THE STUDY OF INTRINSIC AND EXTRINSIC APOPTOSIS PATHWAYS IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF DENGUE VIRUS-INFECTED PATIENTS

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By

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ABSTRACT

THE STUDY OF INTRINSIC AND EXTRINSIC APOPTOSIS PATHWAYS IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF DENGUE VIRUS-INFECTED PATIENTS

Cheong Pei Fen

The global prevalence of dengue virus (DV) has been estimated by WHO to result in around 24,000 deaths worldwide every year. The pathogenesis of DV includes cell death and therefore, the well-defined mechanisms of apoptosis are important. Some studies showed that DV's major target cells are peripheral blood mononuclear cells (PBMC). These PBMC undergo apoptosis but the true mechanisms have yet to be elucidated. Thus, in this study, the intrinsic and extrinsic apoptosis pathways of PBMC from DV-infected patients were determined by studying the levels of the apoptosis regulators. The intrinsic apoptosis regulators, namely hydrogen peroxide (H_2O_2) and B-cell lymphoma protein-2 (Bcl-2) and the extrinsic apoptosis regulator, namely TNF-related apoptosis-inducing ligand (TRAIL), were studied in this research. In addition, caspases-3/7, -8 and -9 which have roles in initiation and execution of apoptosis were also investigated. The apoptosis regulators which were obtained by lysing the PBMC of DV-infected patients and normal healthy controls were measured by using ELISA and also fluorometric methods. The

results revealed that in DV-infected patients, the levels of H_2O_2, TRAIL, caspases-3/7, -8 and -9 were significantly higher than in the controls ($p<0.05$). However, the levels of Bcl-2 was of no significant difference in DV-infected patients when compared to controls ($p>0.05$). These results therefore suggested that the pathways that lead to apoptosis in PBMC of DV-infected patients involved H_2O_2 and TRAIL but not Bcl-2. Caspases-3/7, -8 and -9 were also found to play important roles in apoptosis of PBMC in DV-infected patients. Studies which include more regulators of apoptosis would be needed to complete the understanding of apoptosis mechanisms in PBMC of DV-infected patients.

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APPROVAL SHEET

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SUBMISSION OF THESIS

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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CHEONG PEI FEN

24th October 2011

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LIST OF ABBREVIATIONS

AICD	Activation-induced cell death
AIDS	Acquired Immunodeficiency Syndrome
AIF	Apoptosis-inducing factor
AFC	7-amino-4-trifluoromethyl coumarin
Apaf-1	Apoptosis activating factor-1
Apo2L	Apo2 ligand
Bcl-2	B-cell lymphoma protein-2
BH	Bcl-2 homology
cyt *c*	Cytochrome *c*
CARD	Caspase recruitment domain
CL	Lipid cardiolipin
CMV	Cytomegalovirus
DC	Dendritic cells
DED	Death effector domain
DF	Dengue fever
DHF	Dengue Hemorrhagic fever
DIABLO	Second mitochondria-derived activator of caspase
DNA	Deoxyribonucleic acid
DR6	Death receptor 6
DV	Dengue virus
DSS	Dengue Shock Syndrome
ELISA	Enzyme linked-immunosorbent assay
ER	Endoplasmic Reticulum

Fas	Fibroblast associated
FasL	Fas ligand
FADD	Fas-associated death domain
FITC	Fluorescein isothiocyanate
G	Gap
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HIV +	HIV-positive
HSV-2	Herpes simplex virus 2
H_2O_2	Hydrogen peroxide
IL	Interleukin
IFN	Interferon
JE	Japanese Encaphelitis
kDa	Kilo Dalton
kb	Kilo base
mRNA	Messenger ribonucleic acid
NK cells	Natural killer cells
NS	Non-structural
O_2^-	Superoxide anion
OH^-	Hydroxyl radical
PBMC	Peripheral Blood Mononuclear Cells
PMN	Peripheral mononuclear
RNA	Ribonucleic Acid
ROS	Reactive oxygen species

RNS	Reactive nitrogen species
SLE	Systemic Lupus Erythematosus
smac	Second mitochondria-derived activator of caspase
tBid	Truncated Bid
TNF	Tumour necrosis factor
TNFR1	Tumour Necrosis factor receptor 1
TRAMP	TNF receptor-related apoptosis-mediating protein
TRADD	TNFR1 associated death domain protein
TRAIL	Tumour necrosis factor-Related Apoptosis-Inducing Ligand
TRAILR1	Tumour necrosis factor-Related Apoptosis-Inducing Ligand receptor 1
TMB	Tetra methylbenzidine
Vpr	Viral protein R
WHO	World Helath Organisation

CHAPTER 1

INTRODUCTION

Dengue is a mosquito-borne viral disease that affects millions of individuals per year and imposes a particularly heavy burden on developing countries in tropical and subtropical regions, mainly Southeast and South Asia, Central and South America, and the Caribbean (Kurane, 2007; Schreiber et al., 2007). The disease is now endemic in more than 100 countries predominantly in urban and semi-urban areas and now in rural areas also, placing some 2.5 billion people at risk of infection each year (Kroeger, 2004). The WHO in year 2000 estimated that there may be 50–100 million cases of dengue virus (DV) infections worldwide every year, resulting in 250,000–500,000 cases of dengue hemorrhagic fever (DHF) and approximately 25,000 deaths annually (Gubler & Meltzer, 1999). During epidemics of dengue, attack rates among susceptibles are 40% to 90% (Chaturvedi, Nagar, & Shrivastava, 2006). An estimated 500,000 cases of DHF require hospitalization each year, of whom a very large proportion are children (Chaturvedi et al., 2006). Thus, dengue presents one of the most serious human infectious diseases in recent decades (Kurane, 2007).

People first reported the existence of dengue-like disease in 1779 but it was most likely present long before it first appeared in literature (Kautner, Robinson, & Kuhnle, 1997). Dengue is caused by DV, a member of the *Flavivirus* genus of the *Flaviviridae* family of enveloped, positive-sense single-stranded ribonucleic acid (RNA) viruses (R. Chen et al., 2007; Clyde, Kyle, &

Harris, 2006). The genomic RNA is approximately 11 kb in length and is composed of three structural protein genes that encode the nucleocapsid or core protein, a membrane-associated protein, an envelope protein, and seven nonstructural (NS) protein genes (Kumarasamy, Abdul Wahab, Chua, & Hassan, 2007; Umareddy et al., 2007). Dengue virus are grouped into four serotypes, namely DV-1, DV-2, DV-3 and DV-4 which are transmitted between vertebrate hosts by insect vectors (Catteau, Roué, Yuste, Susin, & Desprès, 2003; Khana et al., 2008; Umareddy et al., 2007). Although DV-1, DV-2, DV-3 and DV-4 are called four serotypes of DV, it is generally accepted that these four DV are antigenically related different species (Kurane, 2007). For human, DV is transmitted through the bites of two mosquito species, namely *Aedes aegypti* and *Aedes albopictus* (Clyde et al., 2006). The virus is transmitted when a mosquito of the *Aedes genus* bites an individual infected with DV. The virus in the blood of the infected individual then infects the mosquito and travels from the mosquito's stomach to its salivary glands were the virus multiplies. The virus is then injected into another person when the mosquito injects anticoagulants that prevent blood clotting during feeding. The mosquito remains its ability to transmit dengue for its entire life (Kautner et al., 1997). Infection with any of the four serotypes of DV is often asymptomatic but can produce clinical manifestations ranging from a self-limiting dengue fever (DF) to severe DHF and a fatal dengue shock syndrome (DSS) (Guy & Almond, 2008).

The mild form of dengue is DF (R. Chen et al., 2007). It is a self-limited febrile illness characterized by biphasic fever, headache, myalgia, eye pain and

rash (Kurane, 2007). DV which is transmitted to human via Aedes mosquito will have an incubation period of 2-7 days (Guy & Almond, 2008). After the incubation period, a sudden onset of fever occurs. The fever is usually accompanied with retro-orbital or frontal headache. Myalgia and bone pain occur soon after the onset of fever (Gubler, 1998; Guzman et al., 1999). A transient macular rash that blanches under pressure, nausea, vomiting, lymphadenopathy, and taste aberrations can develop. These symptoms are accompanied by leukopenia (a decrease in leukocytes) and variable degrees of thrombocytopenia (a decrease in platelets count). One to 2 days after defervescence, a generalized morbilliform maculopapular rash appears. The rash spares palms and soles (Clyde et al., 2006; Kurane, 2007; Limonta, Cap´o, Torres, P´erez, & Guzm´an, 2007). Approximately one-third of DF patients may have hemorrhage manifestations ranging from mild skin hemorrhage, gingival or nasal bleeding, and gastrointestinal bleeding to severe hemorrhage (R. Chen et al., 2007). However, patients usually recover from the symptoms without complications about a week after the onset of disease (Kurane, 2007). The management of DF is supportive with bed rest, control of fever and pain with antipyretics/analgesics, and adequate fluid intake (R. Chen et al., 2007; Guy & Almond, 2008). Immune responses provide complete and probably lifelong protection against homotypic strains, but cross-protection between DV serotypes is limited (Chaturvedi et al., 2006; H. Chen, Hofman, Kung, Lin, & Hsieh, 2007; Guy & Almond, 2008; Rothman, 2004).

Some patients infected with DV demonstrate plasma leakage into interstitial spaces, thrombocytopenia, and also hemorrhagic manifestation (R. Chen et al.,

2007; Schrieber et al., 2008; WHO, 1997). This severe life-threatening syndrome is called DHF. The incubation period of DHF is similar to that of DF. Illness starts with fever, malaise, vomiting, headache, anorexia, and cough. Rapid clinical deterioration and collapse follows after 2–5 days. In the second phase, patients demonstrate cold clammy extremities, warm trunk, flashed face, restlessness, irritability, middle gastric pain, and may progress to a rapid weak pulse, hypotention, and narrow pulse pressure (Chaturvedi et al., 2006; Clyde et al., 2006; Kautner et al., 1997; Khana et al., 2008; Limonta et al., 2007). The crisis lasts for 24–36 hours and the patients recover rapidly once convalescent starts. The haematological manifestations include an increase in hematocrit, thrombocytopenia, a prolonged bleeding time, and an increased prothrombin time (Kurane, 2007). WHO categorizes DHF into four grades on the basis of the clinical presentation and laboratory findings; the mildest is grade I and the most severe is grade IV. Grades III and IV, also referred to as DSS, in which plasma leakage is so profound that shock occurs are fatal (Chaturvedi et al., 2006). At present, there is no specific therapy available for DHF. Appropriate symptomatic treatment has been successful in reducing the mortality of DHF (Chaturvedi et al., 2006; R. Chen et al., 2007). Treatment involves correction of fluid loss, replacement of coagulation factors, and infusion of heparin (Guy & Almond, 2008). Drugs such as corticosteroids or carbazochrome sodium sulfonate are given to stabilize capillary permeability and avoid plasma leakage (Kautner et al., 1997).

The pathogenesis of DHF has been explained by two theories. One theory is based on the virulence of infecting DV; virulent DV strains cause DHF, while

avirulent DV strains cause DF. The other is based on immunopathogenesis. This theory suggests that DHF is mediated by host immune responses including DV-cross-reactive antibodies that augment infections (H. Chen et al. 2007; Kautner et al., 1997; Kurane, 2007). Recovery from infection by one serotype provides long lasting immunity against that serotype but confers only partial and transient protection against subsequent infection by the other three. It has been suggested that sequential infection increases the risk of more serious disease resulting in DHF (Guy & Almond, 2008; Kumarasamy et al., 2007). The reason behind this theory is that the 4 serotypes (DV-1 to DV-4) are different strains of DV that have 60-80% homology between each other (Kautner et al., 1997). The major difference for humans lies in subtle differences in the surface proteins of the different dengue serotypes. After a person is infected with dengue, they develop an immune response to that dengue serotype. The immune response produced specific antibodies to that serotypes's surface proteins that prevents the virus from binding to macrophage cells (the target cell that DV infect) and gaining entry. However, if another serotype of DV infects the individual, the virus will activate the immune system to attack it as if it was the first serotype. The immune system is tricked because the 4 serotypes have very similar surface antigens. The antibodies bind to the surface proteins but do not inactivate the virus. The immune response attracts numerous macrophages, which the virus proceeds to infect because it has not been inactivated. This makes the viral infection much more acute. The body releases cytokines that cause the endothelial tissue to become permeable which results in hemorrhagic fever and fluid loss from the blood vessels. Therefore a vaccine has proven rather difficult to be produced due to

the presence of the four distinct serotypes of the DV (Chaturvedi et al., 2006; R. Chen et al., 2007; Kautner et al., 2007; Kurane, 2007; Rothman, 2004; Schrieber et al., 2008).

