Induction of Apoptosis by Herpes Simplex Virus in Jurkat Cells is Partly Through Caspase-3, -8 and -9 Activation

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Herpes simplex virus (HSV), a large DNA containing virus, is endemic in all human populations investigated. After infection of mucocutaneuos surfaces, HSV establishes a latent infection in nerve cells. Various immune evasion mechanisms have been shown to be utilized by HSV including apoptosis induction in T lymphocytes. However, the mechanisms of T cell infection and apoptosis by HSV are still unknown. The present study investigated the molecular mechanisms of apoptosis induction in T cells by HSV. The Jurkat T cell line was used as a representative for T cells. Apoptosis detection by Annexin V assay demonstrated that both HSV-1 and HSV-2 induced apoptosis in Jurkat cells and caspase-3, -8, and -9 inhibitors blocked apoptosis induced by HSV-1 and HSV-2. The data suggested that HSV-1 and HSV-2 induced apoptosis in T lymphocytes by caspase-dependent pathway. However, apoptosis may occur through other mechanism (s) since caspase inhibitors used in the present study could not completely inhibit apoptosis induced by HSV infection. In addition, the data demonstrated that the number of apoptotic cells induced by HSV-2 was significantly higher than by HSV-1 at 12 hour post-infection (h p.i.) (p= 0.003). Further studies in peripheral blood T cells and the proteins of viruses involved in apoptosis induction should be further performed in order to elucidate the molecular mechanisms of apoptosis induced by these viruses.

Keywords : *Herpes Simplex Virus (HSV), T lymphocyte, Apoptosis, Caspase*

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Herpes simplex virus is a member of the herpesviridae family. The virus frequently infects human beings, causing a range of diseases from mild uncomplicated mucocutaneous infection to fatal HSV encephalitis. HSV-1 is normally associated with orofacial infections and encephalitis, whereas HSV-2 usually causes genital infections and can be transmitted from infected mothers to neonates. Both viruses establish latent infections in sensory neurons and, upon reactivation, cause lesions at or near the point of entry into the body [1,2].

Host immune defenses are critical in the control of HSV infections, most of the evidence suggested that cellular immunity is important [3,4], and severe disease is seen in patients with impaired cellular immunity. Although strong humoral responses are produced in response to HSV infection, and antibodies generated during primary infection can effectively neutralize virus; however, there was little or no evidence that antisera can prevent HSV infection in humans [5]. In addition, there is no evidence that HSV uses antigenic variation to escape the host control, it must use alternative immune escape strategies, if it is, to successfully reactivate and be transmitted. A major evasion mechanism used by HSV is the establishment of latency in dorsal root ganglion, since the nervous system is an immunopriviledged site and viral proteins are not expressed. The virus can persist and avoid the host immune system, upon reactivation, causes shedding and lesions.

However, during the reactivation process, the virus must face the host defenses. For example, HSV expresses receptors for complement and for IgG, and these may affect some degree of resistance to humoral immune response [4]. When the virus reactivates and infects dermal fibroblasts and keratinocytes, it causes down-regulation of MHC class I via inhibition of TAP by ICP47 [6,7], thus interfering with the recognition of these cells by $CD8^+$ CTL [8]. Another host defense is programmed cell death or apoptosis in response to disturbance of cellular machinery by viral proteins or host immune system. Mammalian cells have two major apoptotic pathways. These are the death-receptor pathway and the mitochondrial pathway. Apoptosis presents a major threat to viruses, since apoptotic cells

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are poor hosts for viral replication [9]. HSV protects infected cells from apoptosis through the action of several genes and gene products, including U_S3 [10], U_S5 (gJ) [11], ICP22 [12], ICP27 [13], and LAT [14]. Together, these proteins protect infected cells from apoptosis induced directly by the virus, and also apoptosis induced by external stimuli such as CTL. In addition, there are evidences that HSV inhibits apoptosis in epithelial and fibroblast cells [15,16]. However, some reports suggested that HSV can induce apoptosis in T lymphocytes [17-19]. It is well known that T lymphocytes play important roles in cell-mediated immune response. Thus, apoptosis induced in this cell may be one mechanism of HSV immune evasion.

In the present study, Jurkat, a T lymphocytic cell line, was used as a representative of T cells for investigating mechanisms of apoptosis induced by HSV. Both HSV-1 and HSV-2 were used to compare whether there is any difference in apoptosis induction.

