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Incorporation of the Carbocyclic Analog of 2′-Deoxyguanosine into the DNA of Herpes Simplex Virus and of HEp-2 Cells Infected with Herpes Simplex Virus

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SUMMARY

The carbocyclic analog of 2′-deoxyguanosine (CdG) is active against herpes simplex virus (HSV), human cytomegalovirus, and human hepatitis-B virus. In order to understand the mechanism of action of this compound against HSV, we have evaluated (a) the incorporation of [^{3}H]CdG into viral and host DNA in HEp-2 cells infected with HSV and (b) the interaction of the 5′-triphosphate of CdG (CdG-TP) with the HSV DNA polymerase and human DNA polymerases α, β, and γ (EC 2.7.7.7). Incubation of HSV-1-infected HEp-2 cells with [^{3}H]CdG resulted in the incorporation of CdG into both the HSV and the host cell DNA. These results indicated that CdG-TP was used as a substrate for HSV DNA polymerase and for at least one of the cellular DNA polymerases. Degradation of both viral and host DNA with micrococcal nuclease and spleen phosphodiesterase indicated that CdG was incorporated primarily into internal positions in both DNAs. The viral DNA containing CdG sedimented in neutral and alkaline sucrose gradients in the same way as did viral DNA labeled with [^{3}H]thymidine, indicating that the HSV DNA containing CdG was similar in size to untreated HSV DNA. CdG-TP was a competitive inhibitor of the incorporation of dGTP into DNA by the HSV DNA polymerase (K_i of 0.35 μM) and the human DNA polymerase α (K_i of 1 μM). CdG-TP was not a potent inhibitor of either DNA polymerase β or γ. Using DNA-sequencing technology, CdG-TP was found to be an efficient substrate for HSV DNA polymerase. Incorporation of CdG monophosphate (CdG-MP) into the DNA by HSV DNA polymerase did not interfere with subsequent chain extension. These results suggested that the antiviral activity of CdG was due to its incorporation into the DNA and subsequent disruption of viral functions. In contrast, CdG-TP was not as good as dGTP as a substrate for DNA synthesis by DNA polymerase α, and incorporation of CdG-MP by DNA polymerase α inhibited further DNA chain elongation.

CdG (Fig. 1) is a broad spectrum antiviral agent with *in vitro* activity against HSV-1 and -2 (1), human cytomegalovirus (2), and human hepatitis-B virus (3). In addition, it has also shown *in vivo* antiviral activity against HSV-1 and HSV-2 (4). It is of interest that the L-enantiomer of CdG also had some activity against HSV-1 (5); the MIC_{50} was 36–900-fold greater than that seen with the D-enantiomer. These antiviral activities indicate that CdG is a promising nucleoside analog that may have activity against a number of clinically significant viruses.

As with other anti-HSV guanine nucleoside analogs, the high selectivity for activity against HSV-1 is due, at least in part, to its selective phosphorylation by the HSV-encoded nucleoside kinase (6). Incubation of virus infected HEp-2 cells with 8 μM CdG for 8 hr resulted in a cellular concentration of CdG-TP of 80 μM, whereas only 0.1 μM CdG-TP was detected in cells that were not infected with virus. In addition, CdG was much less active against an HSV-1 strain that is deficient in dThd kinase activity than it was against wild-type virus. These results indicate that phosphorylation of CdG is necessary for its antiviral activity (6). Therefore, in the present work we have studied the action of CdG-TP against the HSV-1 DNA polymerase and human DNA polymerases α, β, and γ, in order to better understand the mechanism of action of the antiviral activity of CdG. A preliminary report of this work has been presented (7).

Experimental Procedures

Materials. K562 cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO, Grand

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ABBREVIATIONS: CdG, the D-enantiomer of the carbocyclic analog of 2′-deoxyguanosine or [1*R*-(1α,3β,4α)]-2-amino-1,9-dihydro-9-[3-hydroxy-4-(hydroxymethyl)cyclopentyl]-6*H*-purin-6-one; ACV, 9-(2-hydroxyethoxymethyl)guanine; DHPG, 9-(1,3-dihydroxy-2-propoxymethyl)guanine; CdG-MP, CdG-TP, ACV-MP, ACV-TP, DHPG-MP, and DHPG-TP, the 5′-monophosphates and 5′-triphosphates of CdG, ACV, and DHPG, respectively; BVdU, (*E*)-5-(2-bromovinyl)-2′-deoxyuridine; F_3dThd, 5-trifluoromethyl-2′-deoxyuridine; 5-I-dUrd, 5-iodo-2′-deoxyuridine; dThd, thymidine; HSV, herpes simplex virus; HPLC, high performance liquid chromatography; MIC_{50}, minimum inhibitory concentration, the concentration required to inhibit virus-induced cytopathogenic effects by 50%; $CCID_{50}$, cell culture infectious dose$_{50}$ (one CCID is equal to the number of virions required to infect 50% of cells).

