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Mechanism of the Protective Effect of Heteropolyoxotungstate against Herpes Simplex Virus Type 2

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Key Words
Anti-herpes simplex virus activity · Herpes simplex attachment · Polymerase chain reaction · Penetration · Polyoxotungstate · Second-round infection

Abstract
The effects of heteropolyoxotungstate ($K_7[PTi_2W_{10}O_{40}]\cdot 6H_2O$; PM-19) on the replication of herpes simplex virus type 2 (HSV-2) were examined using a semiquantitative polymerase chain reaction of intracellular viral DNA established by us and also other methods. Vero cells were infected with HSV-2 strains: either the standard strain 169, or the acyclovir-resistant strain YS-4C-1. PM-19 was added at various stages during the replication of HSV-2. PM-19 strongly inhibited the synthesis of viral genomic DNA when it was added at the time of infection. The addition of PM-19 60–90 min after viral inoculation time-dependently decreased the antiviral activity and increased the relative yield of viral DNA, and the addition of PM-19 was completely ineffective at times later than 90 min. These results suggested that PM-19 inhibited viral penetration but did not affect the synthesis of viral DNA. Furthermore, PM-19 strongly inhibited a second round of infection.

Introduction

Some polyoxometalates (PMs) are potent inhibitors of the replication of DNA and RNA viruses and of tumor growth [1–14]. Keggin-type PMs are effective against herpes simplex virus (HSV). Among these compounds, the keggin heteropolyoxotungstate ($K_7[PTi_2W_{10}O_{40}]\cdot 6H_2O$; PM-19) has been proven to be the most potent inhibitor of the replication of HSV at a non-cytotoxic concentration [13]. The toxicity of PM-19 on host cells was found to be minimal; at 200 μg/ml PM-19 did not affect the growth of Vero cells. The 50% plaque-inhibiting concentrations (EC_{50}) for HSV-2 strains: both the standard strain, 169, and thymidine kinase defective strain, YS-4C-1, were 4.2 μg/ml each. And the presence of PM-19 (over 10 μg/ml) completely inhibited the production of plaques. We have shown that PM-19 inhibits replication at an early stage, such as viral adsorption by or viral penetration of cells, and that it also decreases virus-specific incorporation of ^{3}H-thymidine by cells [4].

While studying the mechanisms of action of antiviral agents, we found that it is difficult to clarify subtle differences in antiviral activity in the early phase of viral replication. Therefore, we established a relative quantitation method using polymerase chain reaction to follow the

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dynamic replication of a small number of virions among the total DNA extracted from infected cells [15]. By using this method, we could evaluate the relative yield during the course of viral DNA synthesis and the effect of an antiviral drug, acyclovir, on the yield.

In the present study, we examined the mechanism of the anti-herpetic action of PM-19 using our semiquantitative PCR method and other methods. We compared the effects of PM-19 with those of a specific inhibitor of herpes DNA replication, acyclovir, and show that PM-19 inhibits viral penetration and a second round of infection of cells, but does not affect the synthesis of viral genomic DNA.

Materials and Methods

Antiviral Compounds

PM-19 was synthesized as described previously [13]. Acyclovir was provided by the Wellcome Foundation Ltd. (UK). These compounds were dissolved in ultrapure water and stored at –80°C until use. Each stock solution was diluted with the experimental medium to be studied.

Cells and Viruses

Vero cells were used throughout the experiments. The cells were cultivated in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co. Inc., Tokyo, Japan) supplemented with 5% fetal bovine serum and nonessential amino acids for MEM, Eagle (Flow Laboratories, N.Y., USA). HSV strains were as follows: KOS as a standard strain of type 1, and 169 as a standard strain of type 2. We also used a variant of HSV-2, YS-4C-1, which was resistant to acyclovir and expressed very little thymidine kinase activity [17, 18]. This variant was kindly given to us by Dr. R. Mori. Viruses were diluted in Hanks' balanced salts solution supplemented with 1% bovine serum albumin (1% BSA-HBSS).

