CHAPTER V

DISCUSSION

A number of in vitro studies of antiviral effects have shown that acyclovir is the most potent inhibitor of herpes simplex virus and it has a primary site of action on the inhibitory of viral DNA-synthesis (10, 11, 12, 21, 31). Since acyclovir has inhibitory effect on HSV-DNA synthesis thus it must have inhibitory effect on the HSV-protein synthesis. However, there is as yet no information available regarding to the effect of acyclovir on the protein synthesis of HSV-2. Therefore, in this study we have sequentially shown that acyclovir had inhibitory effects on the yield of the multiplication of the virus by plaque reduction assay, on the HSV-2 DNA synthesis by ^{3}H-thymidine incorporation technic, and on the HSV-2 protein synthesis revealed by Western blot technic.

The sensitivity of herpes viruses in vitro to acyclovir is dependent on the strain of the virus, the type of the host cell used, and tissue culture conditions (9, 21, 22). There were reports that herpes simplex viruses are 50% inhibited by acyclovir at the concentration of 0.12 ug/mL in HeLa cell (9, 12, 13). Therefore, in our experiment we have set the minimal

concentration of acyclovir at 0.05 ug/mL so that we might be able to observe the inhibitory reaction at this concentration. The oral administration of acyclovir in normal volunteers with a schedule of 200 mg/mL dose every four hours for two days showed a maximum and a minimum peaks of acyclovir concentrations at 0.7 and 0.4 ug/mL, respectively(13). Hence, the maximum concentration of 4.0 ug/mL of acyclovir used in our experiment is much higher than that observed in the plasma concentration. Therefore, the minimum and the maximum concentrations of acyclovir used in this experiment can cover all the range of the previous observation for the sensitivity of acyclovir and maximum plasma concentration of acyclovir.

In cell culture, the inhibition of HSV-2 multiplication by acyclovir appears to be complex depending on many factors including the multiplicity of infection. Therefore, in our experiment, the MOI was set at 2.5, 5, 10, and 25. The result of inhibitory effect of acyclovir on HSV-2 multiplication by plaque reduction assay (Table 1) showed that the yield of virus was completely inhibited at the concentration of 0.1 ug/mL of acyclovir for every MOI tested. Where as the doses of acyclovir for 50 percent inhibition(ID 50) of HSV-2 in HeLa cells were in the range of 0.36-0.74 uM (Mean 0.55 uM = 0.12 ug/mL) (9, 12). This different may partly be due to the different strains of HSV-2 used. Collins showed that the more strains of the viruses were examined, the evidence of variation in

susceptibility was presented (40). In our study, the infection with MOI of 10 was chosen for the rest of the study because it was the suitable MOI for reproducable infection of HSV-2 and many investigators also used this MOI for infection of HSV-2 (6,17,40,43,47,48,52,63,67). However, at MOI 2.5 and 5 in HSV-2 infected cell treated with concentration of 0.05 ug/mL of acyclovir showed the yield of the virus at $2x10^3$ and $1x10^3$ PFU/mL, respectively. Whereas at MOI 10 and 25 in HSV-2 infected cell treated with the same concentration of acyclovir there were no virus detected. This is probably occured by an experimental error of the plaque reduction assay because the virus yield at $2x10^3$ and $1x10^3$ PFU/mL were calculated from the observation of 2 and 1 plaques in the original multi-dishes tissue culture plate. Inhibition of viral multiplication shown by plaque reduction assay in this experiment could be explained in 2 mechanisms; first is the reduction of the DNA synthesis of newly viral progenies, secondly may be due to the reduction in the synthesis of glycoproteins which play major role in the infection of newly produced viral progenies.

One of mechanisn of acyclovir is to inhibit DNA synthesis resulted from the inhibition of DNA polymerase effects on primer template function (38). Thus, we designed a protocol to study the effect of acyclovir at 1.5 h, 6h, and 12 h post infection in order to study the effect of acyclovir at an early stage of infection, time

of DNA synthesis, and time of structural protein synthesis, respectively. The inhibitory effect of acyclovir on HSV-2 multiplication at different time courses of infection (Table 2) showed that while the time of infection prior to adding of acyclovir was increased, the sensitivity to acyclovir was decreased. At 12 h after infection,acyclovir at concentration of 4.0 ug/mL could not completely inhibited the virus whereas at 1.5 h and 6.0 h after infection, acyclovir at concentrations of 0.05 ug/mL and 1.0 ug/mL could completely inhibited the virus. The explanation could be that at 12 h after infection all DNA and structural proteins were synthesized for one cycle, therefore, the drug could not alter the assembly of components has already synthesized. The effect, however, may act on the second cycle. Similar results were shown in the treatment patients with acyclovir. In patients in which the self initiation of the therapy has started as early as possible at the onset of the prodome will have better prognosis than when treatment was started in the clinic 24-48 h within onset of the lesion (13).

