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Zidovudine inhibits hepatitis B virus replication

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Summary

Hepatitis B virus DNA polymerase is a viral enzyme that can use viral DNA as well as viral RNA as a template for DNA synthesis. Since both activities are essential for the production of new virus particles, blocking of this enzyme should reduce viral replication. In the present study the in vitro effect of zidovudine triphosphate on hepatitis B virus DNA polymerase activity and the in vivo effect of zidovudine on viral replication in chronic HBsAg-positive patients are investigated. Zidovudine triphosphate inhibited in vitro DNA polymerase activity by 50% at a concentration of 0.3 μM. Serum DNA polymerase activity was significantly reduced in 7 patients who received zidovudine (200 mg orally 4 times daily) for one week. A dose–response effect was suggested by the results found for 6 patients who received 100 mg, 200 mg and 300 mg orally 4 times daily for one week with 2 drug-free weeks between each course. We conclude that zidovudine may be of value for non-responders to α-interferon therapy or patients with high initial levels of viral replication prior to the start of interferon treatment.

Hepatitis B virus; Zidovudine; DNA polymerase

Introduction

The hepatitis B virus (HBV) has an endogenous DNA polymerase (DNAp) activity that can be detected in the serum of patients with active virus replication (Robinson and Greenman, 1974). This DNAp induces synthesis of

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viral DNA by DNA-dependent DNAp activity and RNA-dependent DNAp activity (Fowler et al., 1984). Both activities are essential for HBV replication. Inhibition of these steps of the replication cycle therefore should reduce the formation of new virus particles.

α-Interferon (α-IFN) can be considered the cornerstone of antiviral therapy for chronic hepatitis B. Treatment for 12–16 weeks with 5 MU daily or 10 MU every other day results in the induction of virus latency (HBe-seroconversion) in approximately 25% of patients (Schalm et al., 1989). Response to α-IFN treatment is related to the initial level of viral replication, with poor results for patients with high levels of replicative activity (De Man et al., 1988). To increase the response rate of α-IFN, it therefore appears rational to add to the treatment regimen an antiviral agent with a different target in the multiplication cycle than the ones influenced by α-IFN. Administration of nucleoside analogues in combination with α-IFN to patients who do not respond to monotherapy or have high initial replication levels prior to the start of treatment may thus increase the response to treatment. Several nucleoside analogues have already been included in the treatment of chronic hepatitis B: acyclovir, with poor success (Alexander et al., 1987) and adenine arabinoside (-monophosphate), with moderate success but severe toxicity (Lok et al., 1984).

Zidovudine (3′-azido-2′3′-dideoxythymidine, AZT) is widely used to inhibit in vivo human immunodeficiency virus (HIV) replication, since it has been demonstrated that survival of AIDS patients is significantly improved (Fischl et al., 1987). Zidovudine triphosphate (AZT TP) has also been reported to inhibit HBV DNAp activity in vitro (Nordenfelt et al., 1987). In order to confirm these findings, we investigated the in vitro effect of AZT TP on HBV DNAp activity and in vivo effects of zidovudine on hepatitis B virus replication.

Materials and Methods

Virus isolation

Hepatitis B viral particles were concentrated from the serum obtained from a patient with chronic HBeAg-positive hepatitis without clinical or serological evidence of other viral infections (conventional tests for antiHDV, antiHIV, IgM antiHAV, IgM anti-EBV and IgM antiCMV were negative). Hepatitis B virus (HBV) particles were concentrated by ultracentrifugation in a SW41 rotor for 3 h at 30000 rpm and 4°C. The pellet was resuspended in phosphate-buffered saline (PBS), layered on a discontinuous gradient of 10 and 20% sucrose in 100 mM sodium, 10 mM Tris-HCl pH 7.5 and 1 mM EDTA (NTE) buffer and centrifuged overnight in a SW27 rotor at 25000 rpm and 4°C. The pellet was resuspended in PBS and immediately assayed for DNAp activity. Negative control samples for the DNAp assay were obtained by pelleting serum of a healthy human donor in the SW41 rotor as described above. These pellets were resuspended in PBS and used in the DNAp assay without further clarification.