Material and Method

Cell lines. Vero cells, African green monkey kidney cell line, were kindly provided by Associate Professor Vimolmas Lipipun, Ph.D., the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Jurkat cells, the human T cell leukemia cell line, were generously provided by Dr. Pokrath Hansasuta, Ph.D., Faculty of Medicine, Chulalongkorn University.

Viruses. HSV-1 strain KOS and HSV-2 strain Baylor (kindly provided by Associate Professor Vimolmas Lipipun, Ph.D.) were grown in Vero cells and their titers were determined by plaque titration assay.

Annexin V staining assay. Jurkat cells (200 µl of $5x10^5$ cells/ml) were plated in a 24 well-plate. The cells were then either mock infected or infected with either HSV-1 or HSV-2 at multiplicity of infection (MOI) 0.5, 1, 5 or 10 and incubated at 37 C for 2, 4, 6, 12, 18 and 24 h.

As a positive control, cells were incubated with camptothecin (Sigma, USA). At the end of the incubation period, cells were collected and annexin V binding assay was performed according to the manufacturer's instruction (Santa Cruz Biotecnology, USA). Cells were analyzed immediately by Flow cytometry.

Inhibition of apoptosis by caspase inhibitors. DEVD-FMK (Aspartyl-glutamyl-valyl-aspartic acid-Fluoromethyl ketone), IETD-FMK (Isoleucyl-glutamyl-threonyl-aspartic acid-Fluoromethyl ketone) and LEHD-FMK (Leucyl-glutamyl-histidyl-aspartic acid-Fluoromethyl ketone) were used as caspase-3, caspase-8 and caspase-9 inhibitors (R&D Systems, USA), respectively. Jurkat cells were either mock infected or infected with MOI 5 of HSV-1 or HSV-2 in the presence or absence of various concentrations of caspase 3, 8 and 9 inhibitors (10 µM, 50 µM and 100 µM were used) as indicated. After 18 h of infection, the cells were collected and apoptosis detection was performed using Annexin V assay as described above.

Combination of caspase inhibitors. Jurkat cells were either mock infected or infected with MOI 5 of HSV-1 or HSV-2 in the presence or absence of combination of caspase 3, 8 and 9 inhibitors (100 µM of each inhibitor were used) as indicated. After 18 h of infection, the cells were collected and apoptosis detection was performed using Annexin V assay as described above.

Statistical analysis. Data were analyzed with the paired sample *t* test, using the statistic package in SPSS for windows version 11.5. A *p* value less than 0.05 was considered significant.

Results

1. Apoptosis of Jurkat cells induced by HSV

Jurkat T cells were infected with various amounts of HSV-1 or HSV-2 for indicated times. Apoptotic cells were then detected using Annexin V binding assay.

The data showed that both HSV-1 and HSV-2 induced apoptosis in Jurkat cells and the induction was dose dependent. As shown in Fig. 1, for HSV-1, there was no significant number of apoptotic cells detected at 12 h p.i. ($p = 0.068$), but the significant numbers were obtained at 18 and 24 h p.i. ($p = 0.007$ and 0.005, respectively). For HSV-2, significant numbers of apoptotic cells compared with non-infected cells, were detected when results from 12, 18 and 24 h p.i. were analyzed ($p = 0.015$, 0.029 and 0.013, respectively). At 12 h p.i., numbers of apoptotic cells were higher when HSV-2 was used than HSV-1 ($p = 0.043$). For 18 h p.i. at 10 MOI, there was significant difference in apoptotic cell numbers from HSV-1 and HSV-2 infection when the data at 18 h p.i. was investigated ($p = 0.021$). However, there was no significant difference when the data at 24 h p.i. was compared ($p = 0.18$).

