นิพนธ์ต้นฉบับ

In vitro effects of acyclovir on herpes simplex type-2 antigens by Western blot assay.

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Panpunnung T, Tiensiwakul P, Pannarugsa V, Desudchit P. In vitro effects of acyclovir on herpes simplex type-2 antigens by Western blot assay. Chula Med J 1990 July; 34(7) : 523-530

This study is to determine the in vitro inhibitory effects of acyclovir on the antigenic polypeptide synthesis of HeLa cell-cultures infected with herpes simplex virus type 2 by Western blot assay. Results obtained showed that at 6h post infection acyclovir in the forms of either pure chemical or intravenous could inhibit overall synthesis of viral polypeptides especially 5 major glycoproteins : gB, gG, gC, gE, gD, and a number of low molecular weight polypeptides. The initial effect was found at the concentration of 0.5 ug/mL of acyclovir. When the concentrations increased up to 2.0-4.0 ug/mL, the synthesis was almost completely diminished. Effect of acyclovir on 12 h-post infected cultures in which structural proteins of the virus reaching peak of synthesis was similar to that of at 6h post infection. Our observation may be used to explain the finding that reduction of antibody response to the viral polypeptides in the serum of primary genital herpes infection may be due to the reduction of viral antigens during acyclovir-therapy. This finding also supports our previous findings in the reduction of viral multiplication and DNA synthesis in the HSV-2 infected cultures treated with acyclovir.

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Received for publication. May 25, 1990.

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ฐิติมา ปาณปุณณัง, พรเทพ เทียนสิวากุล, วรรณา พรรณรักษา, ไพรัช ดีสุดจิต. ผลของอะซัยคลอเวียร์ต่อการสร้างแอนติเจนของเชื้อเฮอร์บีสซิมเพล็กไวรัส ชนิด 2 ในหลอดทดลอง. จุฬาลงกรณ์เวชสาร 2533 กรกฎาคม ; 34 (7) : 523-530

การวิจัยนี้เป็นการศึกษาผลของยาอะซัยคลอเวียร์ต่อการสร้างแอนติเจนในเซลล์เพาะเลี้ยงติดเชื้อเฮอร์บีสซิมเพล็กไวรัสชนิด 2 โดยวิธีเวสเทอร์นบล้อท ผลการทดลองพบว่ายาในรูปสารเคมีบริสุทธิ์และยาฉีดสามารถยับยั้งการสร้างโปรตีนของเชื้อไวรัส ภายหลังการติดเชื้อ 6 ชม. ยามีผลในการยับยั้งการสร้างกลัยโคโปรตีน หลัก 5 ชนิด ได้แก่ จีบี, จีจี, จีซี, จีอี, จีดี, และโปรตีนที่มีน้ำหนักโมเลกุลต่ำอีกจำนวนหนึ่ง ขนาดความเข้มข้นของยาที่มีผลยับยั้งการสร้างโปรตีนของไวรัส เริ่มมีผลที่ความเข้มข้น 0.5 ug/mL และเมื่อความเข้มข้นของยาเพิ่มสูงขึ้นถึง 2.0-4.0 ug/mL มีผลทำให้เกือบไม่มีการสร้างโปรตีน เมื่อใช้ยาภายหลังการติดเชื้อนาน 6 ชม. ซึ่งคล้ายกับผลจากการติดเชื้อนาน 12 ชม. ผลการศึกษาในครั้งนี้สามารถใช้อธิบายข้อสมมุติฐานที่พบการลดลงของการสร้างภูมิคุ้มกันต่อเชื้อไวรัสในผู้ป่วยด้วยการติดเชื้อเฮอร์บีสชนิดปฐมภูมิที่อวัยวะเพศภายหลังจากการรักษาด้วยอะซัยคลอเวียร์ คงจะเกิดจากการลดต่ำลงของแอนติเจนของเชื้อเฮอร์บิสไวรัสจากผลของยาที่ใช้ นอกจากนี้ยังใช้อธิบายถึงการที่เราพบว่ายานี้ทำให้จำนวนไวรัสและการสังเคราะห์ดีเอนเอลดลง

