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Inhibition of the replication of hepatitis B virus by the carbocyclic analogue of 2′-deoxyguanosine

{viral DNA polymerase/episomal DNA/hepatocellular carcinoma/9-[3-hydroxy-4-(hydroxymethyl)cyclopentyl]guanine}

PETER M. PRICE*, RANJIT BANERJEE†, AND GEORGE ACS*†

Departments of *Biochemistry and †Neoplastic Diseases, Mount Sinai School of Medicine, New York, NY 10029

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ABSTRACT **We report that treatment of 2.2.15, a human hepatoblastoma-derived cell line in which hepatitis B virus is actively replicating, with the carbocyclic analogue of 2′-deoxyguanosine [Shealy, Y. F., O'Dell, C. A., Shannon, W. M. & Arnett, G. (1984) *J. Med. Chem.* 27, 1416–1421] resulted in the nearly complete cessation of viral replication, as monitored by the absence of both intracellular episomal and secreted viral DNAs and by the absence of viral DNA polymerase activity. The drug was nontoxic in concentrations up to 200 times the minimum effective inhibitory concentration.**

Hepatitis B virus (HBV) affects nearly 300 million people worldwide (1). Infection with HBV has been associated with a >200-fold increased risk to develop hepatocellular carcinoma (2). The viral DNA genome, ≈3200 nucleotides long, has been cloned in *Escherichia coli* (3–9). HBV and other related hepadnaviruses can be propagated in tissue culture (10–15), making it possible to study various aspects of the viral life cycle. We have established a cell line, 2.2.15, derived from a human hepatoblastoma (Hep G2) in which the HBV genome is stably incorporated into the host genome, and in which HBV is replicated, resulting in the production of infectious virus (16).

The life cycle of HBV (reviewed in ref. 17) involves transcription of a greater-than-genome-length 3.5-kilobase mRNA, which serves as a template for the reverse transcription of a single-stranded DNA. This single-stranded DNA serves as a template for the polymerization of the second strand of the viral genome, the elongation of which is terminated after it is approximately half-finished, resulting in the generation of a partially single- and partially double-stranded circular DNA genome. The viral genome is encapsidated and secreted by the infected cells. The unfinished DNA can be elongated *in vitro* by using the endogenous polymerase activity associated with viral particles (18); this process is a convenient marker for the presence of HBV. We report here that by using this assay, and by monitoring intracellular and secreted HBV DNAs, we can effectively demonstrate the inhibition of HBV replication *in vitro* by (±)-2-amino-1,9-dihydro-9-[(1α,3β,4α)-3-hydroxy-4-(hydroxymethyl)cyclopentyl]-6*H*-purine-6-one (2′-CDG), the carbocyclic analogue of 2′-deoxyguanosine.

MATERIALS AND METHODS

Assay of HBV Polymerase Activity. The 2.2.15 cells were grown to near confluency on 25-cm^2 Petri dishes containing 5 ml of medium (10). Drugs were added to the medium and the cells were incubated for 24 hr before the medium was changed. After another 3 days, the medium was collected and tested for HBV polymerase (7). The medium was centrifuged (10 min, 2000 × *g*) and the supernatant was adjusted to a final concentration of 10% (wt/vol) PEG 8000. The virus was pelleted (10 min, 8000 × *g*) and washed with 10% PEG. The pellet was suspended at 1/20th the original volume in 10 mM Tris, pH 7.5/0.1 M KCl/0.01% 2-mercaptoethanol. The suspension was adjusted to 0.5% Nonidet P-40, 0.4 M KCl, 50 mM Tris (pH 7.5), 20 mM dithiothreitol, 40 mM $MgCl_2$, 0.3 μM (100 μCi; 1 μCi = 37 kBq) [α-^{32}P]dCTP, and 0.5 mM dATP, dGTP, and dTTP. The reaction mixture was incubated 2.5 hr at 37°C, and the viral particles were precipitated by the addition of PEG 8000 to 12.5%. The pellet was collected by centrifugation, washed with PEG, and suspended in 0.9% NaCl. The suspension was adjusted to 1% SDS and 1 mg of proteinase K per ml and incubated 2 hr at 37°C. The digest was extracted with phenol and the DNA precipitated with ethanol. The DNA pellet was dissolved and then electrophoresed in a 1% agarose gel, and the gel was dried and autoradiographed.