2. Induction of Apoptosis by HSV is partly through caspase-3,-8 and -9 activation

To determine whether caspases are involved in HSV-induced apoptosis in Jurkat cells, the effects of the cell permeable caspase-3, -8 and -9 inhibitors on apoptosis induction were examined. Each inhibitor was used at the concentrations of 10, 50 and 100 µM. Jurkat cells were infected with HSV-1 or HSV-2 for 18 h

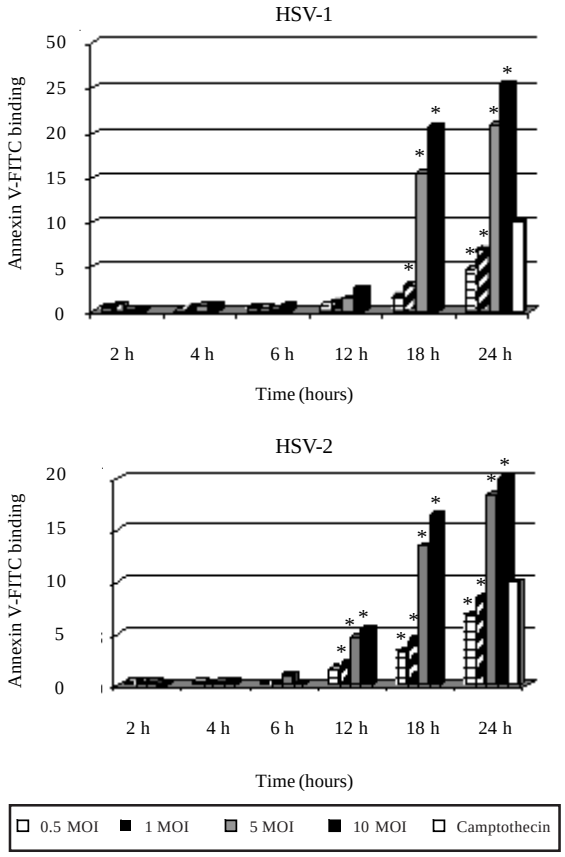

Fig. 1 Apoptosis of Jurkat cells induced by HSV. Jurkat cells were infected with HSV-1 or HSV-2 for 2, 4, 6, 12, 18 and 24 h at 0.5, 1, 5 and 10 MOI as indicated. Cells were subjected to Annexin V-FITC staining assay as described in Materials and Methods. The data shown are mean of numbers of cells positive for Annexin V obtained from three independent experiment. * $p<0.05$

in the presence or absence of caspase inhibitors prior to Annexin V-FITC staining. Fig. 2, 3 and 4 were from experiments using caspase-3, -8 and -9 inhibitors, respectively. Apoptosis inhibition by all caspase inhibitors was dose dependent. All concentrations of all inhibitors reduced the numbers of apoptotic cells. However, only at concentrations 50 and 100 μM of all inhibitors significantly reduced numbers of apoptotic cells induced by HSV-1 or HSV-2 ($p< 0.05$).

According to the data obtained above, apoptosis of Jurkat cells was induced through caspase-3, 8 and -9 activation. The authors further investigated whether the combinations of caspase inhibitors increase the inhibition or provide any additional information. Jurkat cells were infected with HSV-1 or HSV-2 in the presence or absence of combinations of caspase inhibitors for 18 h before AnnexinV-FITC staining was

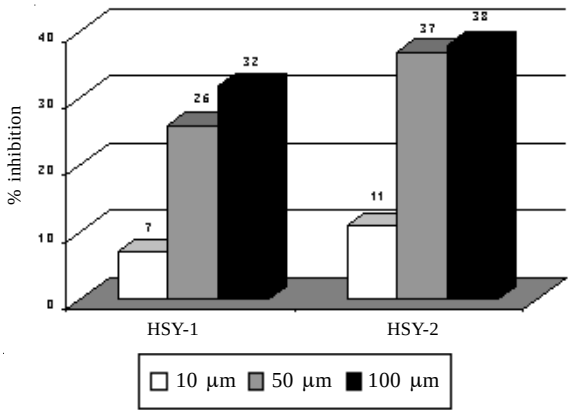

Fig. 2 The percentage of apoptosis inhibition by caspase-3 inhibitors. Data are mean of three independent experiments

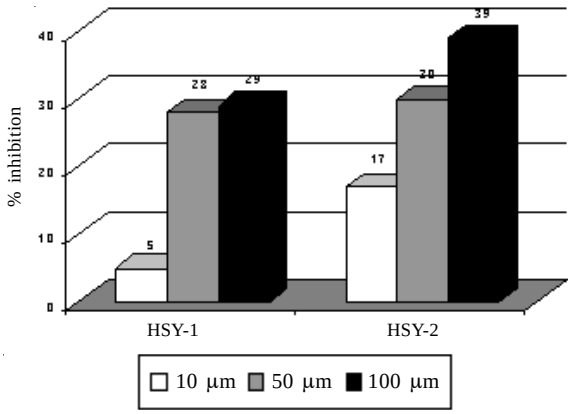

Fig. 3 The percentage of apoptosis inhibition by caspase-8 inhibitors. Data are mean of three independent experiments