The concept of apoptosis or programmed cell death was introduced in 1972 by Kerr, Wyllie, and Currie as a physiologic type of cell death with an important function, complementary to mitosis, in tissue homeostasis. The purpose of it is to remove any cell perceived, rightly or wrongly, as dangerous for the local homeostatic economy (Andoniou, Andrews, Manzur, Ricciardi-Castagnoli, & Degli-Esposti, 2004). This process can be activated in any one of three generic situations, firstly, developmental and/or homeostatic, secondly, as a defence mechanism and thirdly in ageing (Toescu, 1998). The study of apoptotic death has increased in the last few years and many processes and mechanisms specific to apoptosis have been discovered and characterised (Saraste & Pulkki, 2000). Further, apoptosis is related to the progression of an individual cell through the time course of death and the time course can be summarised as the following 3 main phases, namely the initiation phase, the commitment phase and the execution phase (Saraste & Pulkki, 2000). The initiation phase is defined as the phase during which the cell responds to numerous types of signals, with cell-type-specific signal transduction pathways, to transduce the signal for apoptosis to the commitment phase. The commitment phase on the other hand is defined as the phase during which the cell uses a limited number of evolutionarily conserved pathways to integrate the various initiation phase signals into a decision to refrain from apoptosis or commit irreversibly to it, thereby triggering the execution phase, while the execution phase is defined as

the phase during which the cell invokes multiple parallel pathways that lead to the hallmark features of apoptosis (Lazebnik, Cole, Cooke, Nelson, & Earnshaw, 1993; Solary, Bertrand, Kohn, & Pommier, 1993).

Apoptosis can be early detected by the changes in the morphology of the nucleus and in the plasma membrane (Kerr et al., 1972). In the nucleus, a condensation of nuclear chromatin appears and forms a uniformly dark, crescentic mass which migrate towards the nuclear envelope. This process is associated with an abrupt cell shrinkage, during which the specialized surface structures disappear and the cell loses contact with the substrate or its neighbours. Also, convolutions of the plasma membrane begin to appear and progressively develop giving the cell a bubbling or blebbed surface. Shrunken and convoluted cells then begin to pinch at the base of the cytoplasmic blebs and seals afterwards at the point of pinching off. The pinched-off cytoplasmic fragments are known as apoptotic bodies and can contain nuclear fragments, pinched off in a similar manner and enclosed within a double-layered nuclear envelope, and/or other cytoplasmic organelles, which normally do not show morphological alterations (Kerr et al., 1972; Martin et al., 1995; Toescu, 1998; Wyllie, Kerr, & Currie, 1980).

There are two well-characterised apoptosis pathways in mammalian cells. The first one, also called the intrinsic pathway, involves mitochondria, and is triggered and controlled by members of the B-Cell Lymohoma Protein-2 (Bcl-2) family. The second, also known as extrinsic pathway, is mediated by death receptors, a subgroup of the tumor necrosis factor (TNF) receptor superfamily

(Baetu & Hiscott, 2002; Deng, Lin, & Wu, 2002; Falschlehner, Emmerich, Gerlach, & Walczak, 2007; Kandasamy et al., 2003; Petit, Arnoult, Viollet, & Estaquier, 2003). In the mitochondria pathway, death signals lead to changes in mitochondrial membrane permeability and the subsequent release of pro-apoptotic factors involved in various aspects of apoptosis (Green & Reed, 1998). The released factors include cytochrome *c* (cyt *c*), apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac/DIABLO) and endonuclease G (Du, Fang, Li, Li, & Wang, 2000; Li, Luo, & Wang, 2001; Liu, Kim, Yang, Jemmerson, & Wang, 1996; Susin et al., 1999; Verhagen et al., 2000). Cytosolic cyt *c* forms an essential part of the apoptosis complex apoptosome, which is composed of cyt *c*, Apoptosis activation factors-1 (Apaf-1), and procaspase-9. Formation of the apoptosome leads to the activation of caspase-9, which then processes and activates other caspases to orchestrate the biochemical execution of cells (Deng et al., 2002).

The death receptor pathway is initiated by the TNF-α family of cytokines, such as TNF-α, Fas-ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), all of which can act as extracellular activators of apoptosis upon binding to their respective receptors (Armitage, 1994; Cosman, 1994). These "death-inducing" receptors, which include TNF receptor 1 (TNFR1), Fas, TRAIL receptors 1 and 2 (TRAIL-R1 and TRAIL-R2), TNF receptor-related apoptosis-mediating protein (TRAMP) and death receptor 6 (DR6) harbour extracellular cysteine-rich domains as well as cytoplasmic death domains responsible for the recruitment of the death-inducing signaling complex through interaction with adaptor proteins such as Fas associated death domain

protein (FADD) and TNFR1 associated death domain protein (TRADD) (Boldin et al., 1995; Chinnaiyan, O'Rouke, Tewari, & Dixit, 1995; Daniel, Wieder, Sturm, & Schulze-Osthoff, 2001; Hsu, Shu, Pan, & Goedde, 1996). Upon binding of its ligand, TNFR1 recruits FADD through TRADD, while Fas recruits FADD directly. In turn, FADD recruits multiple caspase-8 molecules, resulting in caspase-8 activation through induced proximity (Boldin, Goncharov, Goltsev, & Wallach, 1996; Muzio et al., 1996). Caspase-8 is thought to propagate the apoptosis signaling pathway either by direct processing and activation of caspase-3, or by cleaving Bid, a group III member of the Bcl-2 family; truncated Bid (tBid) in turn targets mitochondria for cyt *c* release. Both the death receptor and the mitochondrial pathways converge at the level of effector caspase-3 activation (Baetu & Hiscott, 2002).

Apoptotic cell death has been implicated as a cytopathological mechanism in response to DV infection both *in vitro* and *in vivo* (Desprès, Flamand, Ceccaldi, & Deubel, 1996). Active replication appears to be a prerequisite for the triggering of the apoptotic process in DV infected cells (Catteau et al., 2003). Although DV can infect a variety of cells, including endothelial and neuronal cells, *in vitro*, only a few cell types have been identified as being infected *in vivo*, and the most recognized target cells for DV in humans are the peripheral blood mononuclear cells (PBMC) (Avirutnan, Malasit, Seliger, Bhakdi, & Husmann, 1998; Jan et al., 2000; Matsuda et al., 2005). Human monocytes appear to be very important in the pathogenesis of dengue infection. They are thought to be the most active sites of virus replication during dengue infection (Kurane & Ennis, 1988). Based on the pathological findings in experimentally

infected rhesus monkeys and in humans with fatal infections, DV is thought to replicate in mononuclear cells *in vivo* and causes cell death (Wang et al., 2002). However, the interactions between DV and the mononuclear cells regarding induction of apoptosis have not been studied (Espina, Valero, Hernandez, & Masquera, 2003). The apoptotic effect of DV towards mononuclear cells could have a beneficial effect by reducing virus production or by removing majority of the infected cells but, it could also at the same time, damage important target cells, with deleterious effect. Therefore, a well-defined mechanism of apoptosis involving DV and PBMC is important due to the fact that cell death is one of the pathogenesis of DV infection. Besides, the information obtained can further lead us to a better understanding of the mechanism of DV infection in human and of course with hope to prevent DV infection.

CHAPTER 2

LITERATURE REVIEW

2.1 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are frequently associated with cytotoxicity, often being described as damaging, harmful or toxic (Carmody & Cotter, 2001). ROS such as superoxide anion (O_2^-), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2), are traditionally thought to be toxic by-products of cellular metabolism (Kannan & Jain, 2000; Zhang & Gutterman, 2006). These radicals can cause serious damage to many biological macromolecules, such as proteins, lipids and nucleic acids, which oxidation leads to a lost of their biological properties and eventually to a cell death (Czarna & Jarmuszkiewicz, 2006). Mitochondria are major sources of ROS and appear to be released both in the matrix and in the intermembrane space (Lenaz, Bovina, Formiggini, & Castelli, 1999). Incomplete scavenging of ROS particularly affects the mitochondrial lipid cardiolipin (CL), triggers the release of mitochondrial cytochrome *c* (cyt *c*), and activates the intrinsic death pathway (Hoye, Davoren, Wipf, Fink, & Kagan, 2008). Mitochondria dysfunction induced by ROS is related to many human diseases and aging (Bai & Cederbaum, 2001). Excess ROS can inflict random cellular damages and alter specific signaling pathways which lead to oxidative stress and often accompanied by loss of cell function, and apoptosis and/or necrosis (Chen, Zhou, Xing, Krysan, & Lou, 2004; Lin, Liao, & Lin, 2004). On the other hand, lowering ROS levels below the homeostasis set

point may interrupt the physiological functions and lead to decreased proliferative response and defective host defenses (Chen et al., 2004). As a consequence, direct or indirect involvement of ROS in numerous diseases has been documented.

ROS is closely linked to degenerative diseases such as Alzheimer's disease, Parkinson's, neuronal death including ischemic and hemorrhagic stroke, acute and chronic degenerative cardiac myocyte death, and cancer (Chen et al., 2004; Hoye et al., 2008). Besides, several studies have shown that viral infections can generate ROS and induce oxidative stress, such as in the case of human immunodeficiency virus (HIV), Sendai virus, cytomegalovirus (CMV), influenza virus, hepatitis B virus (HBV), DV serotype 2, Japanese encaphelitis (JE) virus and tobacco mosaic virus (Cerimele et al., 2005; Lin et al., 2004). It has been shown that generation of ROS in the target cells in response to infection plays a role in virus replication and pathogenesis (Everett & McFadden, 2001).

Machida et al. (2004) in a research involving hepatitis C virus (HCV) showed that patients with chronic hepatitis C often exhibit increased production of tumor necrosis factor (TNF), a cytokine that can produce oxidative stress by stimulating ROS. Further, it was stated that HCV may induce liver damage and hepatocellular carcinoma by a combination of mitochondrial damage and Endoplasmic Reticulum (ER) damage through ROS production. Their studies also provided evidence that HCV alters mitochondrial membrane potential and increases ROS production where the treatment of the HCV-infected cells with

ROS inhibitors effectively prevented mitochondrial damage and production of ROS. This result therefore demonstrated that ROS play a critical role in HCV pathogenesis and, oxidative stress, imposed either directly by the virus or by the host-immune response, is a potentially important pathogenic mechanism HCV disease as well as other chronic liver diseases to initiate and promote multistage carcinogenesis (Machida et al., 2004).

Another study on HCV was also done by Korenaga et al. (2005) where direct incubation of isolated mitochondria with HCV core protein resulted in an increase of calcium influx and ROS production. The investigators also proposed that HCV protein expression caused an increase in mitochondrial ROS production, an oxidation of the mitochondrial glutathione pool, inhibition of electron transport, and an increase in ROS production by mitochondrial electron transport complex I. This change in mitochondrial redox state inhibits complex I activity, further increases ROS production and can create positive feedback loop. Mitochondrial oxidation is therefore associated with enhanced liver injury in alcoholic, toxic, and inflammatory liver diseases, and thus could have significant effects on response to inflammation and the development of progressive liver disease in chronic hepatitis C.

Hagen et al. (1994) in a research involving transgenic animals and HBV have suggested that ROS from activated phagocytes may play a role in the hepatocarcinogeneis of this virus, perhaps similar to the role that ROS play in chemical carcinogenesis (Hagen et al., 1994).

Several studies that relate ROS and apoptosis were also done by many researchers. In a study done by Date et al. (2002), Adenovirus-mediated expression of p35 effectively inhibits a H/R-induced cardiomycyte apoptosis through a reduced level of cellular ROS. This study has proved that a reduced level of ROS can actually prevent apoptosis from occurring.

The DV-infected endothelial cells were also seen to undergo apoptosis in a study done by Yen, Chen, Lin, Shieh, and Wu-Hsieh, (2008). They have showed that endothelial cells infected by DV produce ROS and this production of ROS had a vital role in causing the endothelial cells to undergo apoptosis.

A study on the effect of leptin on ROS production by human monocyte was done by Sánchez-Pozo et al. (2003). This study was based on the fact that leptin can stimulate monocytes and the production of ROS is the result of monocyte activation. They have found that stimulation with leptin produces oxygen radical formation by monocytes. Further, because HIV infection induces the production of ROS, they next investigated the effect of leptin on ROS production in monocytes from HIV-positive (HIV +) subjects. They have also found that monocytes from HIV+ subjects spontaneously produced increased amounts of free radicals.

