (Nordenfelt et al., 1980), but the possible drug toxicity prohibits the clinical application of this inhibitor for the treatment of HBV-associated liver disease. Because the role of HBV-DNA polymerase in viral replication is very important, characterization of the enzyme is necessary for the development of antiviral therapy. But, the method for the preparation of the soluble Dane particle DNA polymerase is not yet available and the origin of HBV-DNA polymerase is still questionable. HBV-DNA polymerase is usually measured in suspension of Dane particles disrupted by a non-ionic detergent, Nonidet P-40 (NP-40), and complete solubilization of the particles results in loss of enzyme activity (Mao et al., 1980). Lability of the HBV-DNA polymerase and difficulty of preparation of a quantity of the enzyme delayed the characterization of its nature at the molecular level. Partial characterization of HBV-DNA polymerase had been
attempted using a chemical reagent by Hirschman and Garfinkel (1977) and by Hess et al. (1981). They found some characteristics of HBV-DNA polymerase which are different from those of the host DNA polymerase.

The present experiment was designed to study the nature of HBV-DNA polymerase in intact virion and the susceptibilities of the enzyme to various enzyme inhibitors in order to evaluate the antiviral action of these drugs.

MATERIALS AND METHODS

Materials

(Methyl, 1', 2'-H)-thymidine 5'-triphosphate (specific activity 101 Ci/m mole) was purchased from Amersham International plc. (U.K.). Unlabeled nucleoside triphosphates (dATP, dCTP, dGTP), Nonidet P-40 (NP-40) and 2-mercaptoethanol were obtained from Sigma Chemical Company (U.S.A.). Ara-ATP (adenine-9′-β-D-arabinofuranoside 5′-triphosphate), ara-CTP (cytosine-β-D-arabinofuranoside 5′-triphosphate), thiamin pyrophosphate (cofactor), phosphonoformic acid (PFA) and phosphonoacetonic acid (PAA), N-ethylmaleimide (NEM) and p-chloromercuribenzoate (sodium form: PCMB) were also obtained from Sigma Chemical Company (U.S.A.).

Preparation of Dane particles

Dane particles were prepared by a modified method of Kaplan et al. (1973). One milliliter of HBe antigen positive serum was diluted with 4 ml of sterilized saline and centrifuged at 2,000 rpm for 15 minutes and the supernate was recentrifuged at 15,000 rpm in a Sorvall RCB centrifuge (SE 12 rotor) for 1 hour at 4°C. The resultant pellets were used for DNA polymerase assay. The presence of Dane particles in the pellets was examined by electron microscope (Fig. 1). The pellets were resuspended in 100 μl of saline, placed on colloidin-coated copper grids, stained with phosphotungstic acid, and examined in the electron microscope at a magnification of 50,000.

Enzyme assay

DNA polymerase activity was determined by the method of Kaplan et al. (1973) with a few modifications. Thirty microliters of 3% NP-40 and 0.2% 2-mercaptoethanol were added to the precipitated Dane particles, mixed well, and kept at room temperature for 5 minutes. Then, 100 μl of nucleotide mixture, containing 13 nmoles each of dATP, dCTP, dGTP and 10 pmoles 4H-dTTP (1 μCi), was added and mixed. Nucleotide mixture was dissolved in 0.16M Tris-0.12M KCl-0.04M MgCl2 buffer, pH 7.4. The volume of the final reaction mixture was 130 μl. The reaction mixture was incubated at 37°C in water bath for 2 hours. The reaction was stopped by adding 1 ml of cold 5% TCA-0.1M sodium pyrophosphate and stood in an ice bath for 1 hour or kept at 4°C overnight. The mixture was centrifuged at 12,000 rpm (Sorvall SE-12 rotor) for 10 minutes and the pellet was washed 4 times with 5% TCA-0.1M sodium pyrophosphate solution. To the final precipitate, 0.7 ml of 1M perchloric acid was added, heated in boiling water bath for 25 minutes, and then centrifuged at 4,000 rpm (Sorvall SE-12 rotor) for 20 minutes. Radioactivity in supernatant containing DNA hydrolysate (0.4 ml) was counted by liquid scintillation counter (Packard TriCarb 300) using 5 ml of absolute ethanol and 10 ml of cocktail solution (2,5-diphenyloxazole (PPO) 5g, 1,4-bis-2-(5-phenyloxazolyl)benzene (POPPOP) 0.1g in 1l of toluene). Enzyme activity was calculated as follows:

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F = \frac{4 \times \text{(1000 mifl)}}{(2.2 \times 10^8 \text{ dpm/mCi}) \text{(1ml) (120min) (specific activity)}}
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In this experiment, 4H-dTTP was used, so that F=0.149×10^-3 (μmol/min/μl of serum)/dpm. From multiplication of dpm obtained from 1 ml of serum by 0.149×10^-3, enzyme activity (μmoles/min/μl of serum) was calculated.

RESULTS

Preparation of Dane particles and distribution of DNA polymerase activity in HBeAg and HBeAg positive serum.