Isolation and Characterization of DNA. Cells were treated for 3 days with 2′-CDG (100 ng/ml), the medium was changed, and the cells were treated another 7 days. Episomal DNA was extracted by the method of Hirt (19). Extracellular DNA was extracted from the medium by PEG precipitation, proteinase K digestion, and phenol extraction as described above. Nuclear DNA from 2.2.15 cells was isolated according to the method of Jeffreys and Flavell (20). After electrophoresis and transfer to membranes, the DNAs were hybridized with ^{32}P-labeled nick-translated HBV DNA (10^8 cpm per μg). The HBV DNA used for nick-translation represented the entire genome (3182 base pairs). After the membranes were washed and dried, autoradiography was performed at −70°C with intensifying screens for 16 hr.

Isolation of RNA. Total cellular RNA was isolated according to Han *et al.* (21) and enriched for polyadenylylated RNAs (22). The RNA (1 μg per lane) was electrophoresed through 1% agarose gel containing 1.1 M formaldehyde (23) and transferred to nylon membrane (24). The immobilized RNAs were hybridized with nick-translated HBV DNA and the membrane was autoradiographed as described above.

RESULTS

Treatment of 2.2.15 cells (10) with as little as 25 ng of 2′-CDG per ml resulted in the almost complete disappearance of replicating HBV as measured by endogenous DNA polymerase activity, extracellular DNA, and episomal intracellular DNA. The maximally effective concentration of 2′-CDG was between 25 ng/ml and 5 μg/ml (data not shown). At 5 ng/ml, the drug inhibited HBV replication, as measured by DNA polymerase activity, by ≈50%, whereas there was no inhibition at 500 pg/ml.

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Abbreviations: HBV, hepatitis B virus; 2′-CDG, carbocyclic analogue of 2′-deoxyguanosine; AZT, 3′-azido-3′-deoxythymidine.

No cytotoxic effect caused by 2′-CDG treatment could be demonstrated either morphologically or by a change in the cell division time or viability at drug concentrations up to 5 μg/ml. Also, at concentrations of 2′-CDG up to 5 μg/ml, no effect could be seen on the cellular protein production or on the integrity of either RNA or DNA isolated from the cells.

At 50 ng/ml, 2′-CDG inhibited the replication HBV DNA, as measured by an endogenous polymerase reaction, whereas neither acyclovir (acycloguanosine), an inhibitor of certain DNA viruses, nor AZT (3′-azido-3′-deoxythymidine), an inhibitor of several viral reverse transcriptases, had any effect (Fig. 1). At 5 μg/ml, acyclovir had no inhibitory effect and AZT only slightly inhibited the HBV polymerase activity.

Since the loss of polymerase is an indirect demonstration of the effect of the drug on HBV replication, viral DNAs, both intra- and extracellular, were assayed (Fig. 2). In both cases, after treatment with 2′-CDG, these DNAs could barely be detected. Episomal DNA isolated from 2′-CDG-treated 2.2.15 cells, by a procedure that included protease digestion prior to phenol extraction, also did not contain HBV DNAs. Omitting the drug during the last 7-day incubation period resulted in an increase of HBV DNAs up to 10% the levels of the untreated cells (data not shown). From comparison with standard amounts of HBV DNAs on the autoradiographs, it was calculated (by assuming 2.6 pg of DNA per 10^6 virions) that the cells contained 108 viral particles and/or episomal HBV genomes per cell. This decreased to 0.2 particles per cell after 2′-CDG treatment and rose to 6.9 particles per cell if the drug was omitted for 7 days. The tissue culture medium contained 55×10^6 particles per ml, which decreased to 3.3×10^6 per ml after drug treatment and which returned to 3.8×10^6 per ml if the drug treatment was interrupted. At the same time, both the amount and the restriction enzyme pattern of the chromosomally integrated HBV genome were unaltered (Fig. 3), indicating that the integrity of the chromosomally integrated DNAs was not affected. The HBV-specific polyadenylylated RNAs (Fig. 4) were only slightly reduced by 2′-CDG treatment, and little effect was seen on the production of the HBV-specific antigens by these cells (HBsAg radioimmunoassay, Connaught; HBeAg enzyme immunoassay, Abbott; data not shown.)