HBV marker assays

DNAp activity measurements were performed in duplo (Howard, 1978) with a modification of the elution of unincorporated tritiated thymidine-5′-triphosphate ([^{3}H]dTTP) (Fang et al., 1981). Forty μl of virus suspension were added to 105 μl of the following reaction mixture: 160 mM Tris-HCl, pH 7.5, 40 mM $MgCl_2$, 120 mM NH_4Cl, 50 μM dATP, 50 μM dGTP, 50 μM dCTP, 0.3 μM [^{3}H]dTTP (50 Ci/mmol, Amersham, UK), 1.0% Nonidet P40, 0.3% 2-mercaptoethanol, 33% distilled water, 9% PBS. Incubation for 3 h at 37°C was followed by spotting 100 μl on a Whatmann 3M chromatography paper which was air-dried for 30 min. DNA was fixed on the filter by overnight incubation in trichloroacetic acid (TCA) (5% w/v) which also eluted the excess [^{3}H]dTTP, and then dried at 80°C for 1 h. Incorporation of [^{3}H]dTTP was assayed by placing the filters in 10 ml scintillation counting fluid (Insta-gel, Packard, IL) in a PW4540 liquid scintillation analyzer and expressed in counts per minute (cpm).

To assay HBV DNAp activity in patient serum, 800 μl samples were diluted to 11 ml in PBS and centrifuged for 4 h at 30 000 rpm and 4°C in a SW41 rotor. The virus pellet was resuspended in 40 μl PBS and added to 105 μl assay mix as described above. Five negative control samples and 2 positive control samples were included in each test run. Samples were considered positive when the cpm exceeded the mean cpm of the negative control samples plus 3 standard deviations (approximately 150 cpm). HBV DNA was measured by liquid phase hybridization (Genostics, Abbott, IL, USA); results exceeding 5 pg/ml were considered positive. Serum HBsAg and HBeAg were tested by RIA (Abbott, IL, USA).

HBV DNAp inhibitors

The sodium salt of 3′-azido 2′3′-dideoxythymidine triphosphate (AZT TP) was donated by Prof. E. de Clercq, Rega Institute, Leuven. A stock solution of AZT TP 200 μM in bidistilled water was kept at −20°C. The concentration of AZT TP in bidistilled water was assessed by high pressure liquid chromatography (Dr. J. Balzarini, Rega Institute, Leuven). The sodium salt of phosphonophormate (foscarnet, PFA) was provided by Dr. M. Kuipers, Astra, the Hague. A stock solution of 10 mM was made in bidistilled water and stored at 4°C.

Patients

Data prior to entry in this study are listed in Table 1. All patients ($n = 7$) had HBsAg-positive hepatitis for more than 1 year, with stable levels of HBeAg (after titration of serum samples, more than 4-fold differences of HBeAg concentration in time were not observed), DNAp activity and HBV DNA in serum as well as HBcAg in the liver. Baseline values for DNAp varied from 508 to 32417 cpm. None of the patients had antiHDV antibodies in serum or HDAg in the liver. Two patients had cirrhosis and 3 were antiHIV-positive with normal CD4-positive cell counts. Patients gave informed consent prior to participation in this study.

TABLE 1

Patient characteristics prior to entry
*DNAp = HBV DNAp activity in counts per minute (cpm), negative controls were 40–60 cpm and the test cut-off was the mean of 5 negative control samples plus 3 times the standard deviation (approximately 150 cpm). nt = not tested.

	HBsAg	HBeAg	DNAp cpm*	antiHD	antiHIV	HIVAg	CD4 pos cells/mm^3
Group I							
Patient 1	+	+	508	–	+	–	> 400
2	+	+	1573	–	–	nt	nt
3	+	+	2140	–	+	–	> 400
4	+	+	3036	–	–	nt	nt
5	+	+	3741	–	–	nt	nt
6	+	+	8753	–	–	nt	nt
7	+	+	32417	–	+	–	> 400
Group II							
Patient 1	+	+	701	–	+	–	> 400
2	+	+	1482	–	–	nt	nt
3	+	+	1504	–	–	nt	nt
4	+	+	1753	–	–	nt	nt
5	+	+	6563	–	–	nt	nt
6	+	+	21522	–	+	–	> 400

Treatment

Zidovudine was given in 100-mg capsules, orally. Seven patients (group I) received 4 × 200 mg zidovudine daily for 1 week. Six patients (group II) then received increasing doses of the drug: 4 × 100 mg, 4 × 200 mg and 4 × 300 mg zidovudine daily for 1 week with 2 drug-free weeks between each course.