Electron micrograph of Dane particles prepared from HBe Ag and DNA polymerase positive serum is shown in Fig. 1. The distribution of HBV-DNA polymerase activity among sera with different serological markers is shown in Fig. 2. Variable amounts of radioactivity (4H-thymine) incorporated into particle associated DNA were observed in HBe Ag positive sera. Most of HBS Ag negative sera gave less than 100 dpm/ml of serum/2 hours, which is considered as a cutting value for the background count. Three out of 14 HBe Ag positive sera were DNA polymerase negative and three out of 6 HBe Ag negative sera were DNA polymerase positive.

Effects of divalent cations

There was a sharp increase in the HBV-DNA
Fig. 1. Electron micrograph of hepatitis B virus particles.
Dane particles (42 nm) were examined in the electron microscope at a magnification of 50,000 and photographic enlargement to 150,000 was made.
Arrow indicates Dane particles.

Fig. 2. Distribution of HBV-DNA polymerase activity in sera with different serological markers.
HBs Ag negative sera were taken from normal healthy persons and all HBs Ag positive sera were taken from chronic hepatitis B patients.

HBV-DNA polymerase activity with the addition of MgCl₂ in the assay mixture (Fig. 3). The incorporation of radioactive nucleotides into HBV-DNA was still elevated by the increment of MgCl₂ to 30 mM with no further elevation with 100 mM MgCl₂, MnCl₂ or CaCl₂ did not stimulate the enzyme activity in the absence of MgCl₂ indicating that Mn++ or Ca++ may not substitute the Mg++ for the HBV-DNA polymerase catalyzed polymerization reaction.

Effect of pH
HBV-DNA polymerase activity was relatively sensitive to the pH change. Optimum pH for the HBVDNA polymerization lies between 6-8 (Fig. 4). Maximum incorporation of radioactive nucleotide was observed at pH 7.0 and the activity decreased as the pH increased further. At pH 9.0, only one-third of the enzyme activity present at pH 7.0 was detected.

Effects of sulfhydryl inhibitors
Sulfhydryl inhibitor, PCMB was a strong inhibitor of HBV-DNA polymerase when tested in the absence of 2-mercaptoethanol (2-ME) in the reaction mixture. However, it had little inhibitory effect on the enzyme in the presence of 2-ME (Fig. 5). N-ethylmaleimide (NEM), another sulfhydryl inhibitor, had a weak inhibitory effect on HBV-DNA polymerase regardless of
2-ME in the assay medium (Fig. 5). Ten mM NEM inhibited about 20% of the original activity in the presence or absence of 2-ME. However, 1 mM PCMB inhibited about 85% of the original activity in the absence of 2-ME with a slight inhibition (~5%) in the presence of 2-ME.

**Effects of phosphocompounds**

Phosphonoformic acid (PFA) was a very powerful inhibitor of the HBV-DNA polymerase, and phosphonoacetic acid (PAA) and sodium pyrophosphate were relatively weak inhibitors of the
enzyme (Fig. 6). Thiamin pyrophosphate, the thiamin coenzyme having pyrophosphate group, did not show any inhibitory effect on the HBV-DNA polymerase (Fig. 6). 100 μM of PFA brought about almost complete inhibition of HBV-DNA polymerase activity but the same concentration of PAA caused only about 30% inhibition. The low concentration of inorganic sodium pyrophosphate (< 100 μM) had a slight inhibitory effect on the HBV-DNA polymerase but the relatively higher concentration (> 250 μM) of it showed a remarkable inhibitory effect (Fig. 6).

Effects of ara-ATP and ara-CTP

Both nucleoside triphosphate analogs (ara-ATP and ara-CTP) were competitive inhibitors with their respective deoxyribonucleoside triphosphates (dATP and dCTP). Double reciprocal plots for the HBV-DNA polymerase activity vs substrate are shown in Fig. 7 & 8. Ara-CTP was a more powerful inhibitor than ara-ATP. When 250 μM of ara-CTP was added to the reaction mixture containing 100 μM of dCTP, the enzyme activity was 0.31 x 10^{-6} μmoles/min/l of serum which
is about 48% of the maximum velocity (Vmax=0.65×10^4 μmoles/min/l of serum). When 250 μM of ara-ATP was added to the reaction mixture containing 100 μM of dATP, the enzyme activity was 0.39×10^4 μmoles/min/l of serum which is about 57% of the maximum velocity (Vmax=0.69×10^4 μmoles/min/l of serum). The Ki values for ara-ATP and ara-CTP were 15 μM and 11.7 μM, respectively.

**DISCUSSION**

The initial step for the HBV-DNA polymerase assay in conventional methods is the separation of Dane particles from the sera of patients by a high speed ultracentrifugation which is inconvenient for routine analysis. In the present study, Dane particles were separated from the diluted serum (×5) by a low speed centrifugation (20,000×g, 1 hour). Electron micrograph of the sediment showed Dane particles predominantly with little contaminations (Fig. 1). The recovery of the HBV-DNA polymerase activity in Dane particles prepared from the diluted serum was approximately 85% of that prepared from the undiluted serum by the conventional method. The separation of Dane particles by a simple low speed centrifuga-
tion has made it possible to simplify the routine analysis of HBV-DNA polymerase.