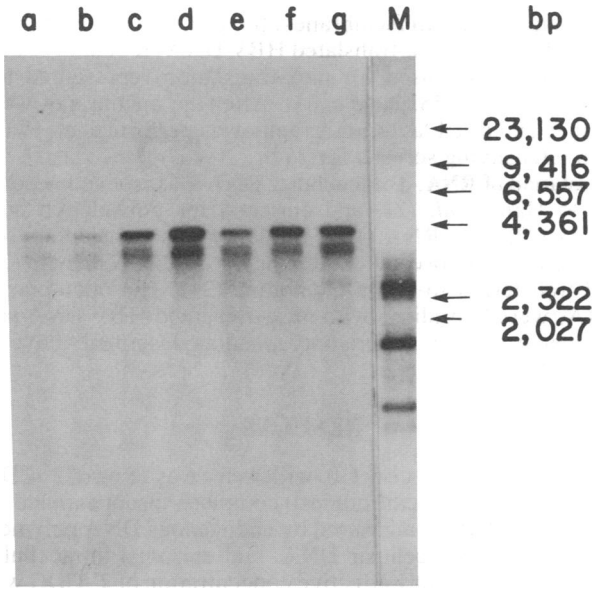

FIG. 1. Endogenous DNA polymerase activity of extracellular HBV. 2′-CDG (lanes a and b), acyclovir (lanes c and d), or AZT (lanes e and f) was present at either 5 μg/ml (lanes a, c, and e) or 50 ng/ml (lanes b, d, and f). Lane g, untreated samples. Lane M, HBV DNA partially digested with *Bam*HI, in which characteristic bands at 2416, 1504, and 912 base pairs (bp) were evident. Size markers at right represent phage λ DNA digested with *Hin*dIII.

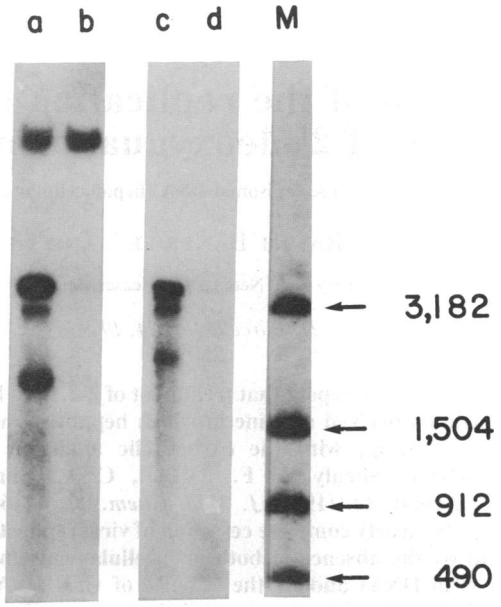

FIG. 2. Episomal intracellular (lanes a and b) and extracellular (lanes c and d) HBV DNAs. The DNAs from 2′-CDG-treated (100 ng/ml; lanes b and d) and untreated (lanes a and c) were electrophoresed in 1% agarose gels, transferred to Zeta-Probe membranes (Bio-Rad) (25), and hybridized with ^{32}P-labeled nick-translated HBV DNA. Each lane represents the DNA isolated from 3.3×10^5 cells (intracellular HBV) or from 1.25 ml of culture medium (extracellular DNA). Size markers (lane M; sizes in base pairs at right) were HBV DNAs digested with *Eco*RI and *Eco*RI plus *Bam*HI.