Statistics

Pre- and posttreatment results were compared by the Wilcoxon sign test.

Results

In vitro results

Inhibition of [^{3}H]dTTP incorporation by foscarnet PFA) was used to identify HBV DNA-specific DNAp activity and inhibition by actinomycin D (act-D) to identify the proportion of activity due to DNA-dependent DNAp activity and RNA-dependent DNAp activity (Tao et al., 1988). A final concentration of 10 μM PFA in the assay mixture resulted in an inhibition of HBV DNAp-specific activity by 91%. Addition of act-D at a final concentration of 100 μM resulted in an inhibition of (DNA dependent) DNAp activity by 83%. A representative experiment expressing the inhibition by AZT TP is presented in Fig. 1. Fifty percent inhibition can be estimated for an AZT TP concentration of 0.3 μM.

TABLE 2

Characteristics of HBV suspension for in vitro assay
PFA = foscarnet; act-D = actinomycin D.

	DNAp activity (cpm)
40-μl sample	1070
Negative control sample	46
40-μl sample + PFA 10 μM	102
40-μl sample + act-D 100 μg/ml	173
Background radiation	22

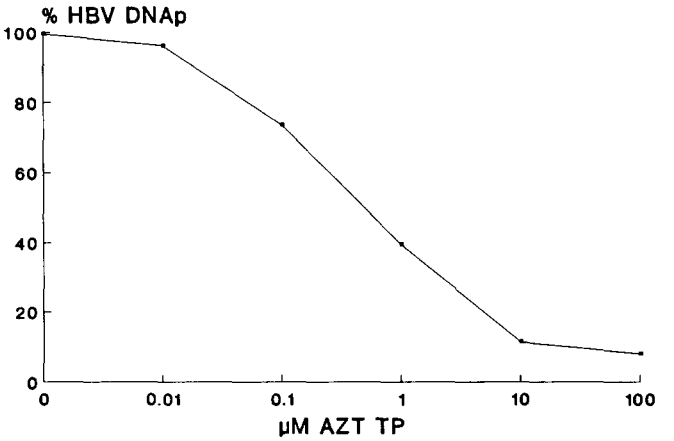

Fig. 1. Percentage DNAp activity of a human HBV serum preparation in the presence of different concentrations of AZT TP.

In vivo results

DNAp activity decreased in all patients of group I after 4 days of treatment and were still low on day 7 (Fig. 2). Median DNAp activity diminished from 3036 cpm on day 0 to 819 cpm on day 4 (median percentual decrease compared to baselines values: 50%, 95% CI 14–73%) and 999 cpm on day 7 (29%, 95% CI 12–72%, $P=0.008$). In group II a dose-response effect was observed; median DNAp activity on days 4 and 7 was 85% (95% CI 54–188%) and 76% (95% CI 57–115%), respectively, of initial activity during 4 × 100 mg, 57% (95% CI 19–107%) and 65% (95% CI 31–108%) during 4 × 200 mg and 40% (95% CI 25–67%) and 54% (95% CI 6.6–136%) during 4 × 300 mg zidovudine daily (Fig. 3). No statistical significance was observed because 1 out of 6 patients did not respond to therapy. There was no difference in initial DNAp activity, no cirrhosis and no antiHIV positivity in the non-responding patient. HBV DNA also tended to decline but no statistical significance could be found because 1 patient did not respond. During 4 × 300 mg zidovudine, the median HBV DNA level decreased from 227 pg/ml on day 0 to 196 pg/ml on day 4 (median percentage compared to baseline values: 105%, 95% CI 62–109%) and 161 pg/ml on day 7 (77%, 95% CI 41–124%). HBeAg levels remained stable in all patients and no HBs-seroconversion was observed.