Preparation of Purified Radiolabeled Virus

HSV-2 virions were purified by centrifugation through dextran gradients as previously described [19]. Briefly, confluent Vero cells were infected with HSV-2 (strain 169 or YS-4C-1) at a multiplicity of infection (MOI) of 3. Five hours after infection, medium containing [^{3}H]leucine at 0.74 Mbq/ml (Amersham Corp.) was added. After incubation at 34°C for 48 h, the infected cells were harvested and lysed, and virus was purified by centrifugation through a dextran gradient (dextran T-10; Pharmacia, Inc.). Aliquots of radiolabeled virus were stored at –80°C until use.

Chemicals and Enzymes

AmpliTaq polymerase, Takara Ex Taq, for PCR and TRIzol® reagent for cellular DNA extraction were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan) and Gibco BRL (N.Y., USA), respectively. Other chemicals were purchased from Iwai Kagakuyakuhin Co. Ltd. (Tokyo, Japan).

Oligonucleotide Primers

Two 22-base oligonucleotides, 5′-CAT CAC CGA CCC GGA GAG GGA C (its position 3140–3161 in HSV-1 and 3309–3330 in HSV-2) and 5′-GGG CCA GGC GCT TGT TGG TGT A (3710–3731 in HSV-1 and 3400–3379 in HSV-2) were designed based on the published sequence of the gene for DNA polymerase of HSV [20]. This set of primers allows amplification of a 92-base pair fragment of the HSV gene for DNA polymerase. These primers were synthesized by Sawady Technology Co. Ltd. (Tokyo, Japan).

Viral Infection

Vero cells were infected with HSV at a MOI of 1. After the adsorption of virus for 60 min at 4°C, Vero cells were washed with phosphate-buffered saline (PBS) to remove unattached virus particles and then were incubated in maintenance medium at 37°C.

Determination of Relative Levels of Cellular Viral DNA by PCR

Total cellular DNA was extracted with TRIzol® Reagent from infected Vero cells at various time points (5, 15 and 19 h) after infection. Concentrations of DNA were calculated from the absorbance at 260 nm, and then the samples of DNA were used directly for amplification by PCR. The PCR was performed in 200 µl of a reaction mixture which contained 25 µl of a template solution, 20 mmol/l Tris-HCl (pH 8.0), 100 mmol/l KCl, 0.1 mmol/l EDTA, 1 mmol/l DTT, 0.5% Tween 20, 0.5% Nonidet P-40, 2 mmol/l $MgCl_2$, 200 mmol/l each of dATP, dCTP, dGTP and dTTP, 1 mmol/l each of sense and antisense primers, and 2.5 U of Taq DNA polymerase. The amplification mixture was overlaid with 20 µl of mineral oil. The reactions were performed for 10–45 cycles in a thermal cycler (Nippon Genetics Co. Ltd., Tokyo, Japan). Each cycle included the following steps: denaturation for 1 min at 94°C, primer annealing for 1 min at 52°C and extension/synthesis for 30 s at 72°C. Each set of amplifications was performed with one negative and one positive control. At the end of the extension step of every cycle, 8 µl of the reaction mixture was collected, and the PCR products were subjected to electrophoresis on 0.7% agarose gels that contained 1.15% synergel in 40 mmol/l Tris, 40 mmol/l acetic acid and 1 mmol/l EDTA (TAE) buffer. Amplified bands were stained with ethidium bromide. The intensity of the fluorescence of each band was determined with a CCD image analyzer and the analysis software ATTO Densito graph (Atto Corp., Tokyo, Japan). To normalize staining of the gel and imaging, a reference DNA solution was loaded in one lane per gel. The intensity of fluorescence of bands was plotted versus the number of cycles of PCR on semilogarithmic graph paper. The linear portion of the kinetic curve indicates the logarithmic phase of amplification before reaching a plateau. The initial amount of template was calculated from the exponential equation: $Y = a \times b^n$, where Y is the intensity of fluorescence, a is the initial amount of template, b is the efficiency of amplification in every cycle, and n is the number of cycles. These data were analyzed using the computer software CA-Cricket Graph III (Computer Associates International Inc., N.Y., USA).

Plaque Assay

Plaque assays were performed according to a method described previously [4]. Virus titers are expressed as plaque-forming units (PFU) and results are expressed as the number of plaques or as plaque inhibition (% of control).

