Antiviral effect of an oligo(nucleoside methylphosphonate) complementary to the splice junction of herpes simplex virus type 1 immediate early pre-mRNAs 4 and 5

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Communicated by Paul C. Zamecnik, November 1, 1985

ABSTRACT
Selective inhibition of regulatory immediate early (IE) genes of herpes simplex virus type 1 (HSV-1) should inhibit virus growth. Treatment of HSV-1-infected cells with the oligo(nucleoside methylphosphonate) d(TpCCTCTG) (deoxyoxynucleoside methylphosphonate residues in italic), which is complementary to the acceptor splice junction of HSV-1 IE pre-mRNA 4 and 5, before (1–24 hr) or at the time of infection caused a dose-dependent inhibition in virus replication. Virus titers were decreased 50% and 90% in cells treated with 25 μM and 75 μM oligomer, respectively; at 300 μM, a 99% reduction in virus production was observed. Viral DNA synthesis was reduced 70–75% and there was a 90% reduction in synthesis of viral proteins, including other IE species and viral functional (130-kDa major DNA-binding) and structural (glycoprotein gB) proteins. In the same concentration range, d(TpCCTCTG) caused a minimal reduction (0–30%) in protein synthesis and growth rates (<40%) of uninfected cells. The data suggest that oligo(nucleoside methylphosphonate)s may be effective in antiviral chemotherapy.

Numerous nucleoside or nucleotide analogues have been screened for antiviral activity (1). Their mode of action exploits differences in the specificity of viral versus host enzymes, thus affecting differentially the rate of viral as compared to host-cell nucleic acid synthesis. However, with rare exceptions, most of them are not effective in experimentally infected animals (2).

An alternative approach to antiviral chemotherapy is the use of sequence-specific oligonucleotides or their analogues to selectively inhibit viral gene expression at the level of mRNA processing or translation. For this purpose we have developed sequence-specific nonionic nucleic acid analogues that contain a 3'-S' methylphosphonate group in place of the negatively charged phosphodiester group normally found in oligonucleotides (3). These analogues are resistant to nuclease hydrolysis and penetrate mammalian cells in culture (4). An oligo(deoxyribonucleobase methylphosphonate) complementary to the Shine-Dalgarno sequence of 16S ribosomal RNA inhibits protein synthesis in Escherichia coli, but not in mammalian cells (5). Oligomers complementary to the initiation codon regions of rabbit globin mRNA inhibit translation in a cell-free system and in rabbit reticulocytes (6), while oligomers complementary to the initiation codon regions of vesicular stomatitis virus mRNAs inhibit viral but not cellular protein synthesis in infected mouse L cells (7).

To explore the possibility that control of gene expression by methylphosphonates could be an effective antiviral modality, we synthesized an oligo(nucleoside methylphosphonate) d(TpCCTCTG); deoxyoxynucleoside methylphosphonate residues in italic) that is complementary to the acceptor splice junction of herpes simplex virus type 1 (HSV-1) immediate early (IE) mRNAs 4 and 5 (8). The rationale for this choice is based on previous findings indicating that (i) HSV proteins form at least three kinetic groups [IE(a); early, E(β); and late, L(γ)] whose synthesis is coordinately regulated and sequentially ordered in a cascade fashion (9), (ii) IE genes play a regulatory role in virus replication (10, 11), and (iii) RNA splicing may be involved in the control of gene expression (12, 13). The effect of this oligo(nucleoside methylphosphonate) on HSV infectivity and on viral protein and DNA synthesis is described.

MATERIALS AND METHODS

Cells and Virus. Vero (African green monkey kidney) and human foreskin fibroblast cells (M. A. Bioproducts, Walkersville, MD) were grown in Eagle's minimal essential medium (MEM) with 25 mM Hepes buffer and 10% fetal bovine serum. The isolation and properties of HSV-1(F) and HSV-2(G) have been described (14).