Island, NY). HEp-2 cells were maintained and grown in 150-cm^2 flasks in Eagle's minimum essential medium containing 10% bovine serum. The S148 strain of HSV-1 was used in these experiments. dATP, dGTP, dCTP, TTP, and T4 polynucleotide kinase were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). [γ-^{32}P]ATP (7000 Ci/mmol) and [8-^{3}H]dGTP (12 Ci/mmol) were obtained from ICN Radiochemicals (Irvine, CA). [^{3}H]CdG (250 mCi/mmol) and [*methyl*-^{3}H]dThd (7 Ci/mmol) were obtained from Moravek Biochemicals Inc. (Brea, CA). The DNA primer (15 or 17 bases long) and the 47-base DNA template were purchased from Genetic Designs, Inc. (Houston, TX). The sequence of the 47-base template DNA was 5′-TTCATTTGGGAAACCCTTGGAACCTGACTGACTGGCCGT-CGTTTTAC-3′, the sequence of the 5-base primer was 5′-GTAAAAC-GACGGCCA-3′, and the sequence of the 17-base primer was 5′-GTAAAACGACGGCCAGT-3′. D-CdG and L-CdG were prepared as described (5). D-CdG-TP and L-CdG-TP were chemically synthesized from their respective nucleosides by Sierra Bioresearch (Tucson, AZ). Micrococcal nuclease was obtained from Worthington Biochemical Corp. (Freehold, NJ). Spleen phosphodiesterase and proteinase K were obtained from Boehringer Mannheim (Indianapolis, IN). CsCl, phosphodiesterase I, DNase I, and alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO).

CsCl density gradient centrifugation of nucleic acid extracts labeled with [^{3}H]CdG. Mock-infected and HSV-infected cells treated with the desired label were collected by centrifugation. The cell pellet was resuspended in 0.5 ml of 10 mM Tris, pH 8.0, 40 mM EDTA, 0.5% sodium dodecyl sulfate, 200 μg/ml proteinase K, and the mixture was incubated at 37° overnight. One hundred microliters of each sample were mixed with 5.0 ml of a CsCl solution, such that the final concentration of the CsCl was exactly 1.75 g/ml. Host DNA labeled with [^{14}C] dThd was included in each sample as an internal control. The samples were centrifuged to equilibrium at 20° (30,000 rpm for 72 hr, with a Ti 70.1 Beckman rotor). The gradients were fractionated, and the DNA in each sample was precipitated onto glass fiber filters with a 5% trichloroacetic acid solution containing 10 mM pyrophosphate. These filters were washed three times with this 5% trichloroacetic acid solution followed by two washes with 95% ethanol, dried, and counted for radioactivity.

To verify that the radioactivity associated with both the host and viral DNA peaks was due to the incorporation of [^{3}H]CdG, the DNA samples were pooled, dialyzed against water to remove the CsCl, lyophilized, and resuspended in 50 μl of DNase I in 50 mM glycine, pH 9.0. After incubation for 2 hr at 37°, 50 μl of 100 units/ml concentrations of both phosphodiesterase I and alkaline phosphatase in 50 mM glycine, pH 9.0, were added to each sample, and the reaction was continued overnight at 37°. The reaction was stopped by boiling for 2 min, the precipitated proteins were removed by centrifugation, and the samples were analyzed by reverse phase HPLC for the appearance of radiolabeled nucleoside. To determine whether the [^{3}H]CdG was incorporated into internal or terminal positions, the DNA after the lyophilization step was digested by the sequential action of micrococcal nuclease (50 units/ml micrococcal nuclease, 5 μg/ml pentostatin, 5 mM $CaCl_2$, 1 mM Tris, pH 9.5, for 2 hr at 37°) and spleen phosphodiesterase (three consecutive additions of 75 μg/ml spleen phosphodiesterase, 1 mM EDTA, in the same buffer, at pH 7.0, for 1 hr each time at 37°), as described by Pelling *et al.* (8). These enzymes specifically cleave RNA and DNA to generate 3′-monophosphates of all internally located nucleotides and the nucleoside of any 3′-terminal nucleotides. After digestion, the samples were analyzed by strong anion exchange HPLC, to separate monophosphates from nucleosides.

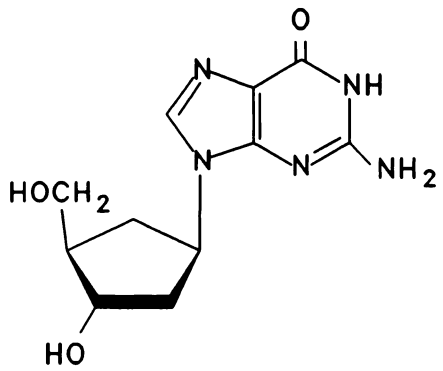

Fig. 1. Structure of CdG.

Neutral and alkaline sucrose gradients of HSV DNA containing [^{3}H]CdG. HSV-infected cells treated with the desired label were collected by centrifugation. The cell pellet was resuspended in 0.25 ml of 10 mM Tris, pH 8.0, 40 mM EDTA, 0.5% sodium dodecyl sulfate, 200 μg/ml proteinase K, and the mixture was incubated at 37° overnight. Forty microliters were mixed with 40 μl of 20% alkaline sucrose, and 75 μl of this solution were layered onto a 5-ml alkaline sucrose gradient (10–20%) prepared as described by McGuirt *et al.* (9). The samples were centrifuged at 100,000 × *g* for 18 hr, in a Beckman SW 50.1 rotor. For the neutral sucrose gradients, 40 μl of the proteinase K solution were mixed with 40 μl of 20% sucrose, and 75 μl of this solution were layered onto a 5-ml neutral sucrose gradient (10–30%) prepared as described by Balzarini *et al.* (10). The samples were centrifuged at 100,000 × *g* for 5 hr in a Beckman SW 50.1 rotor. After centrifugation, 200-μl fractions were collected from the top of the gradients, and the DNA in each sample was precipitated onto glass fiber filters with a 5% trichloroacetic acid solution containing 10 mM pyrophosphate. These filters were washed three times with this 5% trichloroacetic acid solution followed by two washes with 95% ethanol, dried, and counted for radioactivity.