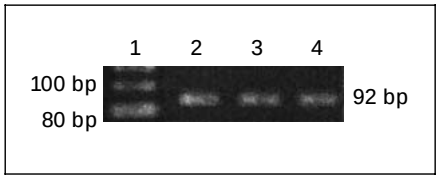

Fig. 1. Detection of HSV genome-specific products of PCR using a single set of primers. Vero cells were infected with each strain of HSV at a MOI of 1. Fifteen hours later, total cellular DNA was extracted from virus-infected cells and used as a template for PCR. As described in the text, HSV DNA-specific bands (92-bp bands) after PCR were examined using agarose gel electrophoresis and staining with ethidium bromide. Fluorescence of bands was measured with an image analyzer. Lane 1 = DNA marker; lane 2 = strain KOS of HSV-1; lane 3 = strain 169 of HSV-2, and lane 4 = strain YS-4C-1 of HSV-2. Lanes 2–4 show products after the 16th cycle of PCR.

Infectious Center Assay

HSV-2-infected Vero cells were propagated at 10 h after the infection. The infected cells were overlaid on normal Vero cell monolayers and then incubated at 37°C. PM-19 was added at various times. After 2 h of incubation, cultures were overlaid with maintenance medium containing 2% methylcellulose, and infectious centers were counted 22 h later after neutral red staining.

Results

Detection of Intracellular Viral Genomic DNA

To detect and quantitate the intracellular viral DNA in HSV-infected cells, we generated virus-specific products of PCR with an identical pair of primers. Vero cells were infected with each of three strains of HSV at a MOI of 1. Fifteen hours later, total DNA from the infected cells was extracted, and this extracted DNA fraction was used as a template for semiquantitative PCR. After PCR, a fluorescent band of virus-specific DNA was detected in all three strains of HSV-2 employed (fig. 1). These bands indicated the amplification of a 92-base pair (bp) fragment of the gene for DNA polymerase of HSV-1 (KOS strain) and HSV-2 (169 and YS-4C-1 strains).

Direct Effect of the Antiviral Agent PM-19 on the PCR

To examine the possible direct effects of PM-19 on the PCR, PM-19 was directly added to the reaction mixture for PCR at various concentrations (10, 30 and 50 μg/ml). PM-19 at concentrations above 30 μg/ml reduced the accumulation of PCR products (data not shown). However, the effects of residual PM-19 could be reduced by washing the treated cells with PBS prior to DNA extraction (data not shown).

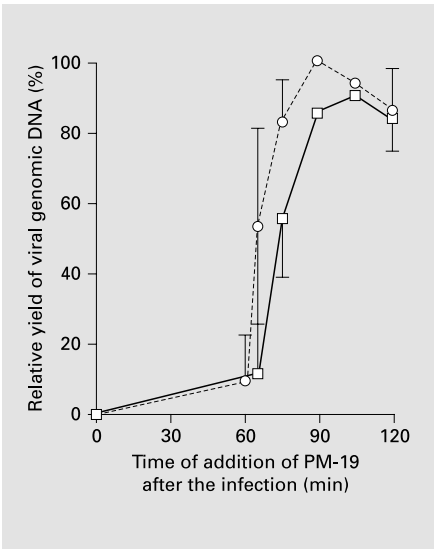

Fig. 2. Relationship between the relative yields of virus DNA and the time of addition of PM-19 during viral attachment and penetration. Vero cells were infected with strain 169 or YS-4C-1 of HSV-2 and treated with 10 μg/ml of PM-19 at various times, and 19 h later, total cellular DNA was prepared. The relative yield of HSV-2 DNA was measured using the quantitative PCR method. □ = Strain 169; ○ = strain YS-4 C-1.

Discrimination between the Effects of PM-19 on Viral Attachment or Penetration and on the Synthesis of Viral Genomic DNA

Vero cells were infected with the 169 or YS-4C-1 strain of HSV-2 at a MOI of 1. PM-19 was added at various times during the attachment to and penetration of the cells, and the cultures were then incubated for 19 h. Using the DNA fraction extracted from the HSV-2-infected cells as a template, the amount of intracellular viral DNA was estimated by quantitative PCR.