Acyclovir (9-(2-hydroxyethoxymethy1) guanine), a nucleoside analog, is probably the most potent and well established antiviral agent for herpes simplex virus. In primary herpes simplex virus infection, the drug reduces the duration of viral shedding, total pain, itching and lesion healing time, but it does not significantly reduce the rate of subsequent recurrences.[1-3] In asymptomatic recurrent genital herpes, however, oral acyclovir treatment does not reduce the viral shedding, and this is not due to the resistance of the isolated strains to the drug.[4] Acyclovir treatment reduces overall humoral antibody responding to HSV-2 polypeptides, including the antibodies to major glycoproteins such as gA, gB, gE and gD of the virus as compared to the placebo controls.[5]

Antiviral mechanisms of acyclovir are shown to be competitive phosphorylation of herpes virus-thymidine kinase,[6,7] a potent inhibitor of HSV-DNA polymerase,[8,9] and a chain terminator of de novo viral DNA synthesis.[10] Outcome of the effects may reduce overall viral polypeptide synthesis. This may be speculated that the reduction of HSV-2 antibody response in the acyclovir treated group is probably due to the reduction of viral antigens affected by the drug.

This report is to investigate the effect of acyclovir for the in vitro antigen synthesis of herpes simplex virus type 2 using Western blot assay. This assay may reveal which components of the viral antigens are affected by the drug. Since each component of the HSV-glycoproteins has its own function, thus the reduction of the particular viral glycoprotein(s) may give additional information on the antiviral mechanisms of acyclovir.

Materials and methods

Cell culture HeLa cell culture, a continuous cell line established from human cervical carcinoma, was obtained from the Department of Medical Sciences, Ministry of Public Health, Thailand. They were grown in a minimal essential medium (MEM, GIBCO) supplemented with 10% fetal calf serum (FCS, Flow Laboratories).

Virus Herpes simplex virus type 2 (HSV-2) strain LB, originally obtained from the University of Illinois at the Health Sciences Center,Chicago, Illinois, U.S.A, was used throughout this experiment.

Chemicals Intravenous acyclovir (Zovirax, Wellcome, lot A 3362-A) and pure chemical acyclovir (Wellcome Reference Substance, batch QA. 0667) were provided by the Wellcome Foundation Ltd, Kent, England.

Treatment of HSV-2 infected HeLa cells with acyclovir HeLa cells, grown in 10% FCS-MEM until reached 80% confluent, were infected with HSV-2 at the multiplicity of infection of 10. After allowing 6 and 12 h for the virus to be absorbed on to the cells, the infected monolayer was rinsed off and either intravenous-or pure chemical acyclovir was added on to the infected monolayer at the concentration ranging from 0.2-4.0 ug/mL. The cultures were incubated at 37°C for 24 h, after which, they were trypsinized with a trypsin-versin solution and washed twice with a phosphate-buffered saline (PBS). The infected cells were packed by centrifugation at 2,500 rpm for 10 min. Finally, the infected cell-pellet was suspended in deionized distilled water, frozen and thawed three times, sonicated in Sonic prep (model 150, MSE,UK) for 5 min. The supernate was determined for protein content by Lowry's method.[11]

Western blot assay The extract of HSV-2 infected HeLa cells after acyclovir treatment was electrophoresed on a discontinuous sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) formed on a 18 × 20 × 0.1 cm vertical slab gel with 3% stacking and 8% resolving gel by the method described by Laemmli.[12]

The electrophoresis was carried out with constant current of 15 mA in stacking gel, followed by 30 mA in resolving gel. Following the SDS-PAGE, the discreted proteins in the gel were electrophoretically transferred onto a nitrocellulose membrane by a modification of the method described by Towbin et al. with the use of Trans-Blot Cell apparatus (Bio-Rad Laboratories).[13] The Western blot assay was carried out at 250 mA for 3h and followed by 50 mA for 18h. After which the nitrocellulose was cut into strips and soaked in 5% non-fat dry milk (Carnation) at 37°C for 3h. An immunostaining for HSV-2 antigens was performed by an incubation with rabbit anti HSV-2 (Dakopatts) diluted 1:20 at 37°C for 3h and overnight at 4°C. Then it was incubated at 37°C for 30 min with 1:50 dilution of peroxidase conjugated swine anti-rabbit globulin (Dakopatts), and finally the color was developed at room-temperature incubation for 30 min in dark with a mixture of 6 mg of 4-chloro-1-naphthol in 2 ml of methanol mixed with 10 ml of TRIS and 5 uL of 30% hydrogen peroxide. The HSV-2 antigens on the nitrocellulose were estimated for molecular weights by calculation from standard molecular weight-markers (Sigma) transferred on to the same nitrocellulose staining with India ink.[14]