Synthesis of d(TpCCTCTG). IE mRNAs 4 and 5 each have a single splice site (Fig. 1). Their common 5' termini lie within the short repeat segments of the genome, as do the 5' and 3' splice junction sites (8, 15). d(TpCCTCTG) is a sequence complementary to the previously described (8) sequence of the 3' acceptor splice junction in HSV-1. It was synthesized by solid-phase techniques on a polystyrene support and purified as described (16). The oligomer, characterized as described by Murakami et al. (17), was dissolved in MEM. Radiolabeling of Infected Cell Proteins. Vero cells were infected with HSV-1 [15–30 plaque-forming units (pfu) per cell] or mock infected with phosphate-buffered saline in the presence or absence of d(TpCCTCTG) and metabolically labeled (1 hr, 37°C) with [35S]methionine (New England Nuclear, 1300 Ci/mmol; 1 Ci = 37 GBq) added at various times postinfection. Duplicate cultures were infected in medium containing 2.8 mM L-canavanine [to restrict gene expression to E(a) and a subclass of E(β) proteins (18)] in the presence or absence of d(TpCCTCTG) added at the time of infection; these cells were metabolically labeled (6–7 hr) with [35S]methionine in medium free of canavanine. Cells were harvested by scraping, washed in cold phosphate-buffered saline, and resuspended in 10 mM Tris-HCl buffer (pH 7.0) with 1 mM phenylmethylsulfonyl fluoride. Suspensions were

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Abbreviations: HSV-1 and HSV-2, herpes simplex virus types 1 and 2; IE, immediate early; ICP, infected-cell protein; pfu, plaque-forming unit(s); hpi, hr postinfection.†To whom reprint requests should be addressed.
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frozen and thawed and then cleared of cell debris by centrifugation (300 × g, 10 min), and the supernatants were analyzed for protein content (19) and for acid-insoluble [35S]methionine incorporation.

**DNA Extraction and Density Gradient Centrifugation.** Vero cells were infected with HSV-1 (20 pfu per cell) in the presence or absence of d(TpCCTCCTG) and incubated at 37°C for 4 hr. They were exposed to [3H]thymidine (25 μCi/ml; New England Nuclear, 40 Ci/mmol) from 4 to 18 hr postinfection (hpi), washed, and lysed with 20 mM EDTA/90 mM NaCl/9 mM sodium citrate, pH 7/1% NaDodSO4. After addition of 0.7 M sodium perchlorate, DNA was extracted twice with chloroform/isoamyl alcohol (24:1), ethanol-precipitated, and centrifuged in a CsCl density gradient at 114,000 × g for 60 hr. Fractions collected from the bottom of the tube were measured for acid-insoluble [3H]thymidine incorporation. Densities were determined by weighing filled micropipettes (25 μl) of known weight. Mock-infected Vero cells were exposed to 10–150 μM d(TpCCTCCTG) and labeled with [3H]thymidine (25 μCi/ml) for 4 hr at 37°C and then harvested onto glass fiber filter strips (Reeve Angel grade 934 AM). The strips were dried and the amount of [3H]thymidine retained on the filters was determined by liquid scintillation spectroscopy.