The inhibitory effect of acyclovir on DNA synthesis of HSV-2 infected cells at MOI of 10 showed that HSV-2 DNA synthesis was seem to be most sensitive to the inhibition by acyclovir at 6.0 h after infection (Table 5). Twenty-eight percents of the DNA synthesis was inhibited as compared to the control at acyclovir concentration of 0.05 ug/mL, and the inhibition was increased up to 75% in the

culture of HSV-2 infected cells treated with acyclovir concentration at 4.0 ug/mL. It has been known that herpes-thymidine kinase (B_2 protein) reaches peak rate of synthesis about 5 to 7 hours after infection (26). This enzyme, presumably, converted acyclovir to acyclovir-monophosphate. Finally, the monophosphate was converted to acyclovir-diphosphate and triphosphate by cellular thymidine kinase. This could inhibits the viral DNA chain elongation by the DNA polymerase. In our study, acyclovir concentration of 4.0 ug/mL could not completely inhibit DNA synthesis in HSV-2 infected cells. In contrast, Furman and Mc Guirt (10, 11) showed that viral DNA synthesis in the infected cells was completely inhibited in the presence of 10 uM acyclovir (2.2 ug/mL) by using cRNA-DNA hybridization technic. The discrepancy may be due to the difference in the technic used since the ^{3}H-thymidine incorporation in our study was to measure the total amount of ^{3}H-thymidine that incorporated into viral DNA and cellular DNA. On the other hand the nucleic hybridization assay used in Furman and Mc Guirt experiment is more specific to the viral DNA. Another possibility is that the ^{3}H-thymidine incorporation counted may be the ^{3}H-thymidine that incorporated into small viral DNA fragments that occured after chain termination. These small DNA fragments were shown by an alkaline sucrose gradient sedimentation technic (11).

Immunoblotting assay, in which antigens separated

by SDS-polyacrylamide gel electrophoresis are electrophoretically transferred onto nitrocellulose sheet has become widely used for the detection of viral antigens or viral antibody. In our experiment, immunoblotting was used to study the inhibitory effects of acyclovir on individual antigenic polypeptides of HSV-2. In the analysis of the Western blot-pattern, we can pin-point to a single component or components of the viral antigens affected by acyclovir. Therefore, we may reveal some additional informations regarding to the inhibitory mechanisms of acyclovir. In addition, the affected antigen(s) probably reflects to the formation of the corresponding antibody. We can, therefore, predict for the disappearance of the corresponding antibody after the administration of acyclovir, and may use it as a marker for the indication of the success or the failure of the treatment.

More than 50 virus-specified proteins have been identified in cultured cells infected with herpesvirus (40, 56, 58), and several investigators have sought to determine which of the more than 50 virus-specified polypeptide antigens are specifically recognized by antibodies in HSV-immune sera (41, 43, 45, 46, 47, 65). In our system, we showed that the rabbit immunoglobulin reacted with HSV-2 polypeptides at molecular weights of 20-130 Kd including five major glycoproteins identified by molecular weight markers they were gB (110-130 Kd), gG (98

Kd), gC (88 Kd), gE (75 Kd), and gD (60 Kd) (Figure 4-6). In addition, our Western blot analysis showed the strong reaction of rabbit anti-HSV-2 serum against 14 polypeptides with molecular weights of 128, 117, 109, 98, 88, 75, 60,46, 40, 30, 24, 22, 21, and 20 Kd. Our findings are in consistent with earlier study using Western blot technic by Bernstein and his colleages, in which hyperimmune rabbit sera reacted with 14 polypeptides of HSV-2 at molecular weights of 125, 110, 100, 92, 84, 66, 59, 49, 47, 45, 42, 37, 32, and 28 Kd (47). There were, however, some variations in antigenic determinants of HSV-2 polypeptides. These may be due to variations in glycosylation of different cell lines used. Bernstein used rabbit skin cells for infection while we used HeLa cells derived from human cervical carcinoma.