Results

Polypeptides of herpesvirus can be classified into 3 groups; alpha-proteins, betaproteins, and gamma-proteins. Their synthesis reach peak-rate at 2-4h, 5-6h, and 12h after infection, respectively. Since the synthesis of individual protein is regulated and sequentially ordered

in a cascade fashion. Therefore, the effect of acyclovir on the synthesis of the viral polypeptides was determined by adding acyclovir in the pure chemical and intravenous forms at 6h and 12h post infection

1. Inhibitory effects of pure chemical acyclovir on HSV-2 Polypeptide synthesis at 6h post infection. The effect of pure chemical acyclovir on the viral polypeptide synthesis was carried out by adding the pure chemical at concentrations of 0.2,0.5, 1.0, 2.0, 3.0 and 4.0 ug/mL into the 6h post infected cultures. As shown in Figure 1, the immunoblot-pattern of HSV-2 infected cultures revealed 5 major viral glycoproteins; gB (109-128 Kd), gG (98 Kd), gC (88 Kd), gE (75 Kd), gD (60 Kd) including p40 (40 Kd) and a number of low molecular-weight polypeptides (LMW) (LaneA). The pure chemical acyclovir at concentration of 0.2 ug/mL (LaneB) did not visibly reduce the intensity of the bands of 5 major viral glycoproteins even though some inhibitory magnitude was shown on the LMW-polypeptides such as 22 Kd- and 24 Kd proteins. The intensity of the 5 major glycoproteins, however, was reduced at the concentrations of 0.5 to 2.0 ug/mL of acyclovir (Lane C, D, and E). The bands almost diminished when the infected cultures were treated at the concentration of 3.0 ug/mL (Lane F) and 4.0 ug/mL (Lane G). Control culture of mock infected cell (Lane H) showed no visible band.

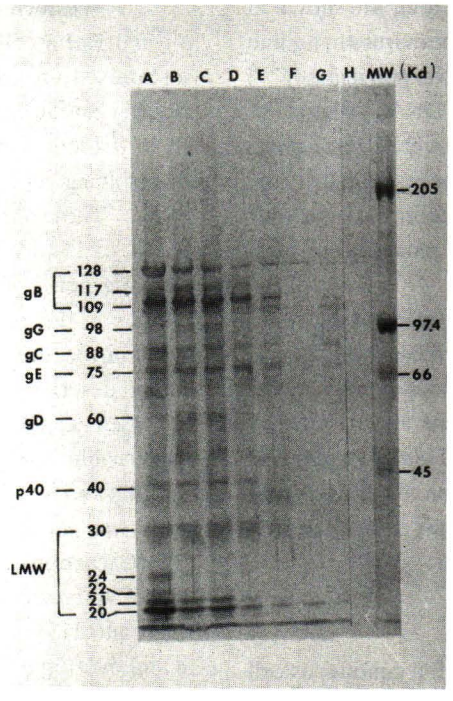

Figure 1. Inhibitory effect of pure chemical acyclovir on HSV-2 polypeptide synthesis in HeLa cells infected with herpes simplex virus at 6h post infection. Six hours post infection of HSV-2 infected cultures were treated with pure chemical acyclovir at concentrations of 0 (lane A), 0.2 (lane B), 0.5 (lane C), 1.0 (lane D), 2.0 (lane E), 3.0 (lane F), and 4.0 (lane G) ug/mL, respectively. Lane H was mock infected culture.

2. Inhibitory effect of intravenous acyclovir on HSV-2 polypeptide synthesis at 6h post infection. Immunoblotting of HSV-2 polypeptides in the cultures of infected HeLa cells treated with intravenous acyclovir at 6h post infection was shown in Figure 2. Lane A showed the pattern of HSV-2 polypeptides without acylovir added in which the 5 major HSV-2 glycoprotein antigens and a number of LMW polypeptides were seen. Lane B to G were HSV-2 polypeptides from the cultures treated with acyclovir at concentrations of 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 ug/mL, respectively. The intensity 5 major HSV-2 glycoprotein antigens was proportionally reduced as the concentration of acyclovir added into the cultures was increased. The degree of inhibition was seen at the concentration of 1.0 ug/mL of acyclovir (Lane D), and almost no visible bands can be seen at the concentration of 3.0 and 4.0 ug/mL of acyclovir (Lane F and G respectively).