**RESULTS**

**d(TpCCTCCTG) Inhibits Virus Growth.** The oligonucleotide methylphosphonate d(TpCCTCCTG) terminates at the 5′ end with a phosphodiester linkage. Its binding site is shown in Fig. 1. Vero cells were exposed to various concentrations (0–300 μM) of d(TpCCTCCTG) before (1–24 hr), during (0 hr), or after (1 hr) infection with HSV-1 (3 pfu per cell), and virus titers were determined 24 hr later. Addition of d(TpCCTCCTG) at the time of infection (Fig. 2A), or at 1–24 hr before infection (Fig. 2B) caused a dose-dependent decrease in HSV-1 titers. In both cases, significant (90%) inhibition was observed in cultures exposed to 75 μM d(TpCCTCCTG). The inhibition did not increase significantly in cultures exposed to higher (100–300 μM) oligomer concentrations, and 25 μM caused 50% inhibition of virus growth. On the other hand, addition of the oligomer at 1 hpi caused only a 40% reduction in virus titers, even at a concentration of 150 μM (Fig. 2B). We interpret these data to indicate that d(TpCCTCCTG) penetrates rapidly into the cells and exerts an inhibitory effect very early in the virus replicative cycle. IE mRNAs 4 and 5 are colinear on the HSV-1 and HSV-2 genomes, and there is a 5-base sequence homology in the acceptor splice junction (23). Consistent with this homology, d(TpCCTCCTG) added at the time of infection caused a
dose-dependent inhibition in the growth of HSV-2 (Fig. 2B).
However, at concentrations of 25–100 μM, its effect was less
(by a factor of 1.5–2) than that observed for HSV-1, so that
50% inhibition in virus growth was observed in cultures
exposed to 50 μM oligomer, as compared to 25 μM for
HSV-1. At 300 μM, the oligomer inhibited the replication of
both viruses equally well.

The inhibitory effect is sequence-specific, as evidenced by
the failure of the oligomer to inhibit virus growth when
modified by the inversion of the two central residues (C and
T). Thus, at the same concentrations (10–75 μM) at which
d(TpCCCTCCTG) caused a 15–90% reduction in virus titers,
d(TpCCTCCTG) did not inhibit virus infectivity (Fig. 2C).

Virus growth was also not inhibited by exposure to the
unrelated hexamer d(TpTTTTTT) at 10–100 μM (Fig. 2A).
Similar results were obtained with human fibroblasts (data
not shown).

Effect of d(TpCCCTCCTG) on the Growth of Uninfected Cells.
Vero cells were seeded (8 × 10^3 cells per well) in 96-well
cluster plates (Falcon and Becton Dickinson, Oxnard, CA) in
the presence or absence of d(TpCCCTCCTG) (10–300 μM).
They were incubated at 37°C for 24 or 48 h, at which times
the cells from replicate wells were trypsinized and counted.
At 24 h after seeding, the total numbers of cells in cultures
grown in the presence of 150 or 300 μM d(TpCCCTCCTG)
were similar to those observed in untreated cultures. At these
high concentrations, growth rates were somewhat decreased
by 48 h after seeding (by factors of 1.3 and 1.6 for 300 and
150 μM, respectively). However, there was no apparent
decrease in the growth rates of cells exposed to lower (10–25
μM) concentrations of d(TpCCCTCCTG) (Fig. 3), even after
five passages in medium containing 10 μM oligomer.

Effect of d(TpCCCTCCTG) on Total Protein Synthesis. Vero
cells infected with HSV-1 (30 pfu per cell) or mock-infected
with phosphate-buffered saline in the presence or absence
of d(TpCCCTCCTG) (250 μM) were labeled by incubation with
[^35S]methionine for 1 h at various times postinfection. Cell
extracts were analyzed for protein content and acid-precipitable
radioactivity, and the results were expressed as cpm/μg of
protein. As described (24), the rate of amino acid incor-
poration into acid-insoluble material in HSV-1-infected cells
increased at 4 hpi, reached maximal levels at 6–7 hpi
(presumably reflecting the synthesis of viral proteins), and
decreased thereafter. On the other hand, the rate of amino acid
incorporation into acid-insoluble material from cells infected
in the presence of d(TpCCCTCCTG) remained relatively stable
(Fig. 4A). Protein synthesis was reduced only 10–30% in
mock-infected cells treated with 250 μM oligomer (Fig. 4B)
and was unaffected in cells treated with 10 μM d(TpCCCT-
CCTG) (data not shown).