Purification of polymerases and measurement of their activity. HSV DNA polymerase was purified from HEp-2 cells infected with HSV-1. Approximately 4 × 10^8 cells were infected with virus at a multplicity of infection of 10 $CCID_{50}$/cell. After a 1-hr adsorption period, the unattached virus was washed from the cells and the cultures were returned to the incubator at 37° for another 8 hr. The cells were collected by centrifugation and mixed with a 0.3 M potassium phosphate buffer, pH 7.5, containing 0.3% Triton X-100 and 10% glycerol. The HSV DNA polymerase was purified approximately 288-fold from this crude extract, as described by Derse *et al.* (11). The specific activity of the HSV polymerase was 4140 units/mg of protein. Human DNA polymerases, α, β, and γ were purified from K562 cells (5 ml of packed cells) grown in cell culture, as previously described (12). The specific activities of DNA polymerase α, β, and γ used in these studies were approximately 34, 1600, and 8 units/mg of protein, respectively. One unit of enzyme activity is defined as the amount of enzyme needed to incorporate 1 nmol of [^{3}H]dGTP into acid-precipitable material per hour at 37°, using gapped duplex DNA as template (13). HSV DNA polymerase activity was measured in 50-μl volumes containing 50 mM Tris, pH 8.0, 3 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 0.2 M KCl, 12.5 μg/ml gapped duplex DNA (13), 10 μm[^{3}H]dGTP (12 Ci/mmol), and 100 μM each of dATP, dCTP, and TTP. DNA polymerase α activity was measured in 50-μl volumes containing 50 mM Tris, pH 8.0, 1 mg/ml bovine serum albumin, 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml gapped duplex DNA (13), 10 μm [^{3}H]dGTP (1 Ci/mmol), and 50 μM each of dATP, dCTP, and TTP. DNA polymerase β and γ activities were measured as described for DNA polymerase α, except that the reactions included 100 mM KCl. After incubation for the desired time, the DNA in each sample was precipitated onto glass fiber filters, using a 5% trichloroacetic acid solution containing 10 mM pyrophosphate. These filters were washed three times with this 5% trichloroacetic acid solution followed by two washes with 95% ethanol, dried, and counted for radioactivity.

Extension of DNA primer annealed to a DNA template by DNA polymerase α. The DNA polymerases were incubated in 10-μl reactions that contained the ingredients listed above, except that the gapped duplex DNA was replaced with 0.24 μg/ml single-strand 47-base DNA oligomer annealed to an equimolar concentration of 5′-^{32}P-labeled primer, prepared as previously described (14). After 30 min, the

reactions were terminated by addition of 20 μl of 90% formamide, 10 mM EDTA, 0.03% bromophenol blue, followed by boiling for 5 min. The extension products of the primers were separated by electrophoresis on a 20% polyacrylamide gel containing 7 M urea and were visualized by autoradiography. Kinetic constants for the incorporation of guanine deoxynucleotides were determined as described previously (12, 14, 15). The kinetic constants for the incorporation of bases after the guanine nucleotide had been incorporated were determined as described (14).

Results

Incorporation of [^{3}H]CdG into HSV and host DNA. The incorporation of [^{3}H]CdG into the DNA of cells infected with HSV was determined at various times after infection, to optimize conditions for the incorporation of CdG into either HSV or host DNA. After incubation with [^{3}H]CdG, the nucleic acids were extracted, and HSV DNA was separated from host DNA by equilibrium density gradient centrifugation in CsCl. When HSV-infected cells were incubated for 9 hr with 8 μM [^{3}H]CdG, starting at the time of infection (Fig. 2), label was found only in host DNA. Very little incorporation of [^{3}H]CdG into DNA was observed in cells that were not infected with virus. These results indicated that [^{3}H]CdG-TP formed from the sequential