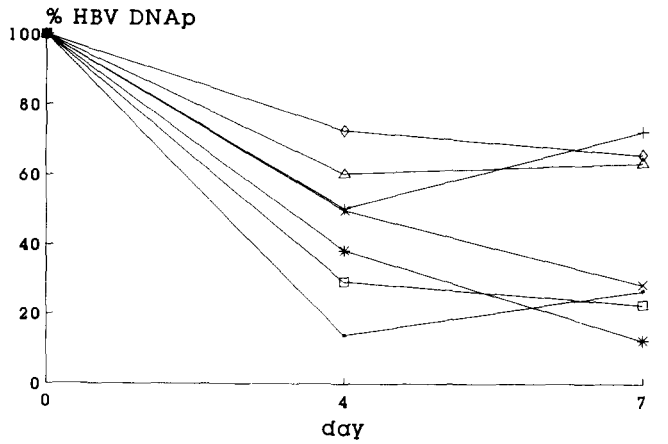

Fig. 2. Percentage residual HBV DNAp activity in serum during treatment with zidovudine 4 times 200 mg orally daily.

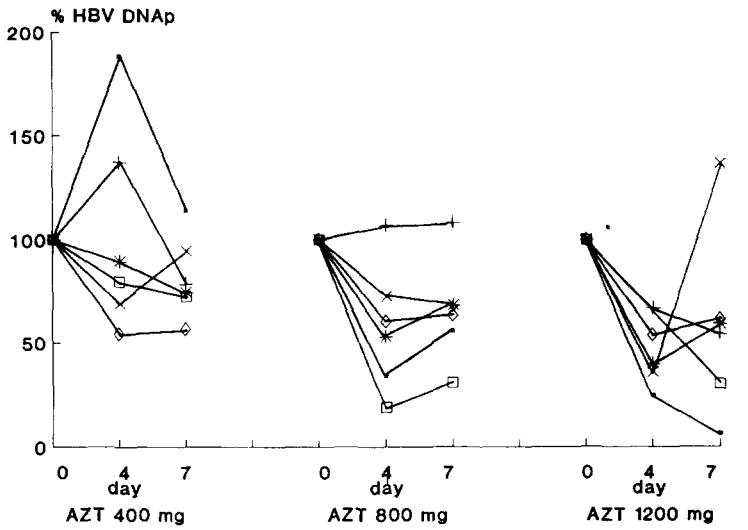

Fig. 3. HBV DNAp activity expressed as percentage of residual activity during treatment with increasing doses of zidovudine, suggesting a dose-response effect. One symbol is used to present the DNAp activity of a patient during the different doses of zidovudine.

Discussion

The results of our in vitro study confirm those reported by Nordenfelt et al. (1987). Since the concentration of AZT TP that yields 50% inhibition is comparable to the concentration of the label used in this assay, the drug has a similar affinity for viral DNAp as the natural substrate thymidine TP. In vitro

AZT TP appears to be a moderately effective inhibitor of DNA-dependent HBV DNAp activity.

The in vivo results show that a statistically significant suppression of HBV DNAp activity is achieved in chronically infected patients by the administration of zidovudine 4 times daily. The repeated 1-week courses with increasing doses ranging from 400 to 1200 mg daily, provides an estimation of the dose–response relationship.

These findings are not in accordance with data of other investigators who found no effect of zidovudine on HBV replication in patients with concurrent HBV and HIV infections (Gilson et al., 1991; Farraye et al., 1989; Marcellin et al., 1989). Such patients usually have high levels of HBV replicative activity. A lack of effect of zidovudine on HBV replication in symptomatic HIV-infected patients may be explained by the use of the insensitive semiquantitative dot-blot methods for HBV DNA measurements (Lin et al., 1987) and, in addition, by the poor clinical condition of the patients (Gilson et al., 1991).

In vitro experiments with HBV-producing cell lines and AZT showed inhibition by non-cytotoxic doses of the drug, indicating that sufficient phosphorylation of AZT occurs in these models (Lampertico, 1991; Ueda, 1989; Galle and Theilmann, 1990). We found the most pronounced inhibition of HBV DNAp on day 4 during each course with no further decline and sometimes even an increase in some patients. An escape mechanism of the virus might explain the lack of further effect on replicative activity. Our results for immunologically competent patients should be confirmed by a randomized placebo-controlled study to evaluate the effect of zidovudine as a single agent on HBV replication. If this study confirms the antiviral effect of zidovudine in chronic hepatitis B, a randomized placebo-controlled study to investigate the effect of zidovudine in combination with α-IFN in patients who do not respond to monotherapy with α-IFN is indicated.

The present results suggest that zidovudine may be effective in lowering HBV replicative activity in immunocompetent chronic HBV-infected patients. Since the drug can be given orally, further study is indicated to assess its clinical efficacy and risks.

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