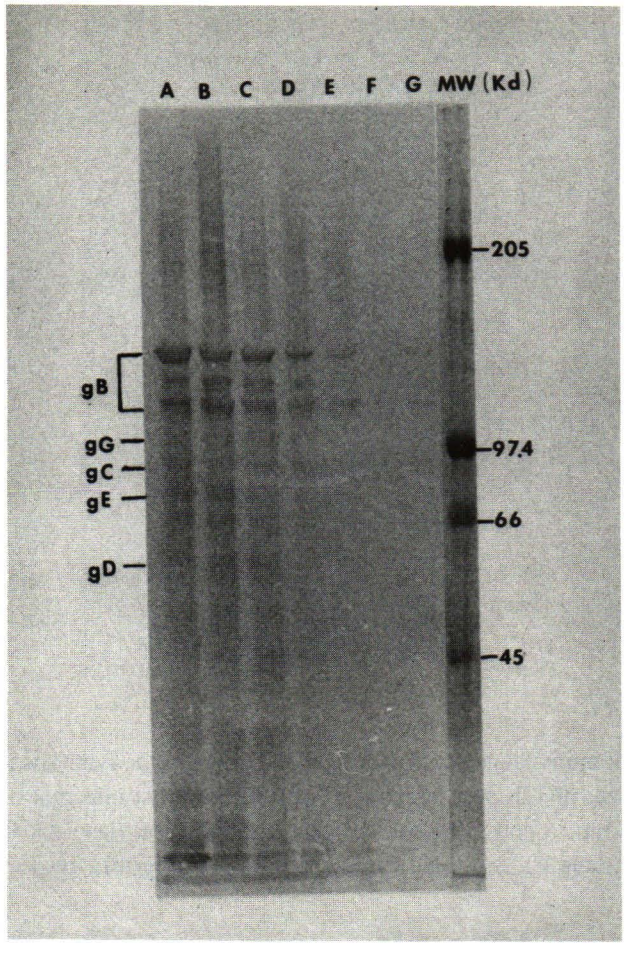

Figure 2. Inhibitory effect of intravanous acyclovir on HSV-2 polypeptide synthesis in HeLa cells infected with herpes simplex virus at 6h post infection. Six hours post infection of HSV-2 infected cultures were treated with intravenous acyclovir at concentrations of 0 (lane A), 0.2 (lane B), 0.5 (lane C), 1.0 (lane D), 2.0 (lane E), 3.0 (lane F), and 4.0 (lane G) ug/mL, respectively.

3. Inhibitory effect of intravenous acyclovir on HSV-2 polypeptide synthesis at 12h post infection. Immunoblotting of HSV-2 polypeptides in the cultures of HSV-2 infected HeLa cells treated with acyclovir at 12h post infection was shown in Figure 3. Lane A was a pattern of HSV-2 polypeptides derived from 12h post infected cultures. Lane B to G were the infected cultures with acyclovir at concentrations of 0.2 to 4.0 ug/mL. In Lane B, concentration of acyclovir at 0.2 ug/mL did not effectively reduce the synthesis of the viral polypeptides as shown by the intensity of the bands being similar to that untreated controls (Lane A). The intensity of the bands, however, was visibly reduced as the concentration of acyclovir increased to 0.5 ug/mL (Lane C). The effectiveness was increased when the concentration of acyclovir was increased to 1.0 ug/mL (Lane D). While major structural protein synthesis almost diminished when the concentrations of the drug were increased upto 2.0 to 4.0 ug/mL (Lane E,F,G). Lane H was mock infected culture.