As shown in figure 2, synthesis of viral genomic DNA of both strains of HSV-2 was markedly inhibited in the presence of PM-19 added at 0–60 min during viral attachment or adsorption. Delaying the time of PM-19 addition until the phases involving virus penetration into cells or synthesis of intracellular viral DNA caused the antiviral activity to decrease gradually, while the relative yields of viral genomic DNA increased.

When PM-19 was added 90 min after viral inoculation which corresponded to 30 min after increasing the temperature (37°C), the relative yields of viral genomic DNA

Table 1. Time course of change in the relative yields of HSV-2 DNA after HSV-2 infection at an MOI of 1

Time, h	Relative yield of HSV-2 DNA
1	0.005
3	0.006
5	0.006
7	0.006
9	0.028
11	0.042
13	0.041
15	0.053
17	0.060
19	0.059
21	0.103
23	0.101

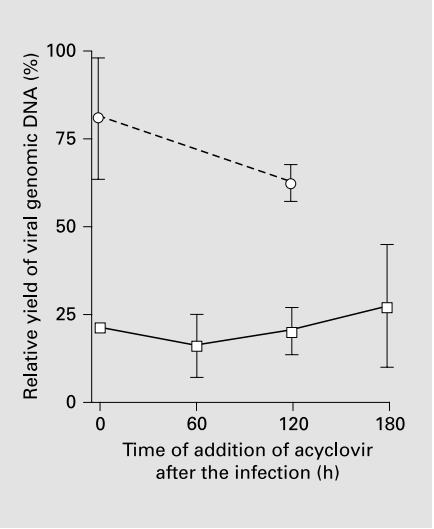

Fig. 3. Relationship between the relative yield of viral DNA and the timing of exposure to acyclovir after HSV-2 infection. Vero cells were infected with the 169 or YS-4C-1 strain of HSV-2 and treated with 2 μg/ml of acyclovir at various times. Nineteen hours later, total cellular DNA was prepared. The relative yield of HSV-2 DNA was measured using the quantitative PCR method. □ = Strain 169; ○ = strain YS-4C-1.

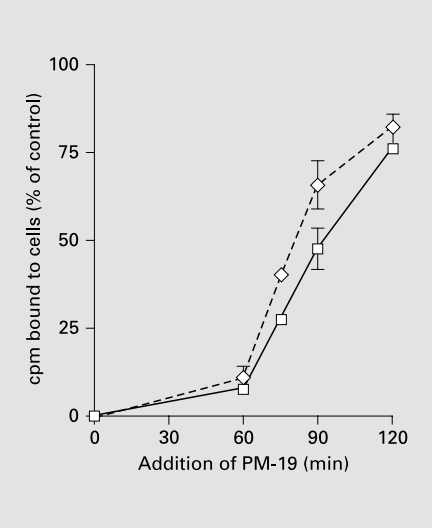

Fig. 4. Relationship between the radioactivity of intracellular radiolabeled virion and the time of addition of PM-19 during viral attachment and penetration. Vero cells were infected with [^{3}H]-labeled HSV-2 (strain 169 or YS-4C-1) and treated with 10 μg/ml of PM-19 at various times, then 3 h later, the radioactivity of intracellular virions was measured using a liquid scintillation counter. □ = Strain 169; ○ = strain YS-4C-1.

were almost completely recovered in both strains. In contrast, acyclovir added under the same conditions as PM-19 reduced the relative yields of viral genomic DNAs of strain 169 by 17–27% and strain YS-5C-1 by 60–80% of viral control, respectively (fig. 3). Results similar to those of figure 2 were obtained in experiments to determine the radioactivity of intracellular virions using [^{3}H]-labeled virions (fig. 4).

Effects of PM-19 prior to the Synthesis of Viral DNA

The time course of the relative yields of HSV-2 DNA after infection was determined. As shown in table 1, the cellular HSV-2 DNA increased in a manner indicating a 6-hour replication cycle followed by a time-lag of 7 h.