Effect of d(TpCCCTCCTG) on the Synthesis of Specific Viral
Proteins. Vero cells were infected with HSV-1 (15 pfu per

Fig. 3. Growth of uninfected cells. Vero cells (8 × 10^3) were
cultured for 24 or 48 hr without (●) or with 10 μM (■), 150 μM (○),
or 300 μM (□) d(TpCCCTCCTG).

d(TpCCTCCTG) (250 μM) at 0 hpi (lane 1) or untreated (lane 2),
and untreated, mock-infected cells (lane 3) were labeled with [^35S]me-
thonine (0–24 hpi). (B) HSV-1(F)-infected Vero cells treated with 50
μM d(TpTTTTTT) (lane 1) or untreated (lane 2) were labeled
with [^35S]methionine (0–24 hpi). (C) Mock-infected Vero cells exposed
to 250 μM d(TpCCCTCCTG) (lane 1) or untreated (lane 2) were labeled
with [^35S]methionine (0–8 hpi). (D) Proteins precipitated by mono-
clonal antibody 20aD4 from HSV-1(F)-infected Vero cells untreated
(lane 1) or treated (lane 2) with 250 μM d(TpCCCTCCTG) and labeled
with [^35S]methionine (0–8 hpi). (E) Vero cells infected with HSV-
1(F) in the presence of 2.8 mM L-canavanine (0–6 hpi) were treated
with 250 μM d(TpCCCTCCTG) (lane 1) or untreated (lane 2) and labeled
with [^35S]methionine from 6 to 7 hpi. Size markers (kDa) are
shown at left or right of each autoradiogram.

Fig. 4. Protein synthesis in HSV-1(F)-infected (A) and mock-
infected (B) Vero cells untreated (○) or treated (●) with 250 μM
d(TpCCCTCCTG) added at 0 hpi. Cultures were labeled for 1 h with
[^35S]methionine.

Fig. 5. Autoradiograms of NaDodSO4/8.5% polyacrylamide
gels. (A) HSV-1(F)-infected Vero cells, either treated with d(Tpc-
CCTCCTG) (250 μM) at 0 hpi (lane 1) or untreated (lane 2),
and untreated, mock-infected cells (lane 3) were labeled with [^35S]me-
thonine (0–24 hpi). (B) HSV-1(F)-infected Vero cells treated with 50
μM d(TpTTTTTT) (lane 1) or untreated (lane 2) were labeled
with [^35S]methionine (0–24 hpi). (C) Mock-infected Vero cells exposed
to 250 μM d(TpCCCTCCTG) (lane 1) or untreated (lane 2) were labeled
with [^35S]methionine (0–8 hpi). (D) Proteins precipitated by mono-
clonal antibody 20aD4 from HSV-1(F)-infected Vero cells untreated
(lane 1) or treated (lane 2) with 250 μM d(TpCCCTCCTG) and labeled
with [^35S]methionine (0–8 hpi). (E) Vero cells infected with HSV-
1(F) in the presence of 2.8 mM L-canavanine (0–6 hpi) were treated
with 250 μM d(TpCCCTCCTG) (lane 1) or untreated (lane 2) and labeled
with [^35S]methionine from 6 to 7 hpi. Size markers (kDa) are
shown at left or right of each autoradiogram.
completely inhibited the synthesis of virtually all the proteins that are normally made (lane 2) under restricted conditions, which allow only IE(a) and a subset of E(β) proteins to be made (18), and significantly (71%) reduced the synthesis of the 130-kDa protein. Viral protein synthesis was not inhibited in d(TpTTTT)-treated infected cells (Fig. 5B). Protein synthesis was only minimally reduced in uninfected cells exposed to high (250 μM) concentrations of d(TpCCTCTG), and the inhibition appeared to be primarily restricted to certain small (12- to 40-kDa) species (Fig. 5C).