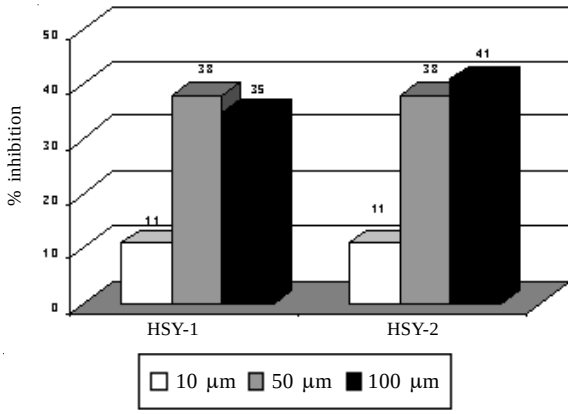

Fig. 4 The percentage of apoptosis inhibition by caspase-9 inhibitors. Data are mean of three independent experiments

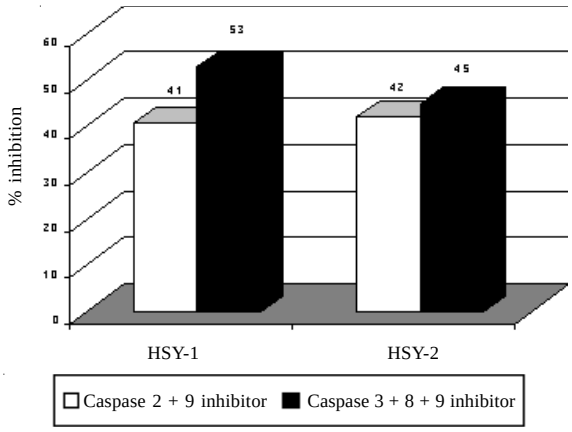

Fig. 5 The percentage of apoptosis inhibition by combination of caspase inhibitors. Data are mean of three independent experiments

performed. As shown in Fig. 5, apoptosis inhibition by the combinations of caspase-3, -8 and -9 inhibitors was only slightly increased and there was no significant difference in inhibition of apoptosis induced by HSV-1 and HSV-2 ($p = 0.253$).

Discussion

Although HSV infection and its ability to establish latent infection have been recognized for a period of time, the molecular mechanisms involved in pathogenesis of this organism is still not clearly understood. The major areas of interest in the study of HSV infection involved mechanisms of reactivation and immune evasion. As mentioned earlier that HSV utilizes various approaches for evasion of immune response and apoptosis induction of T lymphocytes was suggested to be one of those. Apoptosis of T lymphocytes may result in immnosuppression and lymphocytopenia seen in response to HSV infection.

Various evidences on mechanisms of apoptosis inhibition in various cell types were demonstrated, however, there have been a few reports on apoptosis induction and its molecular mechanisms in T lymphocytes. In 1988, Hayward *et al* demonstrated that HSV-1 could replicate in T lymphocytes and this infection induced apoptosis in those cells activated with phytohemagglutinin (PHA) [20]. In 1997, Ito *et al* demonstrated that HSV-1-induced apoptosis in both PHA-activated CD4 and CD8 lymphocytes from cord blood [17]. In 1997, Ito *et al* investigated that HSV-1 induced apoptosis in PHA-activated peripheral blood T lymphocytes and apoptosis induction occurred only in CD4 but not in CD8 lymphocytes [18].