In addition of 5 major glycoproteins, we have observed the HSV-2 polypeptides at molecular weights ranging from 20-50 Kd shown in Figure 4-6. Presently, there is no accepted nomenclature has as yet been applied to these polypeptides, therefore, we will simply refered to LMW (low molecular weights) glycoproteins. Similar observation reported by Bernstein and coworkers (47). They reported the LMW glycoproteins at molecular weights ranging from 28-59 Kd. In contrast, Eberle and Courtney could not detect these LMW glycoproteins with hyperimmune sera (52). It is may be due to the difference in the method employed. We and Bernstein used Western blot

technic while Eberle and Courtney used radioimmunoprecipation technic. In the latter, it was possible that the LMW glycoproteins of HSV-2 were not effective precipitated by any of the rabbit antisera examined. The different results obtains by these two methods has been confirmed by the study of Simmonds and coinvestigators (65).

Acyclovir had been reported to inhibit DNA synthesis but the visual aspect on viral protein synthesis were not determined. The inhibition of DNA synthesis may be extended to the synthesis of some viral proteins. By Western blot assay, individual antigenic proteins inhibited by acyclovir could be visually revealed. In this study we have used Western blot assay to identify the antigens of the infected tissue culture treated with various concentrations of acyclovir. In the Western blot data (Figure 4-6), they were found that the infected cultures treated with acyclovir at concentrations of 0.2-4.0 ug/mL diminished the synthesis of HSV-2 polypeptides in infected cell. Acyclovir reduced the overall synthesis of HSV-2 polypeptides including gB, gG, gC, gE, gD and LMW polypeptides. It is known that the viral glycoproteins are necessarily in the process of infectivity, in particular the glycoprotein gB played role in the penetration. Thus the finding of the marked reduction on the glycoprotein synthesis may explain the observation of the viral plaque reduction experiment to be due to the reduction of viral penetration. Similarly, the

study of effect of acyclovir therapy on the immune response to HSV-2 polypeptides in patients with primary genital HSV-2 infection showed that the amounts of antibodies to individual HSV-2 polypeptides decreased in acute phase serum when compared with those from placebo recipients (16,17,18,62,63,64,). However, all acyclovir-treated patients appeared to develop a full scale of antibody response to these HSV-2 polypeptides in convalescent serum. Moreover, analysis of serum samples obtained 6 to 12 months after prolong suppressive acyclovir therapy had been showed that the antibody responsed to HSV-2 polypeptides was still decreased when compared with placebo control (62). This reduction of antibody response in patient who received acyclovir was most probably due to the reduction in viral antigens exposure. It is,therefore, can be concluded that several HSV-2 polypeptides are inhibited by acyclovir treatment in patient similar to our study *in vitro*. In contrast, *in vitro* system we showed that HSV-2 polypeptide synthesis was almost completely inhibited at concentration of 3-4 ug/mL of acyclovir (lane F, Figure 5-6). One possible explanation for this observation is that the concentrations of 3-4 ug/mL of acyclovir *in vitro* were higher than those possible *in vivo* system in which the levels of acyclovir in plasma peaked at 0.4-0.7 ug/mL. In addition, the HSV-2 could not be completely inhibited by acyclovir *in vivo* because the viral DNA can integrate into host chromosome and become persistent in the ganglions.

The discrepancy of antibodies response to HSV-2 polypeptides was found in the different studies. Bernstein, et al studied the antibodies response to HSV-2 in 21 patients with primary HSV infections and showed that the treatment with oral acyclovir diminished antibodies response to HSV-2 polypeptides at molecular wights 50-100 Kd, a range including those of gD (59 Kd) and gE (80 Kd) (17, 63). While Ashley et al studied the antibodies response in 39 patients treated with acyclovir (oral and intravenous) during their primary episodes of genital HSV-2 infection (16, 18, 62). They found that levels of antibodies to individual proteins were lower in sera from acyclovir treated patients as compared to those from placebo. They were antibodies against the following viral proteins, gB (110-120 Kd),gC/gE (80-90 Kd), VP 16 (66 Kd), gD (60 Kd) and p 45 (45 Kd). However, the antibodies responsed to gG 92 (92 Kd) and to a newly described gG species, gG 70, were not significant difference among the treatment groups. Both groups of the studies could not detect antibodies to LMW polypeptides (20-40 Kd). On the other hand, our system showed that acyclovir inhibited the five major glycoproteins (gB, gG, gC, gE and gD) including several low molecular weight-glycoproteins in the range of 20-40 Kd.