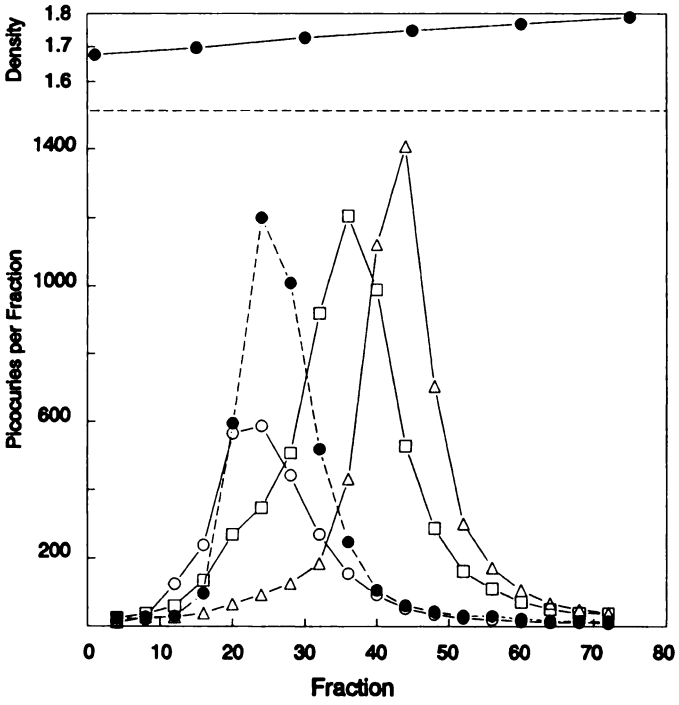

Fig. 2. Incorporation of [^{3}H]CdG into viral and cellular DNA. HSV-1-infected HEp-2 cells were incubated with 8 μM [^{3}H]CdG for 9 hr starting at the time of infection (○), with 1 μM [^{3}H]CdG for 5 hr starting 4 hr after infection (△), or with 8 μM [^{3}H]CdG for 5 hr starting 4 hr after infection (□). The nucleic acids were extracted, and the HSV-1 DNA was separated from the host DNA by centrifugation in CsCl gradients. Twenty-five-microliter fractions were collected from the top of each gradient, and the radioactivity in the acid-insoluble portion in every fourth fraction was determined. Each gradient was plotted in relation to its internal standard, which was the ^{14}C-labeled host DNA (●, *dashed line*). The position of the DNA labeled with 1 μM [^{3}H]CdG for 5 hr starting 4 hr after the infection (△) was the same as that of DNA labeled with [^{3}H]dThd during the same time of infection. The results shown in the figure were from one experiment. However, this experiment has been repeated many times with similar results. Because all gradients were similar, the gradient shown in the figure is an average of all gradients formed in this particular centrifugation.

action of the HSV dThd kinase and host nucleotide kinases (6) was utilized by a host DNA polymerase for DNA synthesis. When HSV-1-infected cells were treated for 5 hr with 1 μM [^{3}H]CdG, starting 4 hr after infection (Fig. 2), label was found primarily in viral DNA. The small amount of label in the host DNA is presumably due to the near-total inhibition of host DNA synthesis by 4 hr of the viral infection. The lack of label in viral DNA incubated with [^{3}H]CdG from 0 to 9 hr of virus infection was probably due to effective inhibition of viral replication by drug treatment (8 μM CdG is 10 times the concentration required to inhibit viral replication by 50%). Although [^{3}H]CdG is undoubtedly incorporated into viral DNA under these conditions, the amount of incorporation is below the level of detection.

The density of viral DNA labeled with 1.0 μM [^{3}H]CdG was the same as that seen with [^{3}H]dThd-labeled viral DNA. However, if the concentration of [^{3}H]CdG was increased to 8 μM, the density of the labeled DNA was decreased, so that the viral DNA banded between the host and viral DNA markers. The reason for this shift in the banding in the CsCl gradient is not known. However, viral DNA obtained from HSV-infected cells treated with 8 μM [^{3}H]CdG sedimented in either neutral or alkaline sucrose gradients in a manner similar to that of viral DNA labeled with [^{3}H]dThd (Fig. 3), indicating that the viral DNA containing CdG was the same size as viral DNA from untreated cells.

The radioactive viral and host DNAs were collected separately from the CsCl gradient, dialyzed, and degraded to nucleosides with DNase I, phosphodiesterase I, and alkaline phosphatase; the nucleosides were analyzed using reverse phase HPLC. All of the radioactivity in the DNA eluted from the reverse phase column with authentic CdG (data not shown), confirming that the radioactivity in the DNA was due to the incorporation of CdG. The viral and host DNA labeled with [^{3}H]CdG were also degraded with micrococcal nuclease and spleen phosphodiesterase I, to generate 3′-monophosphates of all internally located nucleotides and the nucleoside of any terminal nucleotides. The nucleosides and nucleotides were then analyzed by strong anion exchange chromatography. Most of the radioactivity in both the viral and host DNA was converted to the 3′ monophosphate of CdG-Mp (data not shown), indicating that CdG was incorporated into internal linkages by the HSV DNA polymerase and the host polymerase responsible for incorporation. The percentage of CdG incorporated into internal linkages in the DNA chain was the same as that seen in similar experiments using [^{3}H]dThd to label the DNA.

Inhibition of purified HSV DNA polymerase by CdG-TP. Because CdG was incorporated into HSV DNA, the interaction of CdG-TP with HSV DNA polymerase was studied. CdG-TP was a potent competitive inhibitor of the incorporation of [^{3}H]dGTP into DNA by HSV DNA polymerase (K_i, 0.35 μM) (Fig. 4). The K_i/K_m ratio was approximately 1, indicating that the affinity of CdG-TP for the active site of the HSV DNA polymerase was similar to that of dGTP.

These kinetic experiments showed that CdG-TP competes with the natural substrate for DNA synthesis, but they did not indicate what effect this has on DNA synthesis by the enzyme. It is possible that a nucleoside 5′-triphosphate can (a) compete with the natural substrate but not be used as a substrate, (b) substitute for the natural substrate with little effect on subsequent DNA synthesis, or (c) substitute for the natural substrate