Vero cells were infected with HSV-2 strain 169 at a MOI of 1. PM-19 was added to the cells 60 min after viral inoculation and maintained in the culture for 5 h. Then, the total cellular DNA extracted was used as the template for PCR. The initial amount of template DNA was estimated from an exponential equation by reference to the linear portions of kinetic curves (fig. 5a). The relative yields of viral DNA in the presence of PM-19 at 10 and 100 μg/ml were about 80 and 60% of those in nontreated viral control, respectively (fig. 5b).

Effect of PM-19 on Cell-Attached HSV-2 Virions

To determine whether PM-19 could induce the detachment of cell-attached HSV-2 (169 strain) virions, PM-19 was added to Vero cell cultures after virus attachment at 4°C. Virus titers were determined using a plaque assay method. Virus titers of detached virions were at the same levels as in PM-19-treated and control cells. However, the time-dependent lowering of the titer was inhibited in the presence of PM-19 (fig. 6a). The presence of PM-19 (>10 μg/ml) caused detachment of a portion of virions from the cell surface (fig. 6b).

Effects of PM-19 on a Second Round of Infection

Using an infectious center assay, effects of PM-19 on a second round of infection were determined (fig. 7). About 300 infectious centers were plated on normal Vero cells. The presence of PM-19 completely inhibited the production of infectious centers. As shown in figure 8, intracellular virus yield was not influenced by the addition of

Fig. 5. Effect of PM-19 on the synthesis of HSV-2-specific DNA. After the adsorption of virus for 60 min at 4°C, the infected cells were washed with PBS and cultured in the presence of PM-19. Five hours after infection, total cellular DNA was extracted for use as a PCR template. Initial amounts of the template were determined by the quantitative PCR. a Kinetics of the accumulation of HSV-2 DNA-specific product of PCR. ○ = Virus-infected control; ◆ = treated with 10 μg/ml of PM-19; □ = treated with 100 μg/ml of PM-19. b Histogram showing the relative yields of HSV-2 DNA calculated from the exponential equations. Column 1 = Virus control at 5 h; column 2 = PM-19 at 10 μg/ml; column 3 = PM-19 at 100 μg/ml.

Fig. 6. Effect of PM-19 on cell-attached HSV-2 virions. Vero cells were infected with the 169 strain of HSV-2 at a MOI of 1. After the attachment of virus for 60 min at 4°C, Vero cells were washed with PBS to remove unattached virus particles and supplemented with PM-19 (10 μg/ml). The cultures were kept at 4°C. Cell-attached and detached virus titers were determined using a plaque assay and expressed as plaque-forming units (PFU). a Time course of virus titers. □ = Cell-attached virus titer; ○ = detached virus titer; —— = virus control; ····· = PM-19 at 10 μg/ml. b Relation between concentration of PM-19 and virus titers. Results were indicated as percent of control. □ = Cell-attached virus titer; ○ = detached virus titer.

Fig. 7. Effect of PM-19 on a second round of infection. HSV-2 (169 strain)-infected Vero cells were propagated 10 h after the infection. The infected Vero cells were overlaid on normal Vero cell monolayers and then incubated at 37°C. PM-19 (10 μg/ml) was added to normal Vero cells. Two hours after incubation, maintenance medium containing 2% methylcellulose was overlaid, and infectious centers were counted 22 h later after neutral red staining.

Fig. 8. Time courses of increases in the virus yield after HSV-2 infection. Vero cells were infected with HSV-2 (strain 169) at a MOI of 1. PM-19 was added to the cell cultures 2 h after virus infection. Intra- and extracellular virus yields were determined every 2 h by plaque assay and expressed as plaque forming units (PFU). —— = Virus control; ····· = PM-19 at 10 μg/ml; □ = titer of intracellular virus; ○ = titer of extracellular virus.