A conclusion was made by Elbim et al. (1999) where they found that whole-blood monocytes from HIV-infected patients spontaneously produced H_2O_2, to a degree that correlated with viral load and could participate in the overall oxidative injury in these patients. Elbim et al. (1999) also mentioned in their

research that although few blood monocytes are infected, their resulting activation could play a key role in the pathogenesis of HIV disease by modulating their transendothelial migration and inducing the production of ROS. In addition, activated monocytes, together with polymorphonuclear neutrophils, are a major source of ROS. ROS is also said to potentiate the production by monocytes of proinflammatory cytokines, which, in turn, can increase HIV replication. Further in their report, ROS participate in T-lymphocyte depletion by triggering apoptosis and monocytes can also induce their own apoptosis by producing ROS. Based on all the facts and results above, ROS seem to play an important role in apoptosis.

2.2 The B-Cell Lymphoma Protein-2 (Bcl-2)

The Bcl-2 family members are important regulators of the mitochondrial pathway of apoptosis (Gustafsson & Gottlieb, 2007). These proteins consists of two subfamilies; pro-apoptotic members such as Bax, Bad, Bim, Bik, or Bcl-xs which initiate or promote apoptotic signal, and anti-apoptotic members such as Bcl-2, Bcl-xL, Mcl-1, or A1 which block the activation of effector caspases, such as caspase-3 and caspase-2, which transduce the apoptotic signals (Gu¨rsoy, Ergin, Bas_alog˘lu, Koca, & Seyrek, 2008). Both pro-apoptotic and anti-apoptotic members are characterised by the presence of Bcl-2 homology (BH) domains, namely BH1 to BH4 and have been classified into three functional groups (Baetu & Hiscott, 2002; Falschlehner et al., 2007; Loo et al., 2002). Members of group I, such as Bcl-2 that possess anti-apoptotic activity contain all four subtypes of BH domains (BH1-BH4), while members of group

II, such as Bax and Bak which are pro-apoptotic contain only three domains (BH1-BH3) and group III, which are also pro-apoptotic consists of diverse proteins whose only common feature is the presence of the BH3 domain (Baetu & Hiscott, 2002; Galluzzi, Brenner, Morselli, Touat, & Kroemer, 2008). Anti-apoptotic Bcl-2 proteins have been reported to protect cells from many different apoptotic stimuli and are important for cell survival (Gustafsson & Gottlieb, 2007). This protein has the ability to bind to membranes and form, predominantly under non-physiological conditions, ion-conducting channels in synthetic membranes (Belizário, Alves, Occhiucci, Garay-Malpartida, & Sesso, 2007).

Bcl-2, which is present in the outer mitochondria membrane, therefore, has been suggested to prevent apoptosis either by sequestering proforms of death-driving cysteine proteases called caspases (a complex called the apoptosome) or by preventing the release of mitochondrial apoptogenic factors such as cytochrome *c* (cyto *c*) and AIF (apoptosis-inducing factor) into the cytoplasm (Tsujimoto, 1998). Besides, this anti-apoptotic protein also block apoptosis by preventing the matrix swelling, reactive oxygen species (ROS) damage and the loss of membrane potential associated with apoptosis (Gu¨rsoy et al., 2008; Harris & Thompson, 2000).

The anti-apoptotic property of the Bcl-2 protein has been confirmed in a study done by Carthy et al. (2003) whereby it was demonstrated that overexpression of Bcl-2 prevented the release of cyt *c* from mitochondria whilst protecting cells from apoptosis.

Similarly, the anti-apoptotic property of Bcl-2 was also proven in another study done by Zheng, Yang, Guocai, Pauza, and Salvato, (2007). In this research, they showed that HIV Tat protein increases Bcl-2 expression in monocytes which inhibited apoptosis induced by Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL).

On the other hand, Nakamoto, Kaneko, and Kobayashi, (2002) in a research that involved HCV-infected peripheral blood mononuclear cell (PBMC) had confirmed that the increased susceptibility of infected PBMC to apoptosis was associated with diminished intracellular expression of the anti-apoptotic protein Bcl-2. They further demonstrated that the abnormality of PBMC subsets in undergoing apoptosis as a result of the down-regulation of Bcl-2 expression may contribute to viral persistence and progression of liver disease in chronic hepatitis C.

The presence of low amount of Bcl-2 detected in lymphocytes from dengue patient in a study conducted by Azeredo et al. (2006) also proved that Bcl-2 played a critical role in preventing the cells from undergoing apoptosis during DV infection. This was important in determining the possible role of Bcl-2 in clearing the DV during the acute phase of DV infection.

However, a contrasting result was obtained from Carvalho et. al. (2009) where they showed that although apoptosis was induced in human monocytes infected with DV, the level of Bcl-2 did not have any significant changes. The

researchers therefore suggested that other anti-apoptotic protein, such as Bcl-XL was negatively regulated during infection in the monocytes.

2.3 Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL)

TRAIL or known as Apo2 ligand (Apo2L) is a 40 kDa type II transmembrane protein that is structurally related to TNF family of cytokines (Baetu & Hiscott, 2002; Ishikawa, Nakazawa, Yoshinari, & Minami, 2005). The extracellular domain of TRAIL shares the highest amino acid homology with the fibroblast associated ligand (FasL) of which is known to initiate apoptosis of transformed cells and activated lymphocytes through the death receptor pathway. TRAIL was discovered to be able to induce apoptosis rapidly and expressed in variety of cells and tissues (Falschlehner et al., 2007; Matsuyama et al., 2004). TRAIL is mostly expressed by cells of the immune system like T and B cells, natural killer cells (NK cells), dendritic cells (DC), PBMC, spleen and thymus (Baetu & Hiscott, 2002; Yang et al., 2003). Unlike other members of the TNF ligand family, TRAIL has received particular attention because early reports suggest that it induces apoptosis in a variety of cancer cells with little or no effect on normal cells (Mundt et al., 2005). This cytotoxic property of TRAIL towards a variety of tumor cells but not normal cells has made TRAIL as one of the most promising candidates for cancer therapeutics (Horinaka et al., 2005). While TRAIL has received great attention in cancer therapy contexts because it selectively induces apoptosis in tumor cells but not normal cells, it is also proven to be a potent inducer of apoptosis in virally-infected cells that are normally TRAIL-resistant when uninfected (Brincks, Kucaba, Legge, &

Griffith, 2008). TRAIL was therefore shown to play a role in the homeostasis of certain T cells, Natural Killer (NK) cells and T-cell- mediated killing of virally and oncogenically transformed cells (Falschlehner et al., 2007).

Many investigations have shown that various cytokines and virus infection differentially modulate TRAIL activation. In a study done by Sedger et al. (1999) and Clarke et al. (2000), it was shown that human cytomegalovirus (CMV) infection directly up-regulates the expression of TRAIL on virus-infected fibroblast cells. These virus-infected cells become susceptible to apoptosis via TRAIL.

A study done by Ishikawa et al. (2005) on mice have showed clearly that the level of TRAIL mRNA was increased in the lung after exposing the mice to Influenza A virus. Furthermore, the analysis of the expression of TRAIL on the cell surface of lung mononuclear cells revealed that Influenza A virus infection induces TRAIL expression on NK cells and T cells. TRAIL expressed on NK cells after Influenza A virus infection might induce apoptosis of macrophages or DC, which play some role in elimination of the virus infected cells at the early phase of the infection. Thus, Ishikawa et al. (2005) has proposed that TRAIL plays a role in elimination of virus-infected cells and/or in immune modulation after viral infection.

Baetu and Hiscott (2002) also emphasized that TRAIL played an important role in virus induced disease and TRAIL expression is up-regulated as a consequence of infection by multiple viruses. TRAIL may be responsible for

the activation-induced cell death (AICD) of bystander T cells during Acquired Immunodeficiency Syndrome (AIDS) (Baetu & Hiscott, 2002).

TRAIL's function was also observed in another study done by Brincks et al. (2008) where they showed that TRAIL expression is up-regulated on multiple PBMC populations in response to influenza virus (H5N1) stimulation and TRAIL-induced apoptosis is specifically increased in influenza-infected cells compared with uninfected cells. TRAIL up-regulation during the immune response to highly pathogenic influenza strains might significantly contribute to the pathogenicity of these infections. Therefore this study demonstrated that TRAIL-induced apoptosis plays a key role in clearance of virus-infected cells.

A study involving HIV by Kim, Dabrowska, Jenner, and Aldovini (2007) observed an elevated level of TRAIL in serum samples from HIV-infected patients as compared to those of uninfected subjects. Besides the study also indicated that TRAIL has been shown to trigger apoptosis in T cells from HIV-infected individuals, while T cells from uninfected controls are completely resistant to TRAIL-induced apoptosis (Kim et al., 2007).

The level of TRAIL was also being determined in studies involving DV. In a study done by Warke et al. (2008), the human muscle satellite cells were seen to undergo apoptosis after infected with DV and the level of TRAIL was seen to be upregulated as compared to controls. In addition, Warke et al. (2008b) in another research also found similar results where the levels of TRAIL expressions were higher in DV-infected human umbilical veins endothelial

cells, dendritic cells, monocytes and B cells. They also made a conclusion that TRAIL plays an important role in the antiviral response to DV infection and is a candidate for anti-viral interventions against DV.

2.4 Caspases

Apoptosis involves many cellular processes including the sequential activation of caspases (Swanton, Savory, Cosulich, Clarke, & Woodman, 1999). Caspases, a family of cysteine-dependent aspartate-directed proteases, play critical roles in initiation and execution of apoptosis by cleaving a large number of proteins, which in turn lead to the typical morphology of apoptosis (Liu, Wei, & Kwang, 2004). Synthesized as relatively inactive single-chain procaspases or zymogens, caspases are normally present in the cell as proenzymes and must undergo a process of activation during apoptosis (Falschlehner et al., 2007; Wang, Zhi, Wu, & Zhang, 2008). Caspases can be classified into two groups based on their place in the caspase cascade, which are, "initiator caspases" that contain a large prodomain and "executioner caspases" which are characterized by a small prodomain (Belizário et al., 2007; Liu et al., 2004). Initiator caspases possess a longer prodomain which either contains a caspase recruitment domain (CARD), as is the case for caspases-9 or a death effector domain (DED) as is the case for caspase-8. These prodomains enable the caspases to interact with other proteins that regulate their activation (Boutrois et al., 2008). Binding of the initiator caspase precursors to activator molecules appears to promote procaspase oligomerization and autoactivation. Enzymatic activation of initiator caspases leads to proteolytic activation of

downstream effector caspases like caspases-3/7 and cleavage of a number of vital proteins, resulting in the orderly demise and removal of the cell (Green & Reed, 1998; Nuñez, Benedict, Hu, & Inohara, 1998; Sharangpani, Takanohashi, & Bell, 2008) The activated effector caspases will also activate DNases, leading to destruction of the protein scaffold and alterations within the cell membrane which leads to deoxyribonucleic acid (DNA) fragmentation (Boutrois et al., 2008).

Two caspase cascades, initiated by either extracellular (extrinsic) or intracellular (intrinsic) stimuli lead to activation of additional, effector caspases that lead to DNA fragmentation, membrane blebbing and cell death (Sharangpani et al., 2008). Initiating events of the caspase cascade have been well characterized, and one of which is the extrinsic caspase cascade that involves the activation of caspase-8 (Liu et al., 2004; Sharangpani et al., 2008). The extrinsic pathway is characterized by binding of death receptors, such as Fas, tumor necrosis factor receptor (TNF-R) and TNF-related apoptosis inducing ligand receptor (TRAIL-R) to their ligands. This binding leads to interactions of adaptor molecules and procaspases 8, forming the death-inducing signaling complex (DISC), which activates caspase-8 (Hussein, Haemel, & Wood, 2003; Sarmento, Tseggai, Dhingra, & Fu, 2006). Activated caspase-8 subsequently cleaves and activates down-stream caspases, such as caspase-3/7 and subsequently cell death (Peter & Krammer, 1998). Activation of caspase-3/7 represents a certain death by apoptosis for the cell (Luthra et al., 2007).