Detection of DNA polymerase activities in most HBe Ag positive sera but not in all (Fig. 2) suggests that some of HBe Ag positive patients are in the transient phase of seroconversion, and the occurrence of DNA polymerase activity in some of HBs Ag positive with HBe Ag negative sera indicates that HBe Ag alone may not be a good viral marker. Correlation between the Dane particle, DNA polymerase and HBe Ag has been observed by many investigators (Alter et al., 1976; Hindman et al., 1976; Nordenfelt and Andren-Sandberg, 1976), and the Dane particle associated DNA polymerase activity has been shown to be an useful marker for viremia with other serological markers (Krugman et al., 1974). Cram et al. (1973) have reported that sera of 23% of HBe Ag positive patients with chronic hepatitis were HBV-DNA polymerase negative and that changes in viral replication as a result of antiviral therapy were reflected by changes in HBV-DNA polymerase but not by changes in HBe Ag, indicating DNA polymerase activity rather than HBe Ag being a better marker for viral replication.

HBV-DNA polymerase activity increased with increasing concentration of MgCl₂ and a plateau of enzyme activity was not achieved even at 30 mM of MgCl₂ (Fig. 3), which is comparable with the result of Hirschman and Garfinkel (1977) who have reported the optimal concentration of Mg²⁺ to be 80 mM. The requirement of MgCl₂ for the DNA polymerization is universal, but the optimal concentration of Mg²⁺ for HBV-DNA polymerase is quite different from that of the bacterial and mammalian DNA polymerases. The optimal concentration of Mg²⁺ for mammalian, bacterial and some of viral DNA polymerases are in the range of 5 to 10 mM (Weissbach et al., 1973; Hirschman and Garfinkel, 1977; Mao et al., 1980). Salts and divalent cations concentrations far above the optimal levels were found to inhibit mammalian or Herpes virus-induced DNA polymerases (Weissbach et al., 1973; Boezi et al., 1974; Huang, 1975). The insensitivity of HBV-DNA polymerase to a high concentration (100 mM) of divalent cations in the present study is an unusual behavior of DNA polymerase and is assumed to be due to the fact that the enzyme is still particle associated and not yet solubilized as the other polymerases are.

There were different susceptibilities of HBV-DNA polymerase to NEM and PCMB. Ten mM NEM inhibited only 20% of the original HBV-DNA polymerase activity, while 1 mM PCMB exerted a 85% inhibition (Fig. 4). Different susceptibilities of the HBV-DNA polymerase to PCMB and NEM were well agreed upon with the results observed by Hirschman and Garfinkel (1977) or by Hess et al. (1981). It is conceivable that HBV-DNA polymerase requires free sulfhydryl group for the full activity. HBV-DNA polymerase activity was inhibited even at low concentration (< 100 μM) of PFA (Fig. 6), but phosphonoacetic acid (PAA) which has structural similarity to PFA and has the same inhibitory effect on Herpes virus DNA polymerase as PFA (Mao and Robishaw, 1975; Helquist et al., 1978), had a slight inhibitory effect on HBV-DNA polymerase (Fig. 6).

The inhibitory effect of inorganic pyrophosphate on HBV-DNA polymerase in the present study confirmed the earlier observation by Nordenfelt et al. (1980). The selective inhibition by PFA indicates the structural requirement for phosphate group or carboxyl group attached closely each other. The mechanism of inhibition by PAA has been demonstrated to be noncompetitive with regard to substrates in various DNA polymerases (Sabourin et al., 1978; Sundquist and Oberg, 1979, Nordenfelt et al., 1980). The inhibition of HBV-DNA polymerase by PFA seems to be specific and could be used for the discrimination of Dane particle associated DNA polymerase from other DNA polymerases. PFA has been reported to exhibit the lowest cellular toxicity among some antiviral agents such as idoxuridine, vidarabine or ribavirin (Helquist et al., 1978). However, the possible cytotoxic effect of PFA prevent its clinical application at the present time. Therefore, more study on its toxicity is required for the practical application. The inhibition of HBV-DNA polymerase by ara-ATP was competitive with respect to dATP. The same mode of inhibition was observed in DNA polymerases isolated from mammals and viruses (Furth and Cohen, 1967; Muller et al., 1975). It has been reported that viral DNA polymerases (RNA-directed or DNA-directed) were less affected by ara-ATP compared to eukaryotic DNA polymerases and that ara-ATP had no effects on protein synthesizing system in mammalian cells (Muller et al., 1975). However, the idea that ara-ATP is a virus-specific agent came from the fact that it causes a rapid inhibition of Herpes simplex virus (HSV) DNA synthesis and inhibits cellular DNA synthesis only after a lag-phase (Shipman and Drach, 1974). In fact, relatively strong inhibitory effect of ara-ATP on HSV-DNA polymerase over mammalian DNA polymerases has been confirmed by Muller et al. (1977). So, ara-ATP and its monophosphate analog (ara-AMP) are widely used in clinical trials for the therapy of HBV infected chronic liver disease (Bassendine et al., 1981; Cram et al., 1983).
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Properties of Hepatitis B Virus Associated DNA Polymerase

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