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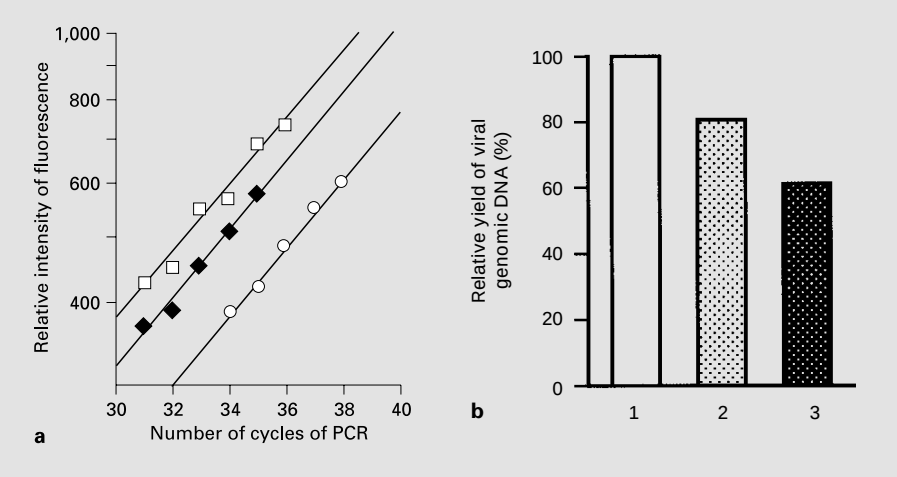

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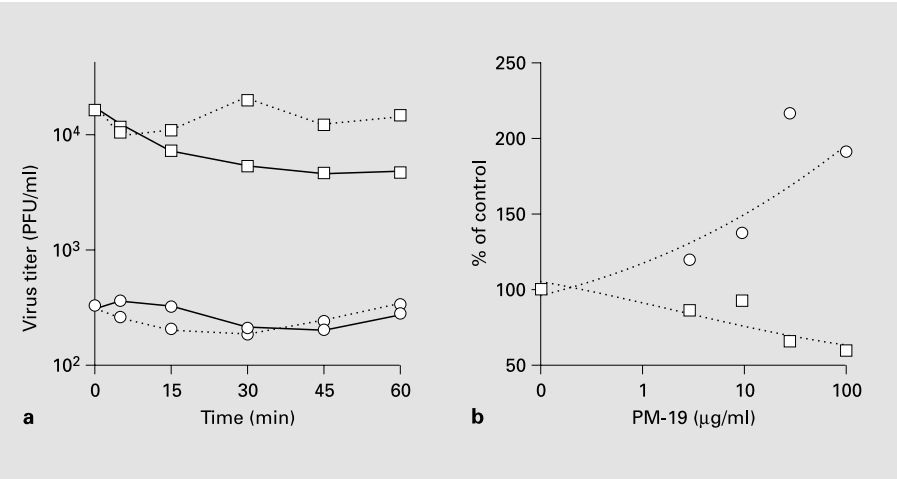

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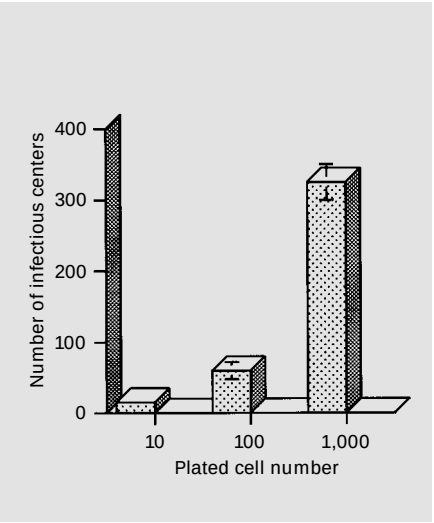

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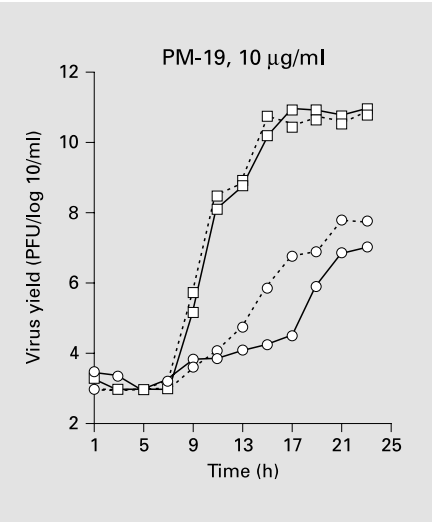

PM-19 after viral penetration (2 h after the infection). On the other hand, extracellular virus yield was higher than virus control from 13 h after the infection.