The intrinsic caspase cascade, initiated by ROS or reactive nitrogen species (RNS), decreased oxygen supply and others, involves activation of caspase-9. In this cascade, procaspase-9, cyt *c* (released from injured mitochondria) and apoptosis protease activating factor-1 (Apaf-1) bind to form the apoptosome complex. When the apoptosome complex dimerizes, procaspase-9 is cleaved to its active form and this leads to activation of effector caspases-3/7 (Liu et al., 2004; Sarmento et al., 2006; St-Louis & Archambault, 2007; Zhang, Zhang, & Herman, 2003).

Caspase-3 and caspase-7, known as executioner caspases, are located down stream of the caspase-8 or caspase-9 activation cascade and represent a point where the intrinsic and extrinsic apoptosis pathways converge (Fischer et al., 2003). Once activated, the executioner caspases are responsible for the proteolytic cleavage of a broad range of cellular proteins (Belizário et al., 2007; Fischer et al., 2003). Proteolysis causes characteristic apoptotic morphological changes and brings about cell death (Zhang et al., 2003).

The involvement of caspases in the intrinsic and extrinsic pathways of apoptosis had been conducted in different researches. In a study done by Chou et al. (2006), it was found that HCV E2 infected Huh-7 cells undergo apoptosis and activated procaspases-3, -8 and -9. The data therefore suggested that the HCV E2 may induce apoptosis through a mitochondrial damage-mediated caspase pathway. Another study on HCV-specific $CD8^+$ T cells done by Radziewicz et al. (2008) showed that the apoptosis of the infected T cells were activated by caspase-9. Remarkably, in the chronic phase of HCV infection, at

the site of infection in the liver, a substantial frequency of caspase- 9-mediated T-cell death was also present. In a similar study on Hepatitis virus, Shi and Guan (2009) found that on HBV-expressing HepG2.2.15 cells, the cells underwent apoptosis and the level of caspase-7 gene expression was increased and therefore responsible for the synergistic induction of apoptosis by interferon-gamma (IFN-γ) and TNF-alpha (TNF-α).

Muthumani et al. (2002) observed that caspase-9, but not caspase-8, was activated following infection of human PBMC with either viral protein R (Vpr)-positive HIV virions or adeno-delivered Vpr. Activation of the caspase-9 pathway resulted in caspase-3 activation and apoptosis in human primary cells. These effects were coincident with the disruption of the mitochondrial transmembrane potential and induction of cyt *c* release by Vpr and concluded that the apoptosis was through the mitochondrial pathway. In another study involving HIV, Peraire et al. (2007) showed that the PBMC in patients with active HIV-1 replication has significantly higher caspase-3 and caspase-9 activity as compared to healthy controls and this brought about the highest overall apoptotic activation in the PBMC of HIV-1 patients.

A research conducted by Sarmento et al. (2006) on rabies virus CVS-B2C showed that activation of caspase-8 and -3, but not of caspase-9, was observed in CVS-B2C-infected BSR cells. The research then concluded that CVS-B2C infection activates the extrinsic, but not the intrinsic, apoptotic pathway of the infected BSR cells. In a separate study on avian reovirus, Chulu et al. (2007) demonstrated that avian reovirus infected BHK-21 cells showed activation of

caspases-9 and -3. These data therefore suggested that the apoptosis of BHK-21 cells infected with avian reovirus was through mitochondria-mediated pathway.

A separate research done on DV by Klomporn, Panyasrivanit, Wikan and Smith (2011) found that DV induced apoptosis in monocytic cells and thereafter activated caspase-8 and caspase-9 and suggested that the DV-infected monocytic cells undergone apoptosis through intrinsic and extrinsic pathways.

A study that involved a nine-residue sequence of the DV protein referred to as ApoptoM also revealed that the ApoptoM-expressing cells had detected caspase-3 like activity (Catteau et al., 2003). However, in that same study, there was no role for caspase-9 in ApoptoM mediated cell death and this data further suggested that a particular mitochondrion-dependant apoptotic pathway may be involved in induction of apoptosis by ApoptoM.

CHAPTER 3

MATERIALS AND METHODS

3.1 Blood Collection

Fifteen mililiter of blood was collected from dengue patients warded at Hospital Besar Kuala Lumpur (HKL), Malaysia with ethical consent approved by the Ministry of Health, Malaysia. Twenty three patients (8 males and 15 females) with mean age of 28 years and with average 6^{th} day of infection were selected for this study. These patients suffered from pure dengue symptoms according to the World Health Organisation (WHO) criteria, which are, fever, myalgia, joint pain, headache, nausea/vomiting, presence of rashes and thrombocytopenia. Besides, these patients were also free from flu, cough, sore throat and any other non-related diseases. There were no dengue haemorrhagic patients recruited in this study as the patients suffered other complications and was totally excluded. Ten normal healthy controls were of healthy people free form diseases and also free from drug and were chosen randomly from blood donation campaigns with ethical consent. The blood from dengue patients and healthy donors were collected in a heparinised tube with EDTA to prevent blood clot.

3.2 PBMC Separation

The blood collected was diluted 1 time with Phosphate Buffered Saline (PBS) (SIGMA®) and mixed slowly to prevent hemolysis. The diluted blood was suspended slowly on top of 3mL Ficoll-Paque (GE Healthcare) solution in a centrifuge tube. The mixture was then centrifuged at 2,400 rpm for 40 minutes. Four different layers were observed after centrifugation. The plasma layer was discarded. The thin layer below the plasma layer was taken as the PBMC. The remaining 2 layers below the PBMC, which are the Ficoll-Paque solution and the red blood cell, were discarded. The PBMC obtained was topped with PBS. The PBMC mixture was then centrifuged at 2,500 rpm for 10 minutes at 18°C to obtain PBMC pellet. The pellet obtained was suspended in PBS and PBMC was subjected to cell count.

3.3 Counting Cells Using A Hemocytometer

The hemocytometer was prepared by washing it with detergent, rinsing with distilled water and thereafter wiping off with alcohol. The alcohol was made sure to be totally wiped off from the surface of the hemocytometer. A cover slip was placed on the hemocytometer and a drop of the PBMC suspension was ejected by using a pipette to fill the space between the coverslip and the hemocytometer. The PBMC would be distributed on the hemocytometer and the viable PBMC which appear round and shining were counted. Too many or too little PBMC present on the hemocytometer were avoided in this study. The PBMC were diluted by adding more PBS to the cell pellet if the cells observed

on the hemocytometer were too compact. The number of PBMC required for subsequent assays was 1 X 10^6. The constant number of PBMC obtained was either used immediately for analysis or stored at -80°C for future use.

3.4 Confirmation of PBMC Apoptosis

3.4.1 By Using Mitocapture™ Apoptosis Detection Kit (Calbiochem)

The apoptosis of PBMC in dengue patients and healthy controls was confirmed by staining the PBMC with Mitocapture™ Reagent as soon as the PBMC was obtained. A constant number of PBMC (1 X 10^6) from dengue patients and healthy donors were used throughout this study. The PBMC was suspended in 1mL Diluted Mitocapture™ Reagent and incubated in a 5% CO_2 incubator at 37°C for 15-20 minutes. The stained PBMC was subjected to centrifugation to obtain PBMC pellet and the supernatant was discarded. The PBMC pellet was suspended in 1mL pre-warmed incubation buffer provided and was analyzed under fluorescent microscopy. The PBMC suspension was placed on a glass slide and covered with a glass coverslip. The PBMC was analyzed immediately under a fluorescent microscope (Olympus) using a band-pass filter that detects fluorescein and rhodamine. The PBMC that appeared green fluorescence was regarded as apoptotic PBMC and the PBMC that appeared red fluorescence was regarded as healthy PBMC.

3.4.2 By Cell Cycle Analysis by Using Flow Cytometry

The apoptosis in PBMC of dengue-infected patients were then being further confirmed by analyzing the PBMC cell cycle using flow cytometry.

3.4.2.1 Fixing of PBMC

The PBMC pellet was suspended in 300µL of cold PBS and 700µL of 100% ethanol and stored at -20°C for 24 hours.

3.4.2.2 Staining of PBMC

The PBMC suspension was centrifuged at 2000 rpm for 1 minute to obtain the PBMC pellet. The PBMC pellet was added with 500µL PBS, 1µL of 20µg/mL propidium iodide and 2µL of 15µg/mL RNase. The PBMC suspension was incubated in dark for 10 minutes.

3.4.2.3 Flow Cytometry Analysis

The stained PBMC were analysed on a FACS Calibur flow cytometer (Beckton Dickinson, US) immediately with a laser emission at 488 nm and the PI fluorescence at 600 nm long pass filter (FL2) in linear mode. The data was obtained by using CellQuest Pro software.

3.5 Dengue NS1 and IgM Confirmation

The serum obtained from dengue patients at HKL were further confirmed to have dengue infection by using Dengue NS1 (PanBio) and Dengue IgM (Calbiochem) kits by following the instructions stated on the kits.

3.6 Hydrogen Peroxide (H_2O_2) Assay by Using Amplex® Red Hydrogen Peroxidase/Peroxidase Assay Kit (Molecular Probes, Invitrogen)

3.6.1 Ten micro Molar Amplex® Red Reagent Stock Solution Preparation

The Amplex® Red Reagent was dissolved in 60µL Dimethyl Sulfoxide (DMSO).

3.6.2 One Time (1X) Reaction Buffer Preparation

Sixteen mililitre of deionized water (dH20) was added to 4mL of 5X Reaction Buffer. This 20mL of Reaction Buffer working solution was sufficient for approximately 100 assays of 100µL each with 10mL excess for making stock solution.

3.6.3 Ten Units per milli Liter Horseradish Peroxidase (HRP) Stock Preparation

The HRP was dissolved in 1mL of 1X Reaction Buffer. Any of the unused HRP stock solution was divided into single-use aliquots and stored frozen at -20°C after the assay.

3.6.4 Working Solution of 100µM Amplex® Red Reagent and 0.2U/mL HRP Preparation

Fifty micro litre of 10mM Amplex® Red reagent stock solution, 100µL of 10U/mL HRP stock solution and 4.85 mL of 1X Reaction Buffer were mixed to obtain the working reagent.

3.6.5 H_2O_2 Assay Procedure

This assay required fresh PBMC, i.e. PBMC obtained as soon as the blood was collected and processed. Fifty micro liters of the Amplex® Red reagent/HRP working solution was added to 1 X 10^6 fresh PBMC. The mixture was incubated for 30 minutes protected from light. The fluorescence was taken by using a microplate reader at ~590nm. The fluorescence intensity in the PBMC of the dengue patients and normal healthy controls were compared.

3.7 Lysing of PBMC

A constant amount of PBMC (1 X 10^6) from confirmed dengue patients and healthy controls were lysed for future assays. The PBMC pellets were resuspended in 1mL of Resuspension Buffer which consisted of 50 mM Tris, containing 5 mM EDTA, 0.2 mM PMSF, 1 µg/ml pepstatin and 0.5 µg/ml leupeptin and adjusted to pH 7.4. Twenty micro liter of Antigen Extraction Agent was added for every 100 mL of cell suspension. The mixture was incubated on ice with occasional vortexing. The extracts were transferred to microcentrifuge tubes and centrifuged for 5 minutes. The clear lysate were aliquoted to clean microfuge tubes. The lysates were either used immediately or stored in -80°C for future use. The lysates were kept in small aliquots to avoid multiple free thaw cycles.

3.8 Bcl-2 Detection by Using Bcl-2 ELISA Kit (Calbiochem)

3.8.1 Wash Buffer (1X) Preparation

Twenty five milliliter Concentrated Wash Buffer solution was added with 475mL of deionised water to obtain 1X Wash Buffer. The mixture was mixed well.

3.8.2 Lyophilised Standard Preparation

Six tubes were obtained and labeled 200, 80, 32, 12.8, 5.12 and 0 U/mL. Three hundred sixty micro liters of Sample diluents were added into each tube except the 200 U/mL tube (the first tube). Six hundred micro liters of solution from the original vial of lyophilized material was removed and added into the first tube. Two hundred forty micro liters of solution was removed from the first tube (200 U/mL) and added to the second tube (80 U/mL) and was mixed gently. This procedure was repeated until the fifth tube (5.12 U/mL). The last tube (0 U/mL) contained only the Sample Diluent. The reconstituted standards were discarded after one use.

3.8.3 Conjugate (1X) Preparation

Sixty micro liters of 200X Conjugate was added to 12 mL of Conjugate Diluent to obtain a 1X Conjugate. The diluted Conjugate was filtered with a 0.2µm syringe filter prior to use.

