CHAPTER VI

DISCUSSION

Growth of HSV has been studied in many cell types. Replication of the viruses has been examined in a number of cell systems. The duration of successive steps in the replication cycle depends upon the type of host cells, the virus strain and the multiplicity of infection (138). The tissue tropism of HSV infection is known to be at skin and mucous cutaneous tissue which comprised of epithelial and fibroblast cell types. We are interested in studying HSV replication in lymphocyte because there are many reports suggested that viral spreading during infection may be mediated by infected lymphocytes and it is believed that replication of virus in leukocytes may represent a very important *in vivo* mechanism of virus in the body.

A feature of HSV type 1 and 2 is their ability to infect a wide variety of animals and animal cells grown in culture. Many animal cell types were evaluated for the isolation of HSV and it was found that Vero cells were the most susceptible cells for HSV isolation (172). The Vero cell line has been employed extensively in virus replication and plaque titration assay (173). HEp-2 cells are also important, it widely used as susceptible hosts for HSV replication as good as Vero cells (172). Since HSV causes diseases in humans, in this study, only human cells were used. However, Vero cells were included as control susceptible cells. Results from the present studies, using MOI of 0.1 PFU/cell revealed that HSV-1 yield in Vero cells at 24 hours after infection was approximately 50 and 2000 times higher than that in HEp-2 and Jurkat cells, respectively (Table 3). The virus yields both in HEp-2 and Jurkat cells remained lower than in Vero cells even when the MOI was increased to 5. Similar to HSV-1, HSV-2 yields in HEp-2 and Jurkat cells also lower than in Vero cells (Table 4). These results confirm the high susceptibility of Vero cells. Moreover, the efficiency of HSV-1 production is better than HSV-2 in Vero and HEp-2 except in Jurkat cells (Table 3 and 4). The distinction of HSV growth might be due to genetic differences among these three cell lines. However, HEp-2 cells, an epithelial cell line, supported HSV replication better than Jurkat cells, T lymphocyte cell line. Previous work reported that in primary chick embryo cells, HSV-1 produced very low yields of progeny virus particles

while HSV-2 replicated very well (174). Our results showed that in Jurkat cells, HSV-2 grew better than HSV-1 (Table3, 4). Interestingly, viruses were detected in supernatant greater than inside cells which was opposite to that observed in Vero and HEp-2 cells (Table 3 and 4). This phenomenon was probably because Jurkat cell grows individually in suspension without adherence like Vero and HEp-2 cells. Thus, releasing of virions will be directly to outside cells, not cell-to-cell spreading liked Vero and HEp-2 cells (15). Our study showed that HSV could replicate in T lymphocytes, Jurkat cells, even its growth was very poor (Table3 and 4). Previously, HSV-2 was shown to replicate in human leukemia T cell line, MT-4 but not MOLT-4 (175). The replication of HSV in these cells is an interesting phenomenon which carries potential biologic relevance. For these reasons, T lymphocytes represent ideal carrier of the virus in the body. Indeed, viremia of patients suffering from generalized HSV disease has been reported (26,176) and infecting the memory lymphocyte, a virus may be able to undermine the antiviral immune response (177).

When Jurkat cells were activated by PHA for one, two and three days before inoculating the virus, the yields of HSV-1 and HSV-2 production were increased significantly (Table 5). Table 6 indicated PHA stimulation enhanced HSV-1 replication. The experiments reported here agree with previous work demonstrating that HSV replication can be enhanced in T lymphocytes activated with PHA (173).

Since HSV can replicate in T lymphocytes, thus, infection of HSV possibly transfers *via* blood transmission. PHA is known to activate T lymphocytes proliferation. This suggests that stimulation of immune response, such as infection by other organisms, leading to T lymphocyte activation may also result in enhancing of HSV replication.

Kinetic expression of HSV (both HSV-1 and HSV-2) proteins in HEp-2 and Jurkat were compared using polyclonal anti HSV-antibodies. The appearance of HSV protein synthesis in HEp-2 cells was similar to previously reports (140-142). However, HSV proteins in Jurkat cells was detected at least two hours delayed (Figure 10) compared to that in HEp-2 and PHA-activated Jurkat cells (Figure 9). This conclusion came from the appearance of cytoplasmic staining observed at six hours in Jurkat cells (Figure 10 c,g) while that staining was seen at four hours in both HEp-2 cells (Figure 9 b,f) and PHA-activated Jurkat cells