Since most studies in T lymphocytes used HSV-1, the authors were interested in comparing apoptosis induction induced by HSV-1 and HSV-2. The significant findings of the present study can be summarized as follows. The authors showed that HSV-1 and HSV-2-infected Jurkat cells underwent apoptosis monitoring by Annexin V-FITC staining, and these processing events were detected in the infected cells for 18 h at 1 MOI for HSV-1 and 12 h at 1 MOI for HSV-2. The data also suggest that HSV-2 induced apoptosis better than HSV-1. In 1999, Galvan *et al.* demonstrated that HSV-1 blocked apoptosis via both caspase-independent and caspase-dependent pathways [21]. The authors demonstrated that HSV-1 and HSV-2 infection induced apoptosis via a pathway which activates and process caspase-dependent. Even though caspase-3 is the downstream effector of caspase-8 and -9, its inhibitor could not inhibit apoptosis more than the inhibitor of caspase-8 or -9. It is probably because there are additional downstream effectors such as caspase-6 and -7. In the present study, HSV could not induce apoptosis in the early time of infection which is probably because infected cells produced anti-apoptotic proteins during 3 and 6 h p.i. In 2003, Goodkin *et al* demonstrated that the nuclear translocation of transcription factor NK-kappaB between 3 and 6 h p.i. was necessary to prevent apoptosis in wild-type HSV-1-infected human HEp-2 cells [22].

Since each inhibitor used in the present study could not completely inhibit apoptosis induced by HSV-1 or HSV-2, the authors were interested in whether the increase in apoptosis inhibition could be observed when combinations of caspase inhibitors were used. The data suggest that other mechanism (s) such as the caspase-independent pathway could be involved since the inhibitors of caspase-8 and -9 and their downstream effector, caspase-3 altogether were not able to completely inhibit apoptosis. Caspase-independent pathway has been shown to execute apoptosis in many types of neuronal injury. In addition, apoptosis-inducing factor (AIF) is an important factor involved in the regulation of this caspase-independent neuronal cell death [23].

Even Jurkat cells have been widely used as a model for T lymphocytes, further study using peripheral blood T lymphocytes should be done to confirm the author's observation. In addition, viral and host proteins involved in apoptosis induction will further elucidate the molecular mechanisms of apoptosis induction. Most genes and gene products of HSV reported are involved in inhibition of apoptosis. For examples, Leopardi *et al* in 1996; demonstrated that a viral protein known as ICP4 blocked apoptosis induced

by HSV-1 in Vero cells [24] and in 1997, they demonstrated that the U_S3 gene of HSV-1 was necessary to prevent apoptosis induced by the virus [10]. A previous study suggested that U_S3 gene of HSV-1 correlated with phosphorylation of BAD, a BH3-only proapoptotic Bcl-2 family member. Phosphorylation of BAD has been demonstrated to abrogate its proapoptotic activity [25]. The HSV latency-associated transcript (LAT) is a complex transcription unit expressed primarily in neurons containing latent genomes and has been shown to inhibit cell death by blocking caspase-8 and caspase-9 pathways [26]. Moreover, Perkin *et al* 2003, reported that ribonucleotide reductase (R1) protein (ICP10 protein kinase) of HSV-2 blocked apoptosis in cultured hippocampal neurons by activating the extracellular signal-regulated kinase (ERK) survival pathway and involving a c-Raf-1-dependent mechanism and induction of antiapoptotic protein Bag-1. In contrast, HSV-1 activated c-Jun N-terminal kinase (JNK), c-Jun,and ATF-2, induces the proapoptotic protein BAD, and trigger apoptosis in hippocampal neurons consistent with disease pathogenesis supporting the evidence that HSV-1 causes encephalitis more than HSV-2 [27]. Other viruses, such as human papillomaviruses, its proteins (E2) are proapoptotic. E2 induces apoptosis through the extrinsic pathway, involving the initiator caspase-8. In addition, E2 is cleaved by caspases during apoptosis, providing an example of an apoptotic inducer, which is itself a target for caspase cleavage. The cleaved E2 protein exhibits an enhanced apoptotic activity, suggesting that it may participate in an amplification loop [28].

Other target sites for HSV is at the level of the antigen-presenting cell (APC). The most potent form of APC known is the dendritic cell (DC). DC plays a key role in the induction of the primary cellular immune response to intracellular pathogens, as they are the main cell type that stimulates naive T cells in the draining lymph nodes [29]. In 2003, Jones CA *et al*, found that HSV induces rapid cell death in murine bone marrow-derived DC, with HSV-2 being more potent than HSV-1 [30]. The present study demonstrated that HSV induces immature DC to undergo apoptosis by a mechanism involving caspase-8. In general, cellular FLICE-inhibitory protein (c-FLIP) inhibits death receptor signal by blocking caspase-8 [31]. It has been demonstrated that HSV-infected immature DC down-regulated long c-FLIP and up-regulate p53 whereas other apoptosis-regulation proteins (e.g. Bcl-2) were not affected [32].