Effect of d(TpCCTCTG) on DNA Synthesis. Vero cells were infected with HSV-1 (20 pfu per cell) in the presence or absence of d(TpCCTCTG) (250 μM) and labeled with [3H]thymidine (25 μCi/ml) at 4–18 hpi. Viral and cellular DNA were separated by CsCl density gradient centrifugation (26). The 3H in the nucleic acid from untreated infected cells localized in one band with a density of 1.725 g/cm³, consistent with that of viral DNA (26, 27). There was virtually no radioactivity in the low-density fraction (1.70 g/cm³) consistent with host-cell DNA (Fig. 6A). On the other hand, the 3H from a similarly centrifuged extract of d(TpCCTCTG)-treated infected cells (Fig. 6B) localized in two peaks with respective densities of viral and host-cell DNA. The level of radioactivity in the viral DNA peak was lower by a factor of 3.5–4 than that in the untreated cells, while there was a 6-fold increase in the amount of 3H in the cellular DNA peak.

DISCUSSION

Oligodeoxyribonucleotides (28–30) and oligo(oligoxyribonucleoside methylphosphonates) (6, 7) can be used to control the expression of mRNA in vitro and in living cells. In this report, we describe the results of our studies with an eight-residue oligomer [d(TpCCTCTG)] that is complementary to the acceptor splice junction of HSV-1 IE mRNAs 4 and 5 (Fig. 1). Considerations leading to its selection include previous findings indicating that (i) HSV IE genes (10), particularly that encoding for ICP22 (11), play a regulatory role in virus growth and (ii) RNA splicing may be involved in the control of gene expression (12, 13). The salient feature of the data is the finding that d(TpCCTCTG) inhibits virus growth, while in the same concentration range (10–75 μM) it has little, if any, deleterious effect on host-cell macromolecular metabolism and growth rate.

HSV-1 replication is inhibited by d(TpCCTCTG) in a dose-dependent fashion; 50% and 90% reductions, respectively, in virus titers are observed in cultures exposed to 25 μM and 75 μM. The inhibitory effect is almost immediate, as evidenced by the reduced level of inhibition in cultures treated at 1 hpi, and it appears to be sequence-specific. Thus, within the same concentration range (10–75 μM), virus growth is not inhibited by the unrelated hexamer d(TpTTTT) or by the oligomer modified by the inversion of the two central nucleosides C and T.

The reduction in virus titers presumably results from the ability of the oligomer to inhibit viral protein synthesis. Indeed, d(TpCCTCTG) [but not d(TpTTTT)] treatment inhibits the synthesis of viral proteins in all three [IE(a), E(β), and L(γ)] [24] kinetic groups including functional proteins (namely, the 130-kDa major DNA-binding protein with DNA-melting activity (20)] and structural virion components (namely, glycoprotein gB). d(TpCCTCTG) treatment also reduces [3H]thymidine incorporation into viral DNA by a factor of 3.5–4. This reduction is not due to dilution resulting from reutilization of the thymine base after degradation of the oligomer [possibly by cleavage of the 5'-terminal phosphodiester linkage or by cleavage of the 3'-terminal thymidine N-glycosyl bond (4)], since (i) infected cells were labeled at 4–18 hpi, at which time the intracellular thymidine pool should have reached equilibrium, (ii) the reduction in [3H]thymidine incorporation into viral DNA was significantly greater (70–75%) than that (35–40%) observed in similarly treated mock-infected cells (data not shown), and (iii) concomitant with the decrease in [3H]thymidine incorporation into viral DNA, [3H] incorporation into cellular DNA was 6-fold increased in d(TpCCTCTG)-treated as compared to untreated cells.

It is tempting to speculate that the effect of d(TpCCTCTG) on virus growth is mediated by its ability to interfere
with the splicing of IE pre-mRNA 4 and 5, thereby inhibiting the synthesis of the respective proteins ICP22 (68 kDa) and ICP47 (12 kDa). However, in the absence of direct evidence that d(TpCCTCCTG) interferes with IE mRNA processing, such conclusions are premature. Further, the regulatory function of these two IE genes is controversial. Thus, the function of ICP47 is unclear, and the protein was not resolved in our studies, presumably due to the insolubility of many IE proteins (31). On the other hand, there is some evidence that ICP22 may play a crucial role in the control of virus growth. Jacquemont et al. (11) found that the failure of HSV-1 to grow in nonpermissive XCI rat cells correlates with an accumulation of nonfunctional IE mRNA 4 and a concomitant failure to express ICP22 but not other IE viral proteins. However, using deletion mutants in this gene, Post and Roizman (32) concluded that ICP22 is not required for virus growth. More recent studies from that laboratory have shown that at least in some cells, the regulatory function of ICP22 is complemented by host-encoded proteins (33).