(Figure 10 j,n). Since the early step of successive HSV-replication involves the expression of IE proteins which known to be synthesized during two to four hours past infection. Thus, the expression of IE protein might be delayed. The expression of three IE proteins (ICP0, ICP22, ICP47) was observed in Jurkat and PHA activated Jurkat cells compared to HEp-2 cell as control. The results of ICP0 expression suggested that the expression of ICP0 correlated with HSV growth in Jurkat and PHA-activated Jurkat cells (Table 3-6 and Fig 11-13). For example, in the case of PHA-activated Jurkat cells, ICP0 could be expressed in HSV-1 infected cells more and faster than in HSV-2 infected cells (Fig 13 and Table7); the results of its kinetic replication (Table 6) showed that HSV-1 grew better than HSV-2. Furthermore, in HSV infected Jurkat cells, ICP0 could be found since two h.p.i. whereas it could be seen at four h.p.i. in HSV-1 infected HEp-2 cells. The difference in time expression of ICP0 between two and four hours might not effect the replication cycle of HSV in HEp-2 cells. Our results indicated that ICP0 was probably not the cause of HSV growth retardation in Jurkat cells but it may have an important role in HSV-2 replication in Jurkat cells. Although many evidences about ICP0 have been accumulated in the past 15 years, until now the biological functions of ICP0 serving in HSV replication are still unknown. However, ICP0 was initially characterized as a promiscuous transcription activator in much as it transactivates both viral and cellular genes (178). Maybe, the early detected ICP0 in any infected cells indicates susceptible replication of HSV in such cells. Recently, Harle *et al.*, 2002 reported that ICP0 is essential for the resistance of HSV to interferon (IFN) - α/β. Based on the results of Harle and other *in vitro* studies (179-82), they proposed that ICP0 provides a mechanism for HSV to override IFN-mediated repression at appropriate times since interferon - α and - β are produced by most cells in the body as an innate response to viral infection (183).

In contrast, ICP22 expression may involve in the delay of HSV-1 and HSV-2 replication in both Jurkat cells and PHA-activated Jurkat cells. The results showed in Figure 12,13 and Table 7,8). Bruni *et al.*, 1999 demonstrated that ICP22 is required *in vivo* and for efficient replication and expression of a subset of late (γ_2) genes in rodent or rabbit skin cell lines and in confluent primary human cell strains (restrictive cell) (53,54,184). Since Jurkat cells are restrictive cells for HSV, it is possible that ICP22 is also required for HSV replication in Jurkat cells and the delay of ICP22 expression might cause the expression of

subset of late (γ_2) genes postponement which affect the completion of HSV virions. However, it has been demonstrated that ICP22 is not required for virus replication in Vero and HEp-2 cell lines (permissive cell) (51). This might be due to the absence of ICP22 including ICP4 and ICP27 allows for prolonged gene expression and cell survival (185) which benefit for viral replication.

As same as ICP0, ICP47 did not implicate with the retard of HSV replication in Jurkat cells (Fig 12, Table 7,8). However, the results demonstrated that the growth of HSV in HEp-2 and PHA-acivated Jurkat cells was related with the efficiency of ICP47 expression in these cells. Especially HSV-1 infected HEp-2 cells that both viral yield production and ICP47 expression were highest. This indicates that ICP47 may have an important function for HEp-2 cells. From our data, ICP47 probably plays an important role in different HSV type replication, especially in HSV-2 infected PHA-activated Jurkat cells due to its absence after four h.p.i. (Figure 13 3a-3d) as well as the delayed appearance of ICP47 in HSV-2 infected all three cell types when compared to that of HSV-1 infected cells (Figure 11,12,13 and Table 7). Tomazin *et al.,* 1998 demonstrated that both HSV-1 ICP47 and HSV-2 ICP47 blocked the major histocompatibility complex (MHC) class I antigen presentation pathway effectively (82). By preventing the expression of viral epitopes, ICP47 prevents the immune recognition of virus infected cells by CD8+ cytotoxic T lymphocytes, which would regularly interact with MHC I.

In summary, the expression of IE proteins (ICP0,22 and 47) were different among cell types. All three proteins were synthesized within two to four hours in HSV-1 infected cells but delayed detection of ICP22 and ICP47 was found in HSV-2 infected cells. The disappearance of ICP47 in HSV-2 infected PHA-activated Jurkat cells may affect the viral multiplication. Thus, the yield production of HSV-2 was low in such cells which was opposite to HSV-1 (Figure 13, Table 5,6).

The numbers of infected cells demonstrated by Flow cytometry. Cells were infected with HSV at MOI 5 and stained by using polyclonal anti-HSV specific type antibodies as primary antibody. The results were shown in Table 9 and Figure 14. The number of HSV-1 and HSV-2 positive cells in HEp-2, Jurkat and PHA-activated Jurkat cells were correlated to

the yield of viral production (Table3-6 and Table 9). The more positive cells were detected the more viral productions were observed. This study confirmed that HSV-2 replication in Jurkat cells was better than HSV-1 (47.15% vs 22.71%) and after PHA activation, HSV-1 could replicate better than HSV-2 (40.07% vs 29.13%). It is very interesting why PHA-activated Jurkat cells could not support HSV-2 growth. It probably related to the expression of ICP47 as discussed above. However, the exact mechanism could not be demonstrated here.