Information on how HSV induces apoptosis in T lymphocytes, which population of T lymphocytes are preferentially infected, where viral antigens are located during a course of infection and which viral and host proteins are involved in those processes are inevitably required for understanding the pathogenic mechanisms of HSV infection.

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ไวรัสเฮอร์ปีส์ซิมเพล็กซ์เหนี่ยวนำให้เกิด apoptosis ใน Jurkat Cells โดยการกระตุ้น caspase-3,-8 และ -9

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ไวรัสเฮอร์ปีส์ซิมเพล็กซ์ (HSV) เป็นไวรัสที่มีสารพันธุกรรมดีเอ็นเอ สามารถพบการติดเชื้อได้ในมนุษย์ทั่วไป หลังจากมีการติดเชื้อของเยื่อบุผิวหนัง ไวรัสเฮอร์ปีส์ซิมเพล็กซ์จะทำให้เกิดการติดเชื้อแอบแฝงในเซลล์ประสาทได้และเมื่อติดเชื้อไวรัสชนิดนี้แล้วจะทำให้เกิดการกลับมาของโรคเป็นซ้ำ ๆ ได้ซึ่งปัจจุบันยังไม่สามารถรักษาให้หายขาด ไวรัสเฮอร์ปีส์ซิมเพล็กซ์มีกลไกมากมายในการหลบเลี่ยงระบบภูมิคุ้มกันของมนุษย์ รวมไปถึงการชักนำให้เกิดกลไกการตาย(apoptosis) ในทีลิมโฟซัยท์ อย่างไรก็ตามกลไกการติดเชื้อและกลไกการตายของทีลิมโฟซัยท์ด้วยไวรัสเฮอร์ปีส์ซิมเพล็กซ์ยังไม่ทราบแน่ชัดในการศึกษาครั้งนี้ ทำการตรวจสอบกลไกระดับเซลล์ของการชักนำให้เกิดกลไกการตายด้วยไวรัสเฮอร์ปีส์ซิมเพล็กซ์ในเซลล์ทีลิมโฟซัยท์ โดยใช้เซลล์ Jurkat มาเป็นตัวแทนทีลิมโฟซัยท์ ผลการศึกษาการเหนี่ยวนำให้เกิด apoptosis จากเชื้อเฮอร์ปีส์ซิมเพล็กซ์ด้วยวิธีการจับด้วย Annexin V พบว่าเชื้อเฮอร์ปีส์ซิมเพล็กทั้งสองชนิดชักนำให้เกิด apoptosis ในเซลล์ Jurkat และสารยับยั้ง caspase-3, -8 และ -9 สามารถยับยั้งการเกิด apoptosis แสดงว่ากลไกที่เฮอร์ปีส์ซิมเพล็กซ์เหนี่ยวนำให้เกิด apoptosis ในทีลิมโฟซัยท์โดยการกระตุ้น caspase ดังกล่าว แต่อย่างไรก็ตามอาจมีวิถีทางอื่นมาเกี่ยวข้องเนื่องจากการยับยั้ง apoptosis ด้วยสารยับยั้ง caspase ที่ใช้ไม่สามารถยับยั้งการเกิด apoptosis ได้อย่างสมบูรณ์ นอกจากนี้ยังพบว่าเชื้อเฮอร์ปีส์ซิมเพล็กซ์ชนิด II สามารถเหนี่ยวนำให้เซลล์ Jurkat เกิด apoptosis ได้มากกว่าเชื้อเฮอร์ปีส์ซิมเพล็กซ์ชนิด I ที่ 12 ชั่วโมงอย่างมีนัยสำคัญทางสถิติ (p=0.003) การศึกษาเพิ่มเติมใน T cells จาก peripheral blood และโปรตีนของไวรัสที่เกี่ยวข้องกับการชักนำให้เกิด apoptosis น่าจะช่วยอธิบายกลไกระดับเซลล์หรือโมเลกุลของการชักนำ apoptosis ด้วยไวรัสชนิดนี้ได้มากขึ้น ซึ่งความรู้ดังกล่าวจะมีประโยชน์ในการเข้าใจกลไกการก่อโรคซึ่งนำไปสู่การพัฒนาวิธีการป้องกันและรักษาโรคติดเชื้อไวรัสชนิดนี้