3.8.4 Assay Procedure

Fifty micro liter of Detector Antibody was added into appropriate microtiter plates. Fifty microliters of PBMC lysates and standards were added into the wells. The plate was covered with a plate sealer and incubated at room temperature for 2 hours. The wells were washed 3 times with washing buffer. Hundred micro liters of diluted conjugate was added into each well and

covered with a plate sealer. The plate was incubated at room temperature for 30 minutes. The wells were washed 3 times with washing buffer and the plate was flooded entirely with distilled water. The contents of wells were removed by inverting the plate and tapping the plate on paper towels. Hundred micro liters of substrate solutions were added into each well and incubated in dark at room temperature for 30 minutes. Hundred micro liters of stop solution was added into each well and the absorbance was read by using a spectrophotometric reader at 450 nm. The plate was read within 30 minutes after the addition of stop solution.

3.9 TRAIL Detection by Using Human TRAIL Immunoassay Kit (Biosource)- ELISA Method

3.9.1 Human TRAIL Standard Preparation

The given standard was reconstituted to 3000 pg/mL with Standard Diluent Buffer. The reconstituted standard was mixed gently by swirling and allowed to sit for 10 minutes to ensure complete reconstitution. The standard was used within 1 hour of reconstitution. Three hundred micro liter of Standard Diluent Buffer was added to each of 6 tubes labeled 1500, 750, 375, 187.5, 93.7 and 46.8 pg/mL of Human TRAIL. Serial dilutions of the standard were conducted as follows. The first tube was the reconstituted standard of 3000pg/mL and 300µL of the 3000pg/mL standard was transferred to the second tube labeled 1500 pg/mL. Three hundred microliters of the mixture from the second tube (1500pg/mL) was transferred to the third tube (750pg/mL). The procedure was

repeated until the last tube (46.8pg/mL) and the last tube (0pg/mL) contained only the Diluent Buffer. Any remaining standard were discarded after the assay.

3.9.2 Straptavidin-HRP Working Solution Preparation

Ten micro liters of the given 100X concentrated Straptavidin-HRP solution was diluted with 1mL of Straptavidin-HRP Diluent for each 8-well strip used in the assay. This was labeled as the Streptavidin-HRP Working Solution.

3.9.3 Wash Buffer Preparation

The 25X Wash Buffer Concentrate was allowed to reach room temperature and mixed to ensure that any precipitated salts had redissolved. One volume of the 25X Wash Buffer Concentrate was diluted with 24 volume of deionised water and labeled as Working Wash Buffer. The Working Wash Buffer was stored in the refrigerator and used within 14 days.

3.9.4 Assay Procedure

Hundred micro liters of PBMC lysates and standards were transferred to appropriate microtiter plates. The side of the plate was gently tapped for mixing the contents. The plate was covered with plate cover and incubated for 2 hours at room temperature. The solutions from wells were thoroughly aspirated and the liquid was discarded. The wells were washed 4 times with

washing buffer. Hundred micro liter of biotinylated anti-TRAIL (Biotin Conjugate) solution was pipette into each well. The side of the plate was tapped gently for mixing the contents. The plate was covered with plate cover and incubated for 1 hour at room temperature. The solution from wells was thoroughly aspirated and the liquid was discarded. The wells were washed 4 times again. Hundred microliters of Streptavidin-HRP Working solution was added to each well. The plate was covered again with plate cover and incubated for 30 minutes at room temperature. The solution from each wells were thoroughly aspirated and the liquid was discarded. The wells were washed again for 4 times. Hundred micro liters of Stabilised Chromogen was added to each well. The liquid in the wells began to turn blue in colour. The plate was incubated in dark for 30 minutes at room temperature. Hundred micro liters of Stop Solution was added into each well. The side of the wells was tapped gently for mixing the contents. The solution in the wells had changed colour from blue to yellow. The absorbance of each well was read at 450nm and the plate was read within 2 hours after the addition of the Stop Solution.

3.10 Caspase-9 Assay by Using Caspase-9 Activity Assay Kit (Calbiochem)

3.10.1 Sample Buffer Preparation

Sixty micro liters of Sample Buffer was added to every 1 X 10^6 PBMC. Ten microliters of the 1M stock DTT solution was added to every 1mL Sample Buffer.

3.10.2 Assay Buffer Preparation

Sixty micro liters of Assay Buffer was added to every 1 X 10^6 PBMC. Ten microliters of the 1M stock DTT solution was added to every 1mL Assay Buffer.

3.10.3 Assay Procedure

A constant number of PBMC (1 X 10^6) from dengue patients and healthy controls was suspended in 50 µL of sample buffer. The mixture was vortexed and incubated on ice for 10 minutes. The mixture was then centrifuged at 500x g for 5 minutes. Fifty microliters of the clear lysate was transferred to a black 96 wells plate and added with 50 µL of Assay buffer. The mixture was added with 10 µL caspase-9 substrate conjugate and was incubated at 37°C for two hours. The plate was read using a fluorescent reader capable of measuring excitation ~400 nm and emission at ~505 nm.

3.11 Caspase-8 Assay by Using Caspase-8 Activity Assay (Calbiochem)

3.11.1 Sample Buffer Preparation

Sixty micro liters of Sample Buffer was added to every 1 X 10^6 PBMC. Ten microliters of the 1M stock DTT solution was added to every 1 mL Sample Buffer.

3.11.2 Assay Buffer Preparation

Sixty micro liters of Assay Buffer was added to every 1 X 10^6 PBMC. Ten microliters of the 1M stock DTT solution was added to every 1mL Assay Buffer.

3.11.3 Assay Procedure

A constant number of PBMC (1 X 10^6) from dengue patients and healthy controls was suspended in 50 µL of sample buffer. The mixture was vortexed and incubated on ice for 10 minutes. The mixture was then centrifuged at 500x g for 5 minutes. Fifty microliters of the clear lysate was transferred to a black 96 wells plate and added with 50 µL of the Assay buffer. The mixture was added with 10 µL caspase-8 substrate conjugate and was incubated at 37°C for two hours. The plate was read using a fluorescent reader capable of measuring excitation ~400 nm and emission at ~505 nm.

3.12 Caspase-3/7 Activity Assay by Using Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega)

3.12.1 Apo-ONE® Caspase-3/7 Reagent Preparation

Hundred microliter of 100X Substrate was added to 9,900 µL Buffer to obtain 1X substrate and this was labeled as Apo-ONE® Caspase-3/7 reagent.

3.12.2 Assay Procedure

Apo-ONE® Caspase-3/7 reagent was added to each well of a black 96 well plate containing 1 X 10^6 PBMC. The contents were gently mixed using a plate shaker at 300-500 rpm from 30 seconds up to reading time and incubated at room temperature for 30 minutes to 18 hour. The fluorescence was measured for each well at excitation wavelength 485 ± 20 nm and emission wavelength 530 ± 25 nm.

3.13 Statistical Analysis

The differences in the levels of apoptotic factors between controls and dengue patients were analysed by using Student T-test where the p-values lesser than 0.05 were taken to have a statistically significant difference between each other. Besides, the correlation between the days of infection and the different apoptotic markers were analysed using the Pearson Correlation Analysis. Similarly, any results with p-value lesser than 0.05 were taken to have a statistically significant correlation with each other. All the statistical analyses of the data were done using SPSS Statistics 19.0.

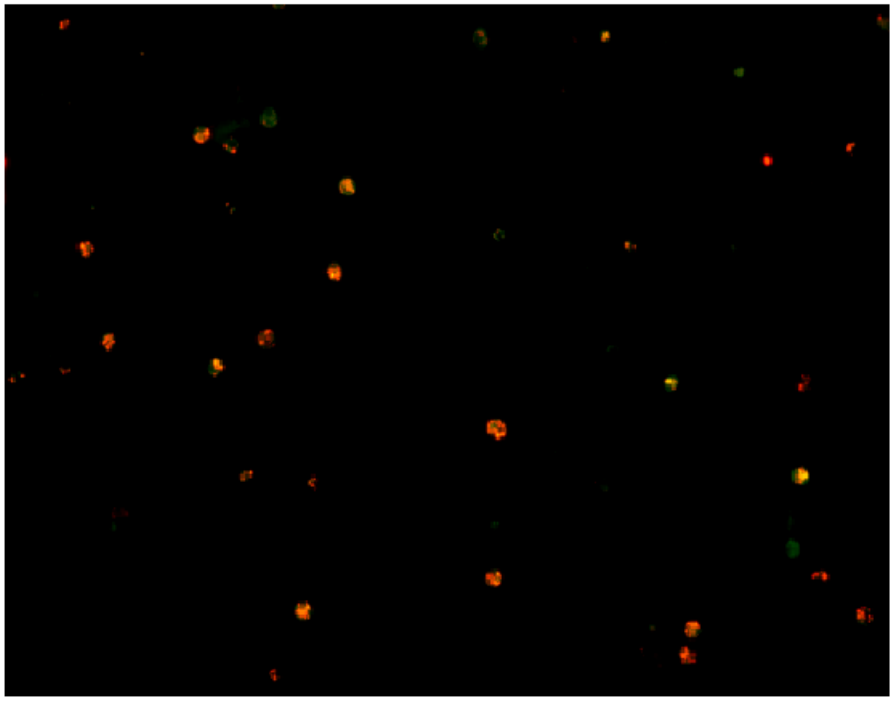
CHAPTER 4

RESULTS

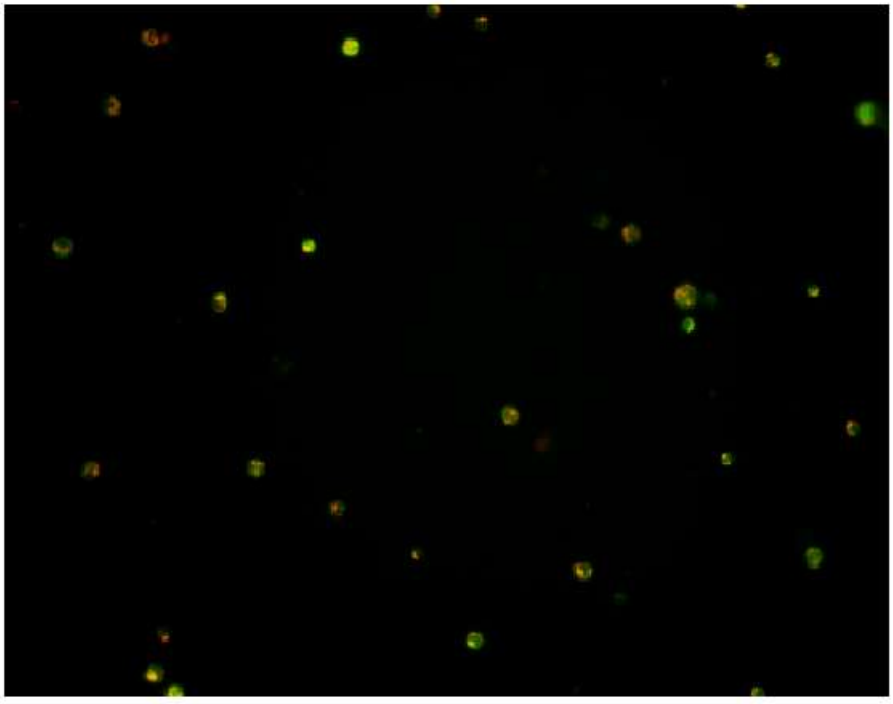
4.1 Confirmation of PBMC Apoptosis

4.1.1 By Using Mitocapture™ Apoptosis Detection Kit

From Figure 4.1, the round and stained cells were the PBMC. The controls PBMC appeared to be in red fluorescent. Controls appeared to have mostly red fluorescent PBMC although a very little amount of green fluorescent PBMC was also observed. However, in patients PBMC, the PBMC appeared to be in green fluorescent. Patients had mostly all green fluorescent PBMC as compared to controls PBMC.



Control



Patient

Figure 4.1: Photos of the control's PBMC and dengue patient's PBMC stained with Mitocapture™ Apoptosis Detection Kit and photographed under a fluorescent microscope (100X).

4.1.2 By Using Cell Cycle Analysis Under Flow Cytometry

From Table 4.1, the controls cell cycle analysis showed that there were higher numbers of PBMC in the G1 phase (80.99%) as compared to other phases. The number of PBMC in the sub G1, S and G2+M phase appeared low as compared to the G1 phase in controls. However, in patient cell cycle analysis, the analysis showed a higher number of PBMC in the sub G1 phase (83.95%) as compared to the other phases. The number of PBMC in the G1, S and G2+M phase appeared low as compared to the sub G1 phase in dengue patients.