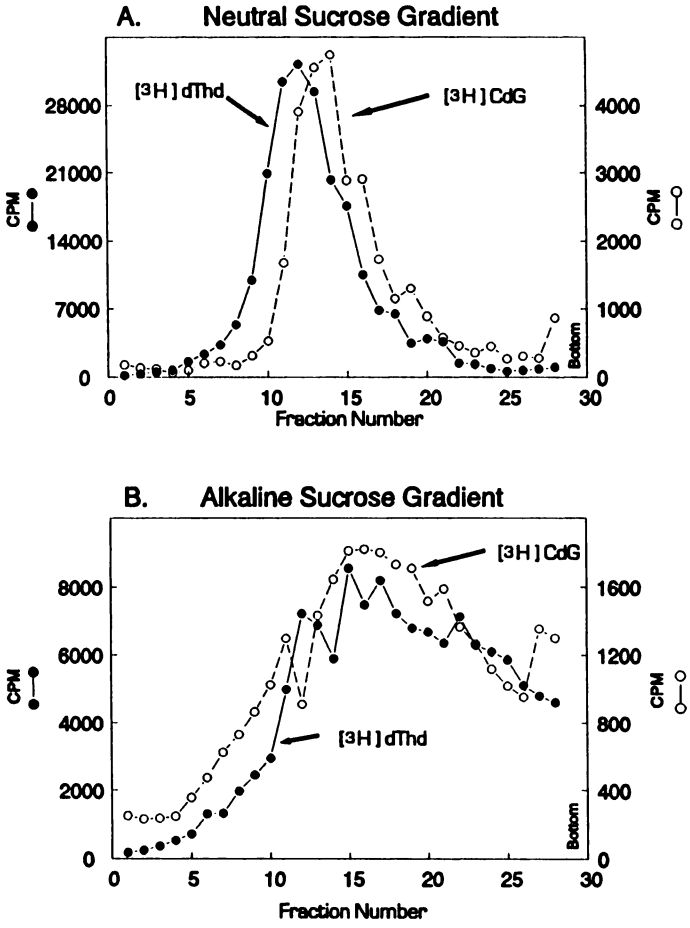

Fig. 3. Sedimentation of CdG-containing HSV DNA in neutral and alkaline sucrose gradients. HSV-infected HEp-2 cells were incubated for 5 hr with either 8 μM [^{3}H]CdG (○) or [^{3}H]dThd (●) starting 4 hr after infection. Nucleic acid extracts were layered onto a 10–30% neutral sucrose gradient (A) or a 10–20% alkaline sucrose gradient (B) and were centrifuged as described in Experimental Procedures. Two-hundred-microliter fractions were collected from the top of each gradient, and the radioactivity of the acid-insoluble portion of each fraction was determined.

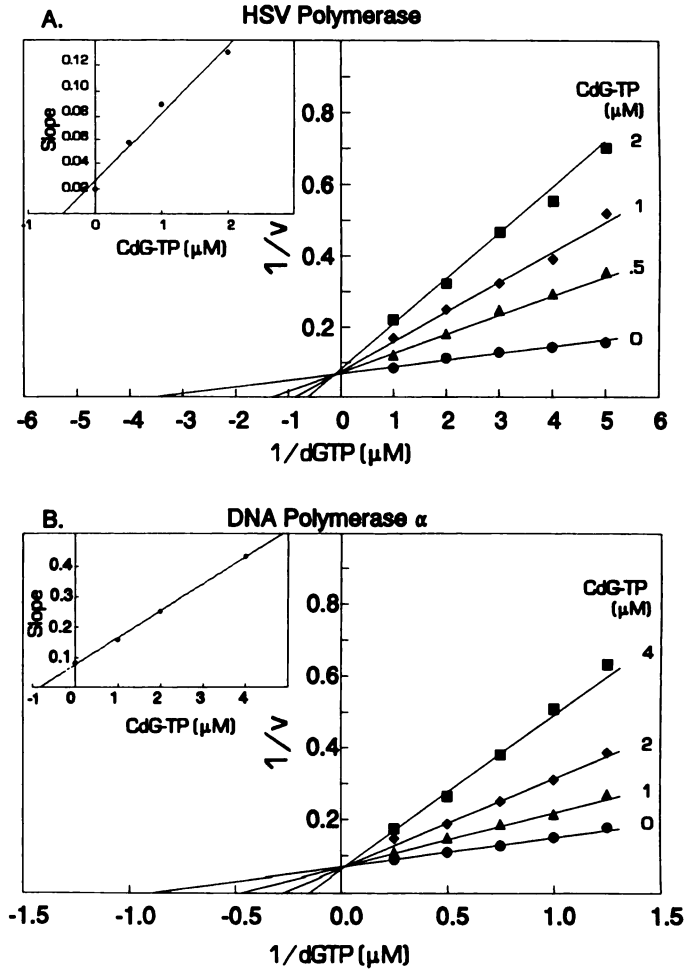

Fig. 4. Competitive inhibition by CdG-TP of the incorporation of [^{3}H] dGTP by HSV DNA polymerase and DNA polymerase α. HSV DNA polymerase (A) and DNA polymerase α (B) were incubated with [^{3}H] dGTP and CdG-TP as shown. The incorporation of [^{3}H]dGTP into acid-insoluble material was determined for each sample, and a double-reciprocal plot of the data is shown. The K_i for CdG-TP for each polymerase was determined from a plot of the slope of each line versus the concentration of CdG-TP (*inset*). The experiment was repeated one time, and nearly identical results were obtained. The average K_i of CdG-TP against the HSV DNA polymerase was 0.35 μM, and with DNA polymerase α it was 0.95 μM.

and interfere with subsequent DNA synthesis. If the nucleotide can substitute for the natural substrate in the DNA polymerase reaction, then it is necessary to determine the kinetic parameters for DNA synthesis, so that the effect of substitution of the nucleotide analog on DNA synthesis can be determined. CdG-TP efficiently substituted for dGTP in the synthesis of DNA by HSV polymerase (Fig. 5). HSV DNA polymerase was able to incorporate CdG-TP into the growing DNA strand and then extend the chain. The template was designed so that the HSV DNA polymerase would first have to incorporate one guanine nucleotide followed by the incorporation of a different nucleotide, then it would have to incorporate two guanine nucleotides in succession before the incorporation of a different nucleotide, and finally it would have to incorporate three guanine nucleotides in succession before the incorporation of a different base. From the results in Fig. 5, it is clear that the HSV DNA polymerase was able to incorporate CdG-MP as many as three times in succession with little effect on chain extension. Under these conditions, the polymerase required more time to extend the primer to full length with CdG-TP, indicating a small effect on the DNA synthesis.