Analysis in this study was extended to compare effect of pure chemical acyclovir and intravenous acyclovir on the yield of HSV-2, the HSV-2 DNA synthesis,

and the HSV-2 antigenic protein synthesis. Because the viral DNA synthesis was greatly inhibited by acyclovir treatment at 6.0 h after infection (Table 5). Therefore, we chose to compare both forms of acyclovir at this time after infection, and it was found that both pure chemical and intravenous acyclovir gave similar results on the yield of HSV-2 by plaque reduction assay (Table 6) and on the HSV-2 DNA synthesis by ^{3}H-thymidine incorporation technic (Table 7). However, they have little variation in each data because the experiments were not carried out at the same time. When HSV-2 polypeptides treated with pure chemical acyclovir was compared with those treated with intravenous form, both forms of acyclovir also gave similar inhibitory effect on polypeptide synthesis of HSV-2 infected cells. (Figure 5, 7). Treatment with both forms of acyclovir ranging from 0.2-4.0 ug/mL reduced the overall synthesis of HSV-2 polypeptides, especially at the concentration of 3.0 ug/mL of acyclovir almost completely diminished the synthesis of HSV-2 polypeptides. However, some differences in the individual HSV-2 polypeptides of each set of experiments was found. The immunoblot of HSV-2 polypeptides treated with intravenous acyclovir had 14 bands with molecular weight of 126, 112, 108, 93, 82, 73, 57, 46, 40, 30, 26, 24, 22, and 20 Kd. While those treated with pure chemical acyclovir had 14 bands with molecular weight of 128, 117, 109, 88, 75, 60, 49, 40, 37, 30, 24, 22, 21, and 20 Kd. This variations of molecular weight of these HSV-2 polypeptides may be due to the error

of molecular weight determination which calculated from the calibration curve plotted from the six standard molecular weights markers.

In conclusion, the findings of this study showed that acyclovir has *in vitro* inhibitory effect on the multiplication of HSV-2 without any effect on the host cells and also confirms that acyclovir has greater effect on the viral DNA synthesis of HSV-2 infected cell at 6 h after infection. Moreover, acyclovir at concentrations of 0.2-4.0 ug/mL reduced the overall synthesis of individual HSV-2 polypeptides, especially the five major glycoproteins.

The result of inhibitory effect of acyclovir on HSV-2 multiplication by plaque reduction assay showed that the yield of virus was completely inhibited at the concentration of 0.1 ug/mL of acyclovir for every MOI tested. In addition, the inhibitory effect of acyclovir on HSV-2 multiplication at different time courses of infection showed that while the time of infection prior to adding of acyclovir was increased, the sensitivity of acyclovir was decreased. The inhibitory effect of acyclovir on DNA synthesis of HSV-2 infected cells at MOI of 10 showed that HSV-2 DNA synthesis was most sensitive to the inhibition by acyclovir at 6.0 h after infection. However, acyclovir concentration of 4.0 ug/mL at 6.0 h after infection could not completely inhibit DNA synthesis in HSV-2 infected cells. By Western blot assay, the rabbit

immunoglobulin reacted with HSV-2 polypeptides at molecular weights of 20-130 Kd including five major glycoproteins identified by molecular weight markers they were gB (110-130 Kd), gG (98 Kd), gC (88 Kd), gE (75 Kd), and gD (60 Kd). In addition to 5 major glycoproteins, we have also observed the HSV-2 polypeptides at molecular weights ranging from 20-40 Kd which refered to LMW glycoproteins. Moreover, our Western blot analysis showed that the strong reaction of rabbit anti-HSV-2 serum against 14 polypeptides with molecular weights of 128, 117, 109, 98, 88, 75, 60, 46, 40, 30, 24, 22, 21, and 20 Kd. In the infected cultures treated with acyclovir at concentrations of 0.2-4.0 ug/mL diminished the synthesis of HSV-2 polypeptides in infected cells. Acyclovir reduced the overall synthesis of HSV-2 polypeptides including gB, gG, gC, gE, gD, and LMW polypeptides. Similar results were obtained at 1.5, 6 and 12 h after infection. In addition, pure chemical and intravenous acyclovir gave similar inhibitory effects on HSV-2 multiplication, DNA synthesis and polypeptide synthesis at 6 h after infection.

Applications of SDS-PAGE and Western blot technic may be useful for further study of herpes simplex virus or other virus and bacteria. At present, this method is a useful tool for the study antibody response to HSV polypeptides in human as well as seroepidemiology of HSV-infection (46, 47, 65, 66, 67, 68, 69). Additionally, HSV

glycoproteins espectially gB and gD have received a great deal of attention as the antigens for a potential vaccine in protection against initial and recurrent genital herpes and use as immunotherapeutic agents for controlling recurrent HSV infection in humans (70, 71, 72). Thus, our finding may be useful for the basic knowledge in the further study of the HSV.