While final conclusions must await the results of studies on IE mRNA processing in d(TpCCTCCTG)-treated cells, our data are consistent with the interpretation that these IE genes play a significant role in the control of virus growth. The oligomer inhibits (or significantly reduces) the synthesis of all viral proteins, while primarily reducing the synthesis of only low molecular weight host protein species. Further, viral DNA synthesis is decreased in d(TpCCTCCTG)-treated cells while host-cell DNA synthesis is increased, consistent with the interpretation that viral functions responsible for inhibiting host-cell DNA synthesis (34) are not made (or are not functional) in oligomer-treated cells. Finally, the inhibitory effect of d(TpCCTCCTG) is observed in both Vero cells and human fibroblasts, and it appears to be sequence-specific. Virus infectivity is not affected by treatment of the infected cells with the unrelated oligomer d(TpTTTTT) or the oligomer modified by inversion of its two central bases. However, it should be stressed that the role of ICP22 in the regulation of virus growth remains unresolved, and the possibility cannot be excluded that d(TpCCTCCTG) inhibits the synthesis of proteins other than ICP22 (viral or host-encoded) that are the significant elements in controlling virus growth.

It is quite probable that a base sequence that is complementary to a relatively short oligomer such as d(TpCCTCCTG) is relatively common in both viral and cellular genes. Presumably the specific inhibition of synthesis of low molecular weight host proteins reflects such similarity. Further, the oligomer is complementary to a 6-base sequence in the acceptor splice junction of IE mRNAs 4 and 5 on the HSV-2 genome (23) and it inhibits the growth of HSV-2 almost as well as that of HSV-1 (Fig. 2). A limited computer search has also shown that HSV-1 is also complementary to a 7-base sequence in the coding region of the mRNA for the L protein of vesicular stomatitis virus and to nucleotides 72-80 in the coding region of rabbit β-globin mRNA. Consistent with these homologies, d(TpCCTCCTG) (150 μM) inhibits (85%) the synthesis of vesicular stomatitis virus in infected mouse L cells, as well as inhibiting the translation of β-globin mRNA in a cell-free rabbit reticulocyte translation system (6).

The concentration-dependence of the observed inhibitory effects is consistent with the nature of the physical binding interaction between the oligomer and HSV mRNA. Thus, the relative magnitude of the inhibitory effect increases more rapidly in the low (0-75 μM) than in the higher (75-300 μM) concentration range. It is apparent from these considerations that effective inhibition could be achieved at very low oligomer concentrations if the oligomer were irrevocably bound to the mRNA by crosslinking or by using the oligomer to cleave the mRNA.

Further information pertaining to the role of ICP22 in the d(TpCCTCCTG)-mediated inhibition of HSV replication must await the results of studies designed to identify RNA species in oligomer-treated and untreated infected cells. However, presently available data indicate that despite its potential limitations relating to specificity for HSV-1 IE mRNAs 4 and 5, d(TpCCTCCTG) selectively inhibits virus growth, with little, if any, deleterious effect on host-cell macromolecular metabolism. Oligo(nucleoside methylphosphonate)s, properly designed to be complementary to sequences that play key regulatory roles in virus replication, may prove effective in antiviral chemotherapy.

We thank Rita Fishelevich and Sharon Spitz for technical assistance and Irene Sluski for help with the manuscript. This work was supported by Grants GM31927 and CA39691 from the National Institutes of Health and by a contract from the Albert Szent-Gyorgi Foundation, Bethesda, MD.