Table 4.1: Table showing the cell cyle phases and the relative average PBMC ± standard deviations of healthy controls and dengue patients.

Cell cycle phases	**Average PBMC ± Standard Deviation (%)**	
	Controls	**Patients**
Sub G1	17.88 ± 1.41	83.95 ± 4.53
G1	80.99 ± 1.54	14.57 ± 4.52
S	0.5 ± 0.19	0.82 ± 0.31
G2+M	0.63 ± 0.21	0.68 ± 0.13

4.2 Determination of H_2O_2 Produced

The concentrations of H_2O_2 detected in PBMC of dengue patients and controls are tabulated in Table 4.2. Dengue patients showed a significant elevated concentration of H_2O_2 as compared to controls ($p < 0.05$).

Table 4.2: Table showing the mean H_2O_2 fluorescence intensity ± standard deviation from PBMC of controls and dengue patients measured under a fluorescent reader at A_{590} and the respective p-values.

PBMC	No. of patients (n)	Mean H_2O_2 fluorescence intensity	p-value
Controls	10	835 ± 518.58	0.01
Patients	23	1414 ± 900.03	

4.3 Determination of Bcl-2 Produced

The concentrations of Bcl-2 detected in PBMC of dengue patients and controls are tabulated and summarised in Table 4.3. There was no significant difference in the concentrations of Bcl-2 in patients and controls as $p>0.05$.

Table 4.3: Table showing the mean absorbance and mean concentration of Bcl-2 ± standard deviation from PBMC of controls and dengue patients measured under a microplate reader at A_{450} and the respective p-values.

PBMC	No. of patients (n)	Mean A_{450}	Mean Concentration of Bcl-2 (units/mL)	p-value
Controls	10	0.09 ± 0.03	9.81 ± 3.40	0.34
Patients	23	0.22 ± 0.61	22.63 ±63.50	

4.4 Determination of TRAIL Produced

The TRAIL concentrations in PBMC from dengue patients and controls were determined and shown in Table 4.4. The TRAIL concentrations in dengue patients had a significant higher level as compared to controls ($p < 0.05$).

Table 4.4: Table showing the mean absorbance and mean concentrations of TRAIL ± standard deviation from PBMC of controls and dengue patients measured under a microplate reader at A_{450} and the respective p-values.

PBMC	No. of patients (n)	Mean A_{450}	Mean Concentration of TRAIL (pg/mL)	p-value
Controls	10	0.62 ± 0.16	158.58 ± 41.19	1.52×10^{-7}
Patients	23	1.14 ± 0.29	293.48 ± 75.47	

4.5 Determination of Caspase-9 Activity

Caspase-9 activities in PBMC of dengue patients and controls were measured under a fluorescence reader and the results were tabulated in Table 4.5. It is shown that the caspase-9 activities in dengue patients were significantly higher than the controls as $p<0.05$.

Table 4.5: Table showing the mean fluorescence intensity of caspase-9 ± standard deviation from PBMC of controls and dengue patients measured under a fluorescence reader at excitation ~400 nm and emission ~505 nm and the respective p-values.

PBMC	No. of patients (n)	Mean caspase-9 fluorescence intensity (RFU)	p-value
Controls	10	5259.80 ± 3296.13	3.09×10^{-6}
Patients	23	21796.76 ± 13302.25	

4.6 Determination of Caspase-8 Activity

The mean fluorescence intensities of caspase-8 which represented the caspase-8 activities in PBMC of dengue patients and controls were measured and tabulated in Table 4.6. There was a significant difference between the concentrations of caspase-8 in dengue patients and controls as the p-value is lesser than 0.05.

Table 4.6: Table showing the mean fluorescence intensity of caspase-8 ± standard deviation from PBMC of controls and dengue patients measured under a fluorescence reader at excitation ~400 nm and emission ~505 nm and the respective p-values.

PBMC	No. of patients (n)	Mean caspase-8 fluorescence intensity (RFU)	p-value
Controls	10	2799.63 ± 2075.62	4.11×10^{-4}
Patients	23	8228.68 ± 6249.67	

4.7 Determination of Caspase-3/7 Activity

The mean concentrations of caspase-3/7 in PBMC of dengue patients and controls were summarised in Table 4.7. The concentrations of caspase-3/7 in dengue patientswere significantly higher as compared to controls ($p<0.05$).

Table 4.7: Table showing the mean fluorescence intensity of caspase-3/7 ± standard deviation from PBMC of controls and dengue patients measured under a fluorescence reader at excitation ~485 ± 20 nm and emission ~530 ± 25 nm and the respective p-values.

PBMC	No. of patients (n)	Mean caspase-3/7 fluorescence intensity (RFU)	p-value
Controls	10	344.10 ± 136.44	4.27×10^{-4}
Patients	23	1239.35 ± 1104.96	

4.8 Pearson Correlation Analysis between Days of Infection and Apoptotic Markers

The correlations between the day of infection and the different apoptotic markers were done by using Pearson Correlation Analysis. From Table 4.8, there were negative correlation between the days of infection and H_2O_2, TRAIL, caspases-8 and -3/7 as seen from the negative r-values but the correlation was not significant as the p-values were more than 0.05. The positive r-values in Bcl-2 and caspase-9 showed that there was a positive correlation between the days of infection and the 2 apoptotic markers but the two groups similarly showed no significant correlation as the p-values were bigger than 0.05.

Table 4.8: Table showing the Pearson Correlation Analysis between day of infection and different apoptotic markers dengue patients.

Apoptotic Markers	Pearson Correlation for different day of infection	
	r-value	p-value
H_2O_2	-0.191	0.382
Bcl-2	0.320	0.136
TRAIL	-0.033	0.879
Caspase 9	0.020	0.927
Caspase 8	-0.289	0.182
Caspase 3 and 7	-0.036	0.870

$p > 0.05$ indicated that the correlation is not significant

CHAPTER 5

DISCUSSION

Dengue virus (DV) has always been a major global health problem in the tropics which causes an estimated 50 million infections annually with no vaccine or antiviral treatment (Warke et al., 2008a). Although DV can infect a variety of cells, including endothelial and neuronal cells, *in vitro* (Avirutnan et al., 1998; Jan et al., 2000), only a few cell types have been identified as being infected *in vivo* and the most recognized target cells for DV in humans are the mononuclear cells (Halstead, O'Rourke, & Allison, 1977). Peripheral blood mononuclear cells (PBMC) like the monocytes, are cells which predominantly support DV infection (Kurane, 2007). DV is thought to replicate in mononuclear phagocytic cells and natural killing of these infected cells may be one of the immune responses which is responsible for controlling primary DV infections *in vivo* (Kurane, 2007; Wang et al., 2002). Therefore, apoptotic cell death or programmed cell death has been implicated as a cytopathological mechanism in response to DV infection both *in vitro* and *in vivo* (Matsuda et al., 2005).

The significances of the present study are direct demonstration of PBMC apoptosis in DV-infected patients and the pathways that is involved in the PBMC apoptosis. The day of infection was seen to have no significance relationship with the apoptosis markers as shown in Table 4.8 as all the p-values were larger than 0.05. Therefore, the apoptosis that was shown was due

to the DV infecting the PBMC in DV-infected patients and not due to other factors. Disruption of mitochondrial transmembrane potential is an early apoptotic event (Belkhiri, Dar, Zaika, Kelly, & El-Rifai, 2008) and is important in identifying apoptosis. This can be proven from the results in Figure 4.1 where it showed apoptosis in PBMC of DV-infected patients but not PBMC from normal controls. In normal controls, most of the PBMC appeared in red fluorescence although there was also very little appearance of green fluorescence PBMC. This is because in normal controls, there were no virus infection and the PBMC was not subjected to any kind of stress. This causes the cationic dye present in the Mitocapture™ reagent which was used to stain the PBMC able to accumulate and aggregate in the mitochondria as the mitochondria membrane potential was not disturbed. This gave off a red fluorescence to the PBMC of normal controls. However, in PBMC of DV-infected patients, almost all the PBMC showed green fluorescence and this is indicative of apoptosis that occurred. PBMC of DV-infected patients had an altered mitochondrial membrane potential due to DV infection and the cationic dye present in the Mitocapture™ reagent was unable to aggregate in the mitochondria. This caused the dye to remain in the cytoplasm in the monomer form, giving off green fluorescence. In PBMC of normal controls, there was some green fluorescence PBMC observed. This is because the normal PBMC, like many other cells, had undergone natural apoptosis which is important in eliminating unwanted cells in cell homeostasis.

The apoptosis of PBMC in DV-infected patients were further confirmed by cell cycle analysis using flow cytometry. From Table 4.1, there were higher

amount of PBMC in the G1 phase in normal controls. This proved that most of the PBMC from normal controls had DNA content at G1 phase as compared to subG1, S and G2+M phases. PBMC are fully differentiated cells and are not able to undergo cell division. Therefore the PBMC will have their cell cycle arrested at the G1 phase where at this stage, the PBMC are fully matured, viable and functioned normally. The majority of PBMC from normal controls appearing at G1 phase indicated in this result therefore proved that the PBMC from normal controls were matured, viable and functioned normally when analysed under flow cytmometry. This is because in normal controls, the PBMC was not subjected to any infections or stresses and therefore the PBMC appeared matured and viable thus giving off a higher number at the G1 phase. However, in PBMC of DV-infected patients, the PBMC appeared mostly at the subG1 phase. This proved that most of the PBMC from DV-infected patients had DNA content at the subG1 phase as compared to other phases. The subG1 phase is also known as the resting G0 phase where the cells in this phase will appear non-viable or senescent as a result of DNA damage or degradation from apoptosis. The majority of PBMC from DV-infected patients appearing at the subG1 phase in this study therefore indicated that the PBMC had either damaged or degraded DNA thus proving that apoptosis had taken place. Hence, both the results from staining the PBMC with Mitocapture^{TM} reagent and cell cycle analysis were keys to the fact that PBMC apoptosis occurred in DV-infected patients and not normal controls.

From Table 4.2, the result showed that there was significant higher H_2O_2 fluorescence intensity in PBMC of DV-infected patients as compared to PBMC

of normal controls. This showed that the PBMC in DV-infected patients produced a higher amount of H_2O_2 as compared to normal controls as the fluorescence intensity was directly proportional to the amount of H_2O_2 produced. This result coincided with several viral researches. In a study done by Dobmeyer et al. (1997), PBMC from Human Immunodeficiency Virus-1 (HIV-1)-infected patients produced a higher amount of H_2O_2 as compared to normal controls. It was also concluded in the study that the H_2O_2 produced by the HIV-infected patients played a role in induction of PBMC apoptosis that contributed to the deletion of lymphocytes and to the pathogenesis of the disease. Another study on HIV was conducted by another group of researchers where it was said that the spontaneous production of H_2O_2 by monocytes in HIV-infected patients were higher when the HIV load was higher and subsequently participated in overall oxidative injury of these patients (Elbim et al., 1999). A separate study done on Hepatitis C Virus (HCV) by Toro, Conesa, Garcia, Bianco, and De Sanctis (1998) also made a similar conclusion. It was found that the H_2O_2 produced by Peripheral Mononuclear Cells (PMN) from HCV-infected patients were significantly higher than normal controls and indicated that H_2O_2 played a role in the oxidative burst in HCV infection.

Reactive oxygen species (ROS) play critical roles for the determination of cell fate by eliciting a wide variety of cellular responses, such as proliferation, differentiation and apoptosis (Fujino, Noguchi, Takeda, & Ichijo, 2006). ROS are broadly defined as oxygen-containing chemical species with reactive chemical properties, and include free radicals such as superoxide and hydroxyl radicals, which contain an unpaired electron, and non-radical molecules such

H_2O_2 and nitric oxide (Mates, 2000; Pelicano, Carney, & Huang, 2004). Further according to Pelicano et al. (2004), during oxidative phosphorylation in mitochondria, electrons are delivered through the respiratory chain, and a proton gradient is established across the inner mitochondrial membrane as energy source for ATP synthesis. One important biochemical event associated with this metabolic process is the production of superoxide (Pelicano et al., 2004). Up to 2% of the oxygen consumed by the mitochondrial respiratory chain undergoes one electron reduction to generate the superoxide radical, and subsequently other ROS such as H_2O_2 (Kowaltowski & Vercesi, 1999). Because superoxide radicals are constantly generated during respiration and can be converted to H_2O_2 and other ROS, mitochondria are therefore considered the major source of cellular ROS (Pelicano et al., 2004). Under conditions in which mitochondrial generation of ROS is increased, such as in viral infection, these ROS may lead to irreversible damage of mitochondrial DNA, membrane lipids and proteins. All these will subsequently cause altered mitochondrial transmembrane potential resulting in mitochondrial dysfunction and ultimately cell death (Kowaltowski & Vercesi, 1999). Also according to Nordberg and Arn´Er (2001), excessive production of ROS may lead to oxidative stress, loss of cell function, and ultimately apoptosis.