To gain precise measurements of the substrate characteristics of CdG-TP, the K_m and V_{max} for incorporation were determined with a primer (15 bases long) in which a guanine nucleotide was the first base incorporated after the primer (Table 1), and the results were compared with those obtained with dGTP. Because both dGTP and CdG-TP can be extended by the HSV polymerase (Fig. 5), TTP, dATP, and dCTP were left out of the reaction, so that the DNA chain could not be extended after the incorporation of guanine nucleotide. The kinetic constants for the incorporation of CdG-MP were similar to those for dGTP. To quantitate the ability of the HSV DNA polymerase to extend the DNA chain after the incorporation of CdG-MP, the kinetic parameters for the extension of the primer by two nucleotides was determined and compared with that seen after the incorporation of dGMP (Table 1). In these experiments, HSV DNA polymerase was incubated with the 15-base primer, CdG-TP or dGTP at 100 μM, and varying concentrations of TTP. No dATP or dCTP was included in the reaction, so that once TMP was incorporated the DNA chain would not

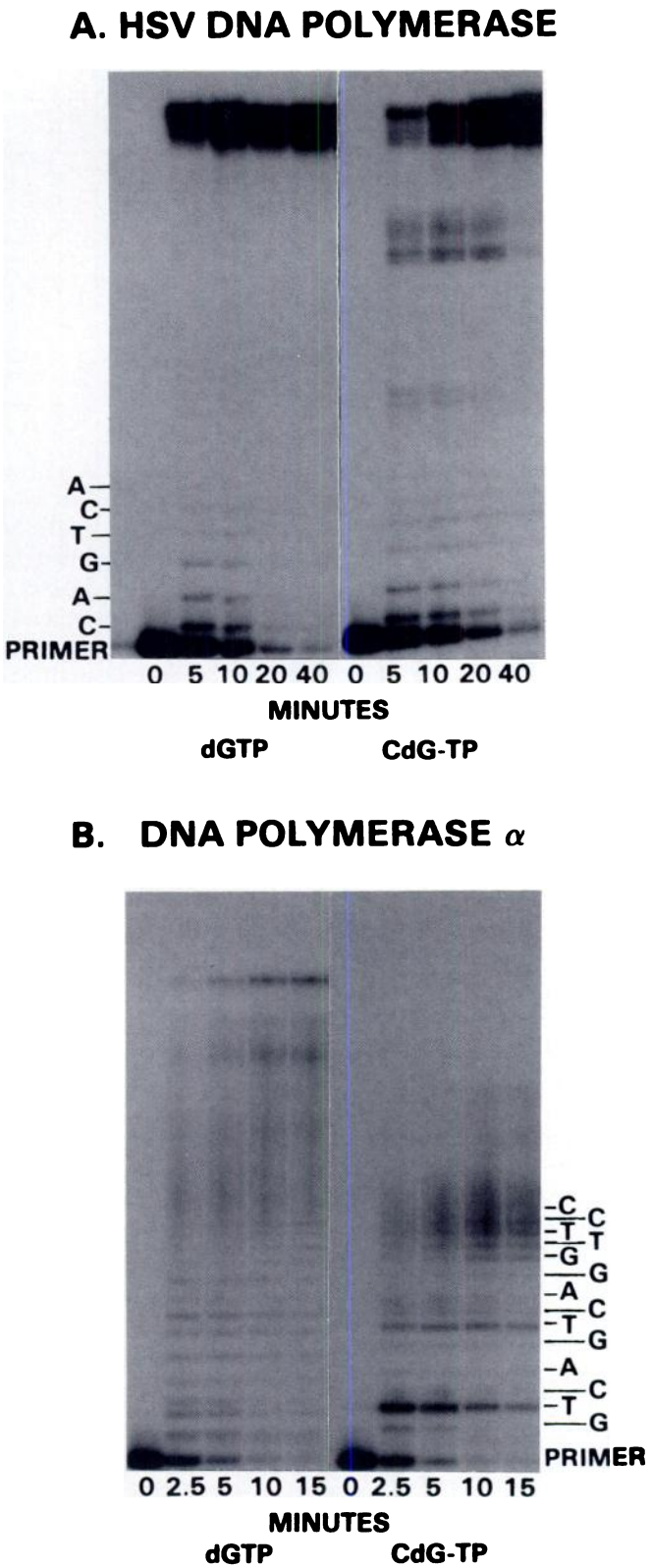

Fig. 5. Incorporation of CdG-TP by HSV DNA polymerase and DNA polymerase α. The HSV DNA polymerase (A) and DNA polymerase α (B) were incubated with a 17-base or 15-base, respectively, ^{32}P-labeled primer annealed to 47-base template, 50 μM each of dATP, dCTP, and TTP, and 50 μM of either dGTP or CdG-TP, as described in Experimental Procedures. Samples were taken at the time shown, the products were separated from the substrate by electrophoresis on a 15% polyacrylamide gel containing 7 M urea, and they were visualized by autoradiography. The sequence of nucleotides incorporated after the 15-base primer was primer-GTCAGTCAGGTTCCAAGGGTTTCCCAAATGAA.

be extended. These values are not kinetic constants for the incorporation of the final nucleotide but represent the velocity constants for the extension of the primer by two bases (14, 15). As seen in Table 1, no differences were observed in the K_m or V_{max} for extension of the primer by two bases, using either CdG-TP or dGTP. These results show that there are only trivial differences between dGTP and CdG-TP as substrates for HSV DNA polymerase.