Although the results described above indicate that the factors of each cells to promote viral growth implicated with the different of HSV growth in each cell type including between types of HSV, from the adsorption experiment the results demonstrated that the ability of adsorption of virus to cells was involved. As shown in Table 10, HEp-2 and PHA-activated Jurkat cells did adsorb HSV-1 better than HSV-2 (93% vs 64% and 97% vs 47%) but the ability of Jurkat cells to adsorb HSV-1 was equal to HSV-2 (89%). Previous results demonstrated that HSV-1 grew better than HSV-2 in HEp-2 and PHA-activated Jurkat cells, suggesting that in those cells HSV-1 could attach and enter cells better than HSV-2. Our results confirm this suggestion (Table 10). HEp-2 cells have all receptors that mediate HSV cell entry i.e., 3-*0*-sulphated heparan sulphate, HIgR, HveA, HveB, HveC and PRR2δ (15). Especially HveC receptors, the most effective HSV receptors which expresse in human tissue target of HSV infection and serve as receptors for all of HSV-1 and HSV-2 strains (15,16,128). In Jurkat cells, even the attachment ability of HSV-1 and HSV-2 in these cells was equal, it has been shown that HSV-2 grew better than HSV-1. This might be due to a restriction of HSV-1 replication in Jurkat cells or to restricted adsorption and entry into these cells. However, it has been shown that after activation with PHA, increasing of HSV-1 adsorption was found whereas the adsorption of HSV-2 in PHA-activated Jurkat cells was decreased. This suggests that PHA may have some roles that involve in HSV entry to Jurkat cells. Montgomery RI *et al.*, reported that PHA activated peripheral T lymphocytes express HveA (15,17,19). HveA is one of HSV receptors. Although it is not the general receptors for HSV, it has been reported that HveA enhanced entry of the wild-type HSV-1 (such as strain KOS, F, 804 and MP) and HSV-2 (strain 333) and mediated HSV entry into activated T cells (17). This finding supported our results why the ability HSV-1 KOS adsorbed to Jurkat cell was increased after stimulated with PHA and increasing of viral replication was found (Table

6, 10) but not for HSV-2 Baylor 186. These results suggest that the increasing of HveA receptor might be a mechanism that supports HSV-1 viral growth in PHA-activated T lymphocytes.

The increase of HveA mRNA expression was demonstrated by RT-PCR. In addition, total RNA was prepared from non activated and PHA-activated Jurkat cells by using the RNeasy kit and the SuperScript ™ First-Strand Synthesis System for RT-PCR was used for reverse transcription. PCR amplification of cDNAs was done with specific primers for HveA. The results in Figure 16 showed that at equal amount of extracted RNA, demonstrated by the equivalent of ß-actin products, PHA-activated Jurkat cells expressed HveA mRNA more than non activated Jurkat cells. Our results demonstrated that the HveA mRNA could be detected at 10 ng in extracted RNA of PHA-activated Jurkat cells whereas it can not observed in non activated Jurkat cells. Although the expression of HveA mRNA at 25 ng and 50 ng of extracted RNA of non activated Jurkat cells can be detected but the amount was lower than in PHA-activated Jurkat cells. It was clearly demonstrated that the expression of HveA mRNA in Jurkat cells was induced by PHA activation and it is possible that the increasing of HveA receptor is a mechanism to support HSV-1 growth in activated T lymphocytes.

However, in the case of HSV-2 Baylor 186, the opposite data was found. Instead of increasing the HSV-2 adsorption after activated with PHA as same as occurred in HSV-1 (KOS) and HSV-2 (333), it was found that the HSV-2 Baylor 186 adsorption ability was decreased from 89% to 47% (Table 16). Caused by this event, the yield production of HSV-2 and the number of positive cells in PHA-activated Jurkat cells was decreased (Table 6 and 9). It might be that HveA receptors were increased but also changes in other cell properties were happened and PHA activation affected the ability of HSV-2 to adsorb to cells. PHA is a mitogen which is well known to affect the cell function such as CD25, CD69, CD71 and HLA-DR expression (186). The cells change not only in increasing HveA expression but also probably other factors involving the entry of HSV-2 Baylor 186. Unfortunately, our study could not clarify those factors.

In conclusion, present studies demonstrated that HSV-1 and HSV-2 could replicate in T lymphocytes. The replication in T lymphocytes was different from that in epithelial cells suggesting delayed replication occurred. Not only cell types but also HSV types played important role in differences of viral growth. Activation of T lymphocytes by PHA mitogen increased HveA receptor for HSV attachment. Thus, the yield of viral production especially HSV-1 strain KOS was increased in PHA-activated Jurkat cells which was opposite to previous observation of HSV-2 strain 333 (17). HSV-2 strain Baylor 186 grew poorly in PHA-activated Jurkat cells. The mechanism for this difference is still unknown.

In general, development of HSV lesions in immunocompetent hosts occur locally, rarely to cause systemic spread through bloodstream. In contrast to infection in immunocompromised hosts, systemic infection can be commonly found. HSV infection is an important opportunistic infection of HIV infected patients and reactivation (187,188). In HIV-infected patients, HSV replication in T lymphocytes may be enhanced and cause generalized infection. However, certain molecular mechanisms of HSV infection and how HSV reactivation, especially in HIV infected patients, can be induced are still unknown.