In this research, high amount of H_2O_2 was produced by PBMC from DV-infected patients because the PBMC was responding to DV infection. PBMC from DV-infected patients activated the generation of H_2O_2 in response to appearance of DV in its cytoplasm (Skulachev, 1998). This increase in H_2O_2 production had brought about PBMC oxidative stress, which subsequently alter

the mitochondrial transmembrane potential, cause lipid peroxidation and initiate DNA fragmentation (Larrosa et al., 2008; Lee et al., 2008). These phenomena will subsequently turned on the process of apoptosis in the DV infected PBMC. Moreover, H_2O_2 produced also diffuses into the adjacent PBMC due to its high membrane permeability and thereafter inducing apoptosis (death of bystander PBMC). So, the infected PBMC and its neighbours which are the most likely to be infected, are eliminated, thus blocking the spreading of the DV infection (Skulachev, 1998). Therefore, the higher amount of H_2O_2 produced in PBMC of DV-infected patients that subsequently lead to PBMC apoptosis was most probably related to the PBMC defense mechanism against DV infection. This therefore had proven that H_2O_2 played a critical role in PBMC apoptosis and the apoptosis pathway of PBMC in DV-infected patients may go through the intrinsic H_2O_2 pathway.

Bcl-2 is another intrinsic apoptosis regulator. From Table 4.3, the mean absorbance and mean concentration of Bcl-2 was higher in PBMC of DV-infected patients as compared to normal controls. However, the amount was not significant as the p-value was higher than 0.05. In this study, there was more Bcl-2 protein being captured by the anti-Bcl-2-antibody located at the bottom of the plate from PBMC of DV-infected patients. However, the insignificancy ($p>0.05$) of the higher amount of Bcl-2 detected in PBMC of DV-infected patients is because the amount of H_2O_2 produced by PBMC in DV-infected patients was only slightly higher than the normal controls and therefore the difference does not give a big impact on the study.

A study done by Carvalho et al. (2009) on DV2-infected human monocytes showed similar result pattern. The level of Bcl-2 produced by DV-infected human monocytes was seen to be of no difference when compared to controls. The data therefore suggested that the anti-apoptotic protein, Bcl-2 have no role in preventing the DV2-infected human monocytes from undergoing apoptosis.

Bcl-2 is an anti-apoptotic member of Bcl-2 member family (Gustafsson & Gottlieb, 2007). Bcl-2 is associated with the mitochondrial outer membrane and stabilizes mitochondrial integrity, thus preserving the integrity of the outer mitochondrial membrane (Mignotte & Vayssierre, 1998). The preserved outer mitochondria membrane will then prevent a pro-apoptotic factor, namely cyto *c* release and thereafter preventing mitochondria-mediated cell death initiation (Falschlehner et al., 2007; Mignotte & Vayssierre, 1998; Swanton et al., 1999). Besides, Bcl-2 can also inhibit apoptosis by blocking the activation of the effector capases which transduce the apoptotic signal (Gu¨rsoy et al., 2008).

In this research, the slightly up-regulated level of Bcl-2 in PBMC of DV-infected patients is probably due to the PBMC responding to viral aggression and in some cases control the infection, resulting in the persistence and clearance of the DV (Aillet et al., 1998; Scallan, Allsopp, & Fazakerley, 1997). The PBMC from DV-infected patients may activate the secretion of Bcl-2 in response to appearance of DV in its cytoplasm. However, the amount of Bcl-2 produced was not sufficient to inhibit the infected PBMC from undergoing apoptosis. Further, it was suggested by Scallan et al. (1997) that Bcl-2 might have direct antiviral properties. It is possible that Bcl-2 acts by interfering with

the viral life cycle, inducing its arrest, or by significantly reducing capacity of the PBMC to support DV infection (Thoulouze et al., 2003). Therefore, the DV-infected PBMC reacted to produce Bcl-2 but in a small amount with the hope to prevent further infection through apoptosis. The apoptosis pathway of PBMC in DV-infected patients were seem to not follow the intrinsic Bcl-2 pathway as the Bcl-2 failed to give a significant result.

From Table 4.4, the mean absorbance and mean concentration of TRAIL was significantly higher in PBMC of DV-infected patients as compared to normal controls. Involvement of TRAIL in apoptosis has been implicated in previous studies involving infection with various viruses, including HIV, reovirus and viral hepatitis (Matsuda et al., 2005). A study done by (Brincks et al., 2008) on influenza virus had showed similar pattern result as this study. Influenza virus-infected PBMC was shown to have an up-regulation of TRAIL as compared to control. This data further supported that TRAIL is a primary mechanism used by influenza-stimulated human PBMC to kill influenza-infected target cells. Another study done by Kim et al., (2007) has shown that TRAIL triggered apoptosis in T cells from HIV-infected individuals, while T cells from uninfected controls are resistant to TRAIL-induced apoptosis. These data further supported the function of TRAIL as apoptosis inducer in viral-infected cells but not normal cells.

TRAIL is involved in the extrinsic pathway of apoptosis and is very unique in the way that it only induces apoptosis in tumor and viral-infected cells but not normal cells (Baetu & Hiscott, 2002). TRAIL is known to initiate apoptosis of

transformed cells and virally-infected cells through the death receptor pathway (Baetu & Hiscott, 2002). TRAIL have been shown to directly induce apoptosis in sensitive target cells on binding to those cognate receptors that are capable of transmitting a caspase-activating signal due to the presence of a so called cytoplasmic Death Domain (DD) (Aggarwal, 2004). Binding of TRAIL to respective receptors located on surface of the cell membrane results in trimerisation of the receptor and formation of the death-inducing signalling complex (DISC). Fas-associated death domain (FADD) is an adapter protein and translocates to the DISC where its DD directly interacts with the DD of TRAIL receptors. Via its second functional domain, the death effector domain (DED), FADD recruits procaspase-8 and -10 to the DISC where these caspases are auto-catalytically activated. This caspase activation is crucial for transmission of the apoptotic signal (Boldin et al., 1996; Falschlehner et al., 2007). However, the exact mechanism of initiator caspase activation at the DISC is not completely understood (Falschlehner et al., 2007).

In this research, the level of TRAIL from DV-infected patients was significantly higher as compared to normal control and this corresponds with the functions of TRAIL. TRAIL functions to induce apoptosis in virally infected cells and not normal cells. The PBMC from normal controls were not subjected to any infection or any stress and therefore they are resistant to TRAIL-mediated apoptosis. However, in PBMC of DV-infected patients, the PBMC was infected with DV and the up-regulation of TRAIL was most probably because of the PBMC defense mechanism. TRAIL was probably responsible to induce apoptosis in PBMC of DV-infected patients to eliminate

DV-infected PBMC (Brincks et al., 2008). The DV-infected PBMC responded to increase TRAIL expression when subjected to DV infection and this increase in TRAIL will cause apoptosis in the infected PBMC. By doing so, the infected PBMC can be eliminated and this can prevent further DV infection to other uninfected bystander PBMC. The viable uninfected PBMC (due to the fact that uninfected PBMC will be TRAIL resistant) will not be infected by DV as the infected PBMC was eliminated by TRAIL and this would be another defense mechanism. The non-infected PBMC was most probably playing its role in defense system against DV and suppresses further DV infection. Therefore, the up-regulation of TRAIL in PBMC of DV-infected patients was to eliminate DV-infected cells and subsequently clearance of the DV. Besides, TRAIL is also an essential tool of an innate defense mechanism against DV and might also be involved in immune modulation after DV infection (Ishikawa et al., 2005; Mundt et al., 2005)

The involvement of the apoptosis regulators mentioned above, if without the activation of a caspase cascade, will not lead to cell death. Caspases play critical roles in initiation and execution of apoptosis by cleaving a large number of proteins, which in turn lead to the typical morphology of apoptosis (Liu et al., 2004). Caspases are divided into initiator (caspases-8 and -9) and executioner (caspases-3 and -7) caspases (Green & Reed, 1998; Liu et al., 2004; Peter & Krammer, 1998). From Table 4.5, the mean fluorescence intensity of the initiator caspases, namely caspase-9 was higher in PBMC of DV-infected patients as compared to the normal controls. The higher caspase-9 fluorescence intensity corresponded to the higher caspase-9 activity. Thus, a

higher caspase-9 cleavage activity was observed in the DV-infected patients as compared to normal controls.

The higher level of caspase-9 activity detected in PBMC of DV-infected patients corresponded to several viral studies. In a study done by Muthumani et al. (2002), it was clearly seen that caspase-9 was involved in the apoptosis pathway of HIV-infected PBMC as the level of caspase-9 was higher in the HIV-infected PBMC as compared to controls. A similar result was also obtained by Radziewicz et al. (2008) where the level of caspase-9 was seen higher in PBMC of HCV patients as compared to controls. These 2 researches supported the results of this study and concluded that caspase-9 was involved in the intrinsic pathway of PBMC apoptosis that may lead to disease progression.

Caspase-9 is the intrinsic initiator caspase that is involved in the initiation of apoptosis (Zhang et al., 2003). As the name implies, caspase-9 activity involves the mitochondria as it is involved in the intrinsic pathway and its function is to initiate apoptosis by activating the effector caspases. Caspase-9 exist in cells as procaspase-9 and is being initiated, either by its own autocatalytic activity (Green & Reed, 1998), or by ROS, reactive nitrogen species (RNS) or a decreased in oxygen supply that caused stress to the mitochondria (Al-Molawi, Beardmore, Carter, Kass, & Roberts, 2003; St-Louis & Archambault, 2007). The procaspase-9 together with the cyt *c* produced from the stressed or injured mitochondria and also apoptosis protease activating factor-1 (Apaf-1) will then bind and form a complex known as

apoptosome. When the apoptosome complex dimerizes, procaspase-9 is cleaved to its active form, caspase-9 and this lead to the activation of effector caspases (caspases-3 or -7). The activation of effector caspases will then subsequently lead to apoptosis (Swanton et al., 1999).

In this research, the level of caspase-9 activity was higher in PBMC of DV-infected patients as compared to normal controls. This result actually corresponded to the presence of high levels of H_2O_2 shown previously. This is because caspase-9 is involved in the intrinsic pathway and H_2O_2 is one of the factors held responsible in the intrinsic apoptosis pathway (Dai, Dent, & Grant, 2003). In this study, the PBMC of DV-infected patients responded to DV by generating a large amount of H_2O_2 (Skulachev, 1998). These H_2O_2 produced caused a change in the mitochondria membrane permeability and subsequently the release of cyt *c* from the mitochondria (Lee et al., 2008). The released cyt *c* was then associated with Apaf-1 and this interaction actually caused the Apaf-1 complex to recruit several inactive procaspase-9 molecules to form the so-called apoptosome. The apoptosome formed then cleaved procaspase-9 into its active form, caspase-9 (Sharangpani et al., 2008; Swanton et al., 1999) and hence a higher caspase-9 activity was observed in this study. This activated caspase-9 will subsequently initiate the effector caspases that lead to apoptosis of PBMC in DV-infected patients which was proven through the Mitocapture™ staining and also cell cycle analysis. Therefore, the predicted intrinsic pathway of apoptosis in PBMC of DV-infected patients in this study was partially determined as the high level of caspase-9 activity was associated with high levels of H_2O_2. Besides, another intrinsic apoptotic factor in this

study, which is the Bcl-2 showed no significance in it role in this intrinsic pathway. The slightly higher Bcl-2 level which is an anti-apoptotic member obtained in this study from the PBMC of DV-infected patients was not able to inhibit apoptosis like its function and apoptosis were still observed. This could also be another probable factor that contributed to the high levels of caspase-9 activity as the altered functions of Bcl-2 that was supposed to inhibit apoptosis were lost during DV infection (Su et al., 2001) and forced the PBMC to undergo apoptosis. The apoptosis that occurred due to this altered function of Bcl-2 was through the intrinsic pathway and in order for intrinsic apoptosis pathway to take place, the initiator intrinsic caspase-9 needed to be activated and this activation was proven through a high caspase-9 activity.