Inhibition of human DNA polymerases by CdG-TP. In an attempt to determine the host enzyme responsible for the incorporation of CdG into the DNA, we determined the ability of CdG-TP to inhibit the human DNA polymerases α, β, or γ (Table 2). Under similar incubation conditions, the concentration of CdG-TP required to inhibit the incorporation of 1 μM [^{3}H]dGTP into the DNA by 50% was 2 μM with DNA polymerase α but was 2200 and 300 μM for DNA polymerase β and γ, respectively. The L-enantiomer of CdG-TP inhibited DNA polymerase β and γ at concentrations similar to or less than that required for inhibition by the D-enantiomer. These results suggested that neither DNA polymerase β nor γ was responsible for the incorporation of [^{3}H]CdG into host DNA observed above in virus-infected cells. Therefore, characterization of the interaction of CdG-TP with only DNA polymerase α was done. CdG-TP competively inhibited the incorporation of [^{3}H]dGTP into DNA by DNA polymerase α, with a K_i of 1 μM (Fig. 4). The K_i/K_m ratio was 1, indicating that the affinities of dGTP and CdG-TP for the active site of the enzyme were similar.

Primer extension studies indicated that CdG-TP was used as a substrate for DNA synthesis by DNA polymerase α but, once it was incorporated, the further elongation of the DNA chain was inhibited (Fig. 5). Product accumulated that was two bases longer than the primer, indicating that DNA polymerase α was able to incorporate the next natural nucleotide (TMP) after the incorporation of CdG-MP but then had difficulty extending the DNA chain. Incorporation of CdG into the DNA chain did not cause an absolute block to further extension by DNA polymerase α. Inhibition of DNA chain extension was also seen after the incorporation of CdG-MP farther from the primer. Quantitative determinations of the kinetic parameters for the incorporation of CdG-MP and the extension of the DNA chain are summarized in Table 1. CdG-TP was not as good a substrate for DNA polymerase α as was dGTP. The K_m for incorporation was 3 times that seen with dGTP, and the V_{max} was only 30% of that seen with dGTP. Consistent with the results in Fig. 5, the kinetic constants for the extension of the primer by two bases were only moderately affected. However, the K_m for extension of the primer by three nucleotides with CdG-TP was approximately 20-fold that seen with dGTP; the V_{max} for extension of the primer by three nucleotides with CdG-TP was the same as that with dGTP.

Discussion

In this work we have evaluated the effect of CdG on DNA synthesis by the HSV and human DNA polymerases. In whole-cell experiments, it was clear that CdG was incorporated into both the host cell and HSV DNA in HSV-infected cells treated with CdG. Centrifugation of CdG-containing HSV DNA in neutral and alkaline sucrose gradients indicated that the viral DNA synthesized in the presence of 8 μM CdG (a concentration that inhibited viral replication by >90%) was of full genome size. Consistent with this observation, *in vitro* studies with the

TABLE 1

Kinetic parameters for the incorporation of CdG-MP into DNA

DNA polymerases were incubated with the 15-base 5′-^{32}P-labeled primer annealed to the 47-base template. Products were separated from substrate by electrophoresis on a 15% polyacrylamide gel containing 7 M urea, and they were visualized by autoradiography. The kinetics of incorporation of the various nucleotides were determined as described (14, 15). Each value represents the mean and standard deviation from three separate experiments.

	Incorporation of guanine nucleotide		Extension of primer by two nucleotides		Extension of primer by three nucleotides	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
	μM	%/min	μM	%/min	μM	%/min
HSV polymerase						
dGTP	0.5 ± 0.2	0.8 ± 0.2	1.4 ± 0.7	0.49 ± 0.30	ND[a]	ND
CdG-TP	1.0 ± 0.2	0.6 ± 0.2	1.3 ± 0.4	0.34 ± 0.17	ND	ND
DNA polymerase α						
dGTP	0.6 ± 0.2	1.4 ± 0.2	4.2 ± 2.4	0.84 ± 0.14	1.6 ± 0.7	1.0 ± 0.4
CdG-TP	1.9 ± 1.1	0.4 ± 0.2	8.2 ± 4.1	0.78 ± 0.07	29 ± 18	0.6 ± 0.02

[a] ND, not done.

TABLE 2

Inhibition of DNA polymerases by CdG-TP

The DNA polymerases were incubated with varying concentrations of either the D- or L-enantiomer of CdG-TP in the presence of 1 μM [^{3}H]dGTP, and the concentration of CdG-TP required to inhibit by 50% the incorporation of [^{3}H]dGTP into DNA was determined. Each value represents the best estimate from 2 or more experiments.

	IC_{50}	
	D-CdG-TP	L-CdG-TP
	μM	
HSV DNA polymerase	1.5	460
DNA polymerase α	2.0	470
DNA polymerase β	2200	1400
DNA polymerase γ	300	215

isolated HSV DNA polymerase indicated that CdG-TP was utilized for DNA synthesis almost as efficiently as dGTP and that HSV DNA polymerase was able to extend the DNA chain easily after the incorporation of CdG-MP. Previously (6), we have shown that treatment of HSV-1-infected cells with 8 μM CdG results in the production of a cellular concentration CdG-TP of 80 μM, which was considerably greater than the concentration of dGTP in these cells, indicating that under these conditions a considerable amount of CdG-MP would be incorporated into the HSV DNA. These results indicated that DNA synthesis by HSV DNA polymerase is not adversely affected by CdG-TP and suggested that inhibition of viral replication is due to the incorporation of CdG-MP into the viral genome, with subsequent disruption of some function vital to viral replication. In this regard, the mechanism of anti-HSV activity of CdG is similar to that seen with BVdU, F_3dThd, and 5-I-dUrd (16, 17) but is different from that of ACV and DHPG, which preferentially inhibit HSV-induced DNA polymerase activity.