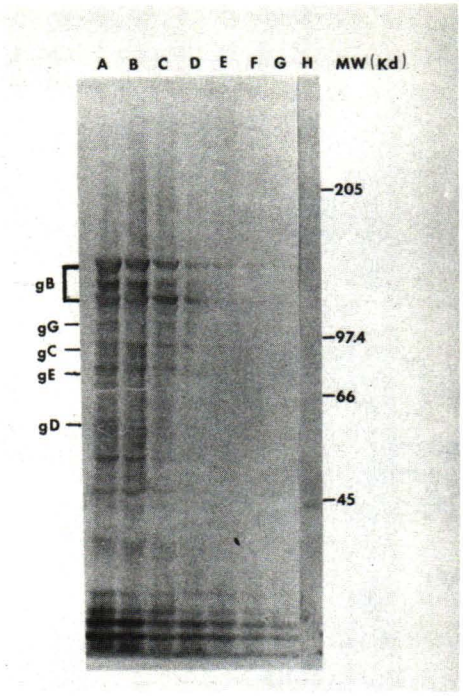

Figure 3. Inhibitory effect of intravanous acyclovir on HSV-2 polypeptide synthesis in HeLa cells infected with herpes simplex virus at 12h post infection. Twelve hours post infection of HSV-2 infected cultures were treated with intravenous acyclovir at concentrations of 0 (lane A), 0.2 (lane B), 0.5 (lane C), 1.0 (lane D), 2.0 (lane E), 3.0 (lane F), and 4.0 (lane G) ug/mL, respectively. Lane H was mock infected culture.

Discussion

In preliminary experiments, we found that acyclovir had inhibitory effects on the yield of the virus by plaque reduction assay and on the DNA synthesis by ^{3}H-thymidine incorporation technic. In this study, we extended our study to determine its effect on the viral protein synthesis with the use of Western blot assay.

Western blot assay, in which antigens separated by SDS-PAGE are electrophoretically transferred onto nitrocellulose sheet, has become widely used for the detection of viral antigens or antibodies. By the analysis of Western blot-pattern, we can pin-point to a single component or components of the viral antigens affected by acyclovir. We may, therefore, reveal some additional information regarding the inhibitory mechanisms of acyclovir. In addition, the affected antigen (s) probably reflects the formation of the corresponding antibody. Thus, we can predict for the disappearance of the corresponding antibody after the administration of acyclovir and may use it as a marker for the indications of the success or the failure of the treatment.

Results obtained from the 6h post infection of HSV-2 infected cultures treated with either pure chemical- or intravenous acyclovir (Zivirax) showed that the initial inhibitory effect of the drug appeared to be at the concentration of 1.0 ug/mL. The effect was pronounced at 2.0 ug/mL. The polypeptide synthesis was almost completely inhibited at the concentrations of 3.0 to 4.0 ug/mL. It has been known that acyclovir inhibits herpes-thymidine kinase (B_2 protein) which reaches peak-rate of synthesis about 5 to 7 hours after infection.[15] The enzyme, presumably, converted acyclovir to acyclovir monophosphate, and the latter was converted to acyclovir-diphosphate, and triphosphate by cellular thymidine kinase. This effect could inhibit the viral DNA chain elongation by DNA polymerase. This is consistent with our finding for the reduction of HSV-2 DNA synthesis and the viral multiplication (data not shown).

At 12h post infection in which gamma proteins of herpes virus reach the peak of synthesis, the acyclovir had similar inhibitory effect to that observed in 6h post infection (Figure 3). Acyclovir reduced overall synthesis of HSV-2 polypeptides including gB, gG, gC, gE, gD and LMW polypeptides.

These findings may be used to explain the reduction of antibody response to individual HSV-2 polypeptides in the acute phase serum of primary genital herpes infection with acyclovir is most probably due to

the reduction in viral antigen-exposure. Application of SDS-PAGE and Western blot technic may be useful for further study of the infection by herpes simplex virus or other viruses and bacteria. At present, this method is a useful tool for the study of antibody-response to HSV-infection.[15-20] In addition HSV-glycoproteins, especially gB and gD has received a great deal of attention as the antigens for potential vaccine in the prevention of initial and recurrent herpes infection. Thus our findings may be useful for the further study of herpes infection.

In couclusion, this study showed that acyclovir at the concentration ranging from 0.5 - 40 ug/mL has in vitro inhibitory effect for the overall synthesis of HSV-2 polypeptides, especially the 5 major structural glycoproteins and LMW polypeptides.

Acknowledgement

This work was supported by Rachadapisek-sompoj Research Fund (China Medical Board) Chula-longkorn University.

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