Discussion

The mechanisms of action of a novel antiherpetic agent, PM-19, were determined using a semiquantitative PCR method for HSV DNA and other methods.

In a previous study, we established a semiquantitative PCR method for intracellular HSV-2 DNA based on a kinetic analysis of the accumulation of PCR products [15]. We demonstrated that this method allowed an estimation of the activity of a specific antiherpetic drug, ACV, against viral DNA synthesis.

In the present study, we examined whether it was possible to distinguish between the antiviral effects of PM-19 on virus attachment or penetration and the synthesis of viral genomic DNA using our semiquantitative method. In cells infected with HSV-1 (strain KOS) and HSV-2 (strains 169 and YS-4C-1), the intracellular viral genomic DNA could be detected by PCR with an identical set of primers (fig. 1). Addition of PM-19 at concentrations above 30 μg/ml to the reaction mixture for PCR reduced the accumulation of PCR products (data not shown). It has been reported that other PM-related compounds are inhibitors of various DNA polymerases [21, 22]. If PM-19 can enter the cell, it will act as an inhibitor of DNA polymerase. However, the contamination by PM-19 of the reaction mixture could be reduced to an ineffective level by washing cells with PBS before DNA extraction (data not shown). This shows that the direct influence of PM-19 on this assay system can be eliminated.

PM-19 markedly inhibited the synthesis of viral genomic DNA of HSV-2 (strains 169 and YS-5C-1) when it was added to infected cells during the phase of viral attachment and penetration: 0–60 min after viral inoculation. Delaying the time of addition of PM-19, however, caused the inhibitory activity to gradually decrease, and it was completely lost by 90 min after viral inoculation, as shown by the fact that the relative yield of viral genomic DNA was recovered (fig. 2). Similar results were obtained in experiments to measure the radioactivity of intracellular virions using [^{3}H]-labeled virions (fig. 4). In contrast, acyclovir, a specific inhibitor of HSV DNA, inhibited DNA synthesis under the same treatment conditions as PM-19 (fig. 3). From these results, it is obvious that PM-19 primarily acts on the viral penetration of cells under conditions of synchronized viral replication.

In a previous study, we showed that PM-19 significantly and concentration-dependently inhibited the incorporation of ^{3}H-thymidine into viral DNA 5 h after infection when added at >5 μg/ml after HSV-1 adsorption and that PM-19 at <25 μg/ml did not interfere with virus adsorption by human embryo lung cells [4]. In the present study, the relative yield of viral genomic DNA 5 h after the infection was not yet increased (table 1). Five hours after the infection, the relative yield of viral genomic DNA after treatment of cells with PM-19 decreased concentration-dependently (fig. 5). Therefore, the antiviral activity of PM-19 on HSV appears to be mainly due to the inhibition of viral penetration, rather than to an effect on the synthesis of viral genomic DNA. Recently, several cellular molecules such as HveA, HveB (nectin-2), HveC (nectin-1), and 3-O-sulfated heparan sulfate were reported to act as cofactors at the step of viral entry [23–27]. PM-19 may have some association with these cofactors, but not the activity of direct inactivation against the HSV virions. Furthermore, PM-19 have an influence on HSV glycoprotein D-mediated virus entry into the cells (in determination).

As shown in figure 6, PM-19 at concentrations of >10 μg/ml could remove a portion of cell-attached virions and the time-dependent decrease in virus titer of the cell-attached virions was inhibited in the presence of PM-19. This suggested that PM-19 caused the retention of cell-attached virions at the cell surface.

Using the infectious center assay, it was possible to estimate the effect of PM-19 on the second round of infection (fig. 7). PM-19 completely inhibited the second round of infection.

As shown in figure 8, extracellular virus yield from PM-19-treated cells was higher than that from the virus control at times later than 13 h after the infection, indicating that the presence of PM-19 after viral penetration caused the retention of the released virions at the extracellular surface.

This evidence suggests that PM-19 may be useful for local applications, since we have shown the therapeutic effect of PM-19 on herpetic exanthem in vivo.

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