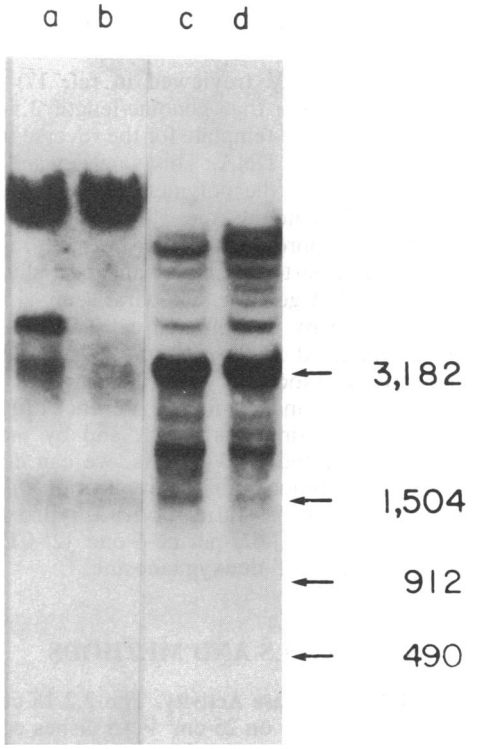

FIG. 3. Chromosomal HBV DNA. Cells were either untreated (lanes a and c) or treated with 2′-CDG (100 ng/ml; lanes b and d) as described in Fig. 2. DNA (10 μg per lane), either digested with *Eco*RI (lanes c and d) or undigested (lanes a and b), was electrophoresed and hybridized as described in Fig. 2. Size markers were HBV DNAs digested with *Eco*RI and *Eco*RI plus *Bam*HI.

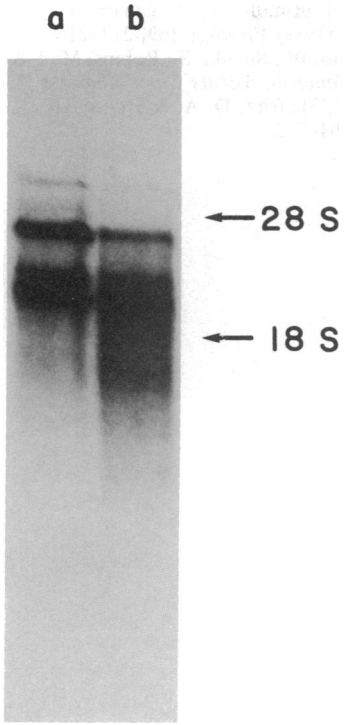

FIG. 4. Polyadenylylated HBV RNA. Cells were either untreated (lane a) or treated with 2'-CDG (100 ng/ml; lane b) as described in Fig. 2. Positions of 18S and 28S ribosomal RNAs are indicated.

DISCUSSION

Effective vaccines are available that can prevent the spread of HBV (reviewed in ref. 26), but effective drugs are not available that can treat patients already having the disease. One promising treatment involves an immune stimulation followed by administration of α-interferon (27). This treatment, however, can be life-threatening to patients with advanced liver disease (28). Other treatments, involving adenine arabinonucleoside monophosphate (29) and acyclovir (30), have proven to be ineffective. Several drugs, including interferon, acyclovir, adenine arabinonucleoside, cytosine arabinonucleoside, and AZT, have been tested using tissue culture models of HBV replication (31) but either have not been effective in reducing HBV DNA replication or have been found to be effective only at concentrations close to toxic levels. The 2',3'-dideoxynucleosides have also been tested in tissue culture systems (31, 32) with varying success, possibly as the result of inactivation by components in the culture medium. In the best case, the concentration of drug at maximal effectiveness was 50 times that of the 2'-CDG in our studies.

In contrast to the effectiveness of the 2'-CDG in inhibiting HBV replication, the drug did not affect the integrated HBV DNAs. In 2.2.15 cells, the RNA replicative intermediates are being transcribed from this integrated DNA; the cells are constantly being replenished with replicating HBV virions. Therefore, it was not surprising that interruption of the drug treatment resulted in a return of HBV virus to both intra- and extracellular populations. Since relatively few natural HBV infections result in this genomic integration, we assume that 2'-CDG would be even more effective in these cases.

Regardless of the method of treatment of HBV infection, the major problem that must be addressed is the speed and efficacy of the therapy. One of the major health hazards resulting from HBV infection is its progression, in 2–4% of patients, to hepatocellular carcinoma. Preceding this development, and predisposing the occurrence of carcinoma (33), is the integration of the HBV genome into the chromosomes of the host cells. Since 2'-CDG eliminates the HBV-specific episomal DNAs, which are precursors to the integrated DNAs, this drug has the potential to inhibit the progression to HBV DNA integration, thereby preventing HBV-induced carcinoma.

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