Viral DNA isolated from HSV-infected cells incubated with a high concentration of CdG (8 μM) was of lower density than viral DNA containing only natural nucleotides. The reason for this change in density is not known, but it is unlikely that the difference in molecular weight between CH_2 and O is sufficient to account for this shift in density. It is possible that multiple substitution of dGMP by CdG-MP causes perturbation of the three-dimensional structure of the DNA double helix, resulting in a change in the bouyant density in CsCl gradients.

Because CdG, ACV, and DHPG are all inhibitors of HSV replication, it is of interest to compare the effect of the three corresponding nucleotides on DNA synthesis by the HSV DNA polymerase. The 5′-triphosphates of both ACV and DHPG are competitive inhibitors of the HSV DNA polymerase with respect to dGTP. The K_i for inhibition of the HSV DNA polymerase by ACV-TP (18–20) ranges from 0.003 to 1.23 μM, and for DHPG-TP it is 0.03 μM (21). These values are similar to those observed with CdG-TP. Reardon (22) has determined the kinetic constants for the incorporation of ACV-TP and DHPG-TP by HSV-1 DNA polymerase and human DNA polymerase α, using defined primer/templates and ^{3}H-nucleotide analog. Because the kinetic constants determined at specific positions can be dependent on the specific site of incorporation, comparisons between his work and ours will be made in reference to the kinetic values at the specific site for dGTP. The kinetic values for the incorporation of all three nucleotide analogs by the HSV DNA polymerase are similar. The K_m for incorporation of CdG-MP and DHPG-MP is approximately 2-fold that for the incorporation of dGMP, whereas the K_m for incorporation of ACV-MP is the same as that for dGMP. The V_{max} values for the incorporation of CdG-MP, DHPG-MP, and ACV-MP are 100%, 21%, and 40% of that seen with dGMP, respectively. However, once incorporated, the three nucleotide analogs have quite different effects on DNA chain elongation. Incorporation of ACV-MP results in chain termination, due to the lack of a 3′-OH for extension of the DNA chain. Furthermore, the potent inhibition of HSV-1 DNA polymerase by ACV-TP is due to the formation of a dead-end complex upon binding of the next nucleotide triphosphate (23). Extension of the DNA chain after the incorporation of either DHPG-MP or CdG-MP is possible because both nucleotides contain a 3′-OH. However, Reardon (22) showed that the incorporation of the next base after the incorporation of DHPG-MP is inhibited with respect to incorporation after the normal nucleotide dGMP. In two experiments, the K_m for incorporation of the next base was 36% or 420% of control, depending on the position incorporated. However, the V_{max} for incorporation of the next base after the incorporation of DHPG-MP at both positions was 1% of that seen after the incorporation of the natural nucleotide. Further work by Reid *et al.* (24), using DNA-sequencing technology similar to that in this work, showed that HSV DNA polymerase stalled after the addition of one natural nucleotide after the incorporation of DHPG-MP. These results indicated that elongation of the DNA chain after the incorporation of DHPG-MP is greatly inhibited. In contrast, CdG-TP is used as a substrate by the HSV DNA polymerase almost as efficently as dGTP. The K_m and V_{max} for incorporation of CdG-TP are similar to those of dGTP, and the HSV DNA polymerase extended the DNA chain past the incorporation of CdG-MP as easily as it did after the incorporation of dGMP. These results indicate

that the antiviral mechanisms of action of these three guanine nucleotide analogs are quite different. Whereas ACV and DHPG inhibit the ability of the HSV polymerase to replicate the viral DNA, CdG is incorporated into the viral DNA, potentially resulting in a nonfunctional genome.

In contrast to these results with the HSV DNA polymerase, incorporation of CdG-MP by DNA polymerase α inhibits further chain elongation after one normal base has been added. This is an interesting mechanism of inhibition of chain elongation that has not been seen with other inhibitors of DNA polymerase α, although similar results were seen after the incorporation of DHPG-MP by HSV DNA polymerase (24). Reid *et al.* (24) have suggested two possible mechanisms for the disruption of DNA synthesis in this manner. It is possible that interactions of the polymerase with the second nucleotide from the 3′-end of a DNA chain may be important to the translocation step in processive synthesis of DNA and that the incorporation of some nucleoside analogs may interfere with this particular binding. If this is the case, then our data suggest that the recognition of the DNA chain by DNA polymerase α is different from that of the HSV DNA polymerase. A second possibility is that the CdG-deoxycytidine base pairs may be held together by DNA polymerase α but once the polymerase has incorporated the next base then fraying of the CdG-deoxycytidine base pairs may occur, disrupting further elongation of the DNA chain. ACV-TP, DHPG-TP, and CdG-TP are all competitive inhibitors of DNA polymerase α with respect to dGTP. Derse *et al.* (20) found that the K_i for ACV-TP was 0.18 μM, and with DHPG-TP it was 0.12 μM. Furman *et al.* (19) found that the K_i of ACV-TP in three different preparations of DNA polymerase α was approximately 2.8 μM. All of these guanine nucleotides were substrates for DNA synthesis by DNA polymerase α and inhibited further elongation of the DNA once they were incorporated into the DNA chain (22).

The ribonucleotide pools in HSV-infected cells treated with CdG were smaller than those in HSV-infected cells (6), which suggested that CdG caused toxicity to HSV-infected host cells. In this work we showed that CdG was incorporated into the DNA of cells infected with virus and that CdG-TP was a potent inhibitor of DNA synthesis by DNA polymerase α. These results suggest that the cytotoxicity of CdG to HSV-infected cells may be a result of the inhibition of DNA synthesis due to the inhibition of DNA polymerase α. It is possible that the selective destruction of HSV-infected cells may also be a factor in the antiviral activity of CdG.

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