Another initiator caspase that is involved in the apoptosis pathway is actually the extrinsic initiator caspase, caspase-8. From Table 4.6, the mean caspase-8 fluorescence intensity was seen to be significantly higher in the PBMC of DV-infected patients as compared to normal controls. The fluorescence intensity, like in caspase-9, represented the caspase-8 activity. Thus, the higher caspase-8 fluorescence indicated the higher the caspase-8 activity.

Several studies have shown similar results as this study. A research done on HCV by Chou et al. (2005) showed an increase in the concentration of caspase-8 in HCV-infected Huh7 cells as compared to controls. They also concluded that caspase-8 was activated via TRAIL and the apoptosis was dependant upon the extrinsic pathway. In another study done by Kondo et al. (2009) on HCV, the study mentioned that the level of caspase-8 was also higher in HCV-

infected T-cells and proved that the apoptosis of the T-cells was via extrinsic pathway through initiation of caspase-8 downstream pathway.

Classically, the extrinsic caspase cascade involves the activation of caspases-8 (Thorburn, 2004). Binding of circulating ligands to specific transmembrane receptors leads to intracellular aggregation of proteins within the cell called DD (Hussein et al., 2003). The receptor and DD are then associated with procaspase-8 and this DISC leads to cleavage of the procaspase to its activated form (Sarmento et al., 2006). Activated caspase-8 then activates the effector caspase resulting in apoptosis.

In this research, the level of caspase-8 activity was significantly higher in PBMC of DV-infected patients as compared to normal controls. This result corresponded to the presence of high levels of TRAIL shown in this study. This is because TRAIL is involved in inducing apoptosis through the death receptor pathway or extrinsic pathway and caspase-8 is the initiator caspase that is involved in the said pathway. The initiation of the extrinsic apoptosis pathway is dependant upon the binding of TRAIL to the receptor located on the surface of the cell membrane and subsequently the activation of caspase-8. In this study, the PBMC of DV-infected patients up-regulated the expression of TRAIL as a response to DV infection as TRAIL is known to initiate apoptosis in virally infected cells but not normal cells (Matsuyama et al., 2004). This caused the expressed TRAIL to bind to the TRAIL receptor on the surface membrane and this binding will cause trimerization of the receptor and formation of DISC. The DD involved, namely the FADD was translocated to

the DISC where its DD directly interacts with the DD of TRAIL receptors. The FADD then recruited procaspase-8 to the DISC via its second functional domain, the DED, where the procaspase-8 is auto-catalytically activated. The activated caspase-8 in turn initiated the effector caspase-3 or -7 to undergo activation that subsequently lead to apoptosis. This result therefore showed the relationship between TRAIL and caspase-8 whereby a high expression of TRAIL will subsequently lead to high expression of caspase-8 and further proved that the apoptosis that occur in the PBMC of DV-infected patients was through the extrinsic pathway.

A caspase cascade will not be complete and apoptosis will not occur if there is absence of the effector caspase. Effector caspases are the key regulators of apoptosis whereby they play important roles in execution of apoptosis through initiation of morphological changes that lead to apoptosis (Liu et al., 2004). The effector caspases, like caspases-3 and -7 are activated via the actions of the initiators caspases and this activation is crucial in determining the cell to undergo apoptosis (Peter & Krammer, 1998). In this study, the mean fluorescence intensity of caspase-3/7 was seen higher in PBMC of DV-infected patients as compared to normal controls as shown in Table 4.7. The higher intensity in fluorescence represented a higher caspase-3/7 activity. This result corresponded to several studies done by some researchers. Vásquez et al. (2009) did a research on DV and concluded that caspase-3 activation was seen in the apoptosis of microvascular endothelial cells of the brains and intestinal serosa in the DV-infected patients. On the other hand, Schrieber et al. (2008) in a research involving Hepatitis C virus, mentioned that the plasma in HCV-

infected patients has higher caspase-3/7 activity as compared to controls. These results thereby showed the involvement of caspase-3/7 in executing apoptosis in different viral infections.

Effector caspases, like other initiator caspases, exist in cell as procaspases or zymogens (Kirsch et al., 1999). They are located down stream of the caspase-8 or caspase-9 activation cascade and represent a point where the intrinsic and extrinsic apoptosis pathways converge (Fischer et al., 2003). They are activated via the action of initiator caspases. The activated initiator caspases specifically activate effector caspases, like caspases- 3 and -7, which can degrade a large number of cellular proteins (Wang et al., 2008). The degradation or proteolysis of cellular proteins will eventually bring about the characteristic apoptotic morphological changes that results in cell death (Zhang et al., 2003)

In this study the higher caspase-3/7 activity observed in PBMC of DV-infected patients corresponded to all the results summarised above. Firstly, apoptosis of PBMC was seen in DV-patients and this was proven through the Mitocapture staining and cell cycle analysis. Furthermore, the level of H_2O_2 that was significantly higher in PBMC of DV-infected patients and subsequently a higher caspase-9 activity further confirmed that the intrinsic pathway was involved in this study. The intrinsic pathway of apoptosis would not be executed without the activation of caspase-3/7 as these caspases are held responsible for the morphological changes that lead to apoptosis. On the other hand, the results of TRAIL showed a significant higher level in PBMC of DV-

infected patients as compared to normal controls. In order to further confirm the extrinsic pathway, caspase-8 was tested and it was found that the level of caspase-8 activity was also higher in PBMC of DV-infected patients as compared to normal controls. The significant higher levels of caspase-3/7 activity in this study thus completed the execution of apoptosis via the extrinsic pathway. In other words, in order for apoptosis to occur intrinsicly or extrinsicly via the caspase cascade, the effector caspase-3/7 must be activated and the high levels of caspase-3/7 activity detected in this study therefore proved that the whole caspase cascade in apoptosis pathway had completed.

Based on all the results above, a proposed mechanism of apoptosis in PBMC of DV-infected patients were as follows. In DV-infected patients, the PBMC detected DV in its cytoplasm and responded by producing a large amount of H_2O_2 (Skulachev, 1998) and this was proven through a higher level of H_2O_2 detected in the PBMC of DV-infected patients. The H_2O_2 secreted caused an increased in calcium concentration in the cytoplasm and subsequently caused calcium influx into the mitochondria (Ermak & Davies, 2001). The influx of calcium into mitochondria then induced the permeability transition pore in the mitochondria membrane and therefore changed the membrane permeability (Mattson & Chan, 2003) as proven in the Mitocapture staining. In the results shown above, the mitocapture stains could not aggregrate in the mitochondria due to the altered membrane permeability and subsequently altered the mitochondrial membrane potential and therefore remained in the cytoplasm in its monomer form, thus appearing in green fluorescence. This altered membrane permeability allowed the release of cyt *c* where the released cyt *c*

bound to the IP3 receptor on the endoplasmic reticulum (ER) and thus enhanced the release of more calcium to the cytoplasm (Mattson & Chan, 2003). This will positively regulate the calcium influx into mitochondria and caused more cyt *c* to be released (Pretorius & Bornman; 2005). This cyt *c* released was then associated with APAF-1 and this interaction actually caused the APAF-1 complex to recruit several inactive procaspase-9 molecules to form the so-called apoptosome (Bayir et al., 2006). The apoptosome formed then cleaved procaspase-9 and hence activated caspase-9 into its active form (Sharangpani et al., 2008; Swanton et al., 1999) which was proven through high levels of caspase-9 activity detected in this study. The activated caspase-9 then cleaved procaspase-3 into active caspase-3 (Kanthasamy et al., 2006), which was proven again in this study through a detection of high level of caspase-3/7 activity in the PBMC of DV-infected patients, which subsequently lead to the PBMC death in DV-infected patients. Apoptosis in PBMC of DV-infected patients by H_2O_2 was crucial as the infected PBMC and its neighbors which were most likely to be infected, were eliminated and this could block the spreading of the DV infection to prevent further infection (Skulachev, 1998). As for Bcl-2, the function as an anti-apoptotic regulator was probably diminished by the DV infection (Su et al., 2001) as there was a slight increase in the secretion of Bcl-2 by the PBMC in DV-infected patients. This slight increase was supposed to inhibit apoptosis of PBMC in DV-infected patients but it failed to do so and this was proven through the Mitocapture staining where it showed that almost all the cells underwent apoptosis and also proven through cell cycle where almost all the cells were arrested at the subG1 phase. The appearance of the PBMC at the subG1 phase indicated that the PBMC had

either damaged or degraded DNA and was a further proof that the PBMC had undergone apoptosis. The up-regulation of Bcl-2 in this study was most probably due to the PBMC responding to DV aggression (Carvalho et al., 2009) or could be because the PBMC was trying to control and clear the DV infection (Thoulouze et al., 2003). Besides, the Bcl-2 was said to have anti viral properties where it could probably interfere with the viral life cycle, induced its arrest, or by significantly reducing the capacity of the PBMC to support DV infection (Thoulouze et al., 2003).

On the other hand, when PBMC encountered DV in its cytoplasm, it simultaneously up regulated the secretion of TRAIL (Brincks et al., 2008), which was proven through a high amount of TRAIL secreted by PBMC of DV-infected patients, as TRAIL functions to induce apoptosis in virally infected cells but rarely normal cells (Baetu & Hiscott, 2002). TRAIL, when being secreted by PBMC in DV-infected patients, bound to TRAIL receptors located on the surface membrane and caused trimerisation of the receptor and formation of DISC (Aggarwal, Bhardwaj, & Takada, 2004). This caused the FADD to translocate to the DISC where its DD directly interacted with the DD of TRAIL receptors (Falschlehner et al., 2007). FADD then recruited pro-caspase-8 to the DISC via its second functional domain, the DED where caspase-8 was auto-catalytically activated (Sarmento et al., 2006). The activated caspase-8 (which was again proven through a high level of caspase-8 detected in the PBMC of DV-infected patients), then cleaved procaspase-3 into active caspase-3 (proven through a detection of high level of caspase-3/7 activity in the PBMC of DV-infected patients) which subsequently caused the

PBMC to undergo apoptosis in DV-infected patients (Ashkenazi & Dixit, 1998; Peter & Krammer, 1998; Schulze-Osthoff, Ferrari, Los, Wesselborg, & Peter, 1998). The PBMC apoptosis in DV-infected patients through TRAIL was a necessary defense mechanism towards the DV-infection as the elimination of PBMC in the DV-infected patients and at the same time keeping the uninfected PBMC alive could accelerate the clearance of the DV infection and at the same time defending the cell from further DV-infection by the viable PBMC (Ishikawa et al., 2005, Mundt et al., 2005). Apoptosis of PBMC in DV-infected patients was therefore concluded as a necessary defence mechanism in suppressing further infection and contributed to the clearance of the DV. The apoptosis of PBMC in DV-infected patients was seen to follow through the intrinsic pathway via H_2O_2, extrinsic pathway via TRAIL and also through a caspase cascade (caspase-9, caspase-8 and subsequently caspase-3/7). Bcl-2 on the other hand played no significant role in apoptosis of PBMC of DV-infected patients.

This proposed mechanism therefore can be served as additional information towards the mechanism of DV infection and can be used in the future for the mapping of the complete apoptosis pathway. The complete apoptosis pathway can then be used to determine the pathogenesis of DV towards human PBMC, which is considered one of the first line defences in our body so that DV infection can be prevented.

CHAPTER 6

CONCLUSION

As a conclusion, the mean levels of hydrogen peroxide (H_2O_2) and Tumour necrosis factor-related apoptosis inducing ligand (TRAIL), caspases-9, -8 and -3/7 activities were higher in PBMC of DV-infected patients as compared to normal controls. The mean level of Bcl-2 although showed a slight increase in PBMC of DV-infected patients as compared to normal controls, the increase was not significant. Therefore, the apoptosis pathway of PBMC in DV-infected patients was said to follow the intrinsic pathway through H_2O_2 and extrinsic pathway through TRAIL. The apoptosis pathway was confirmed to go through the caspase cascade where there was involvement of caspase-9, -8 and -3/7. Bcl-2 showed no significant role in the apoptosis pathway of PBMC in DV-infected patients.

However, further studies need to be conducted in order to complete the whole apoptosis map in DV-infected patients. Additional apoptosis regulators like Fas ligand, other oxygen radicals like reactive nitrogen species and other Bcl-2 members like Bax, Bid and Bak should be studied in order to determine the exact apoptosis pathway of PBMC in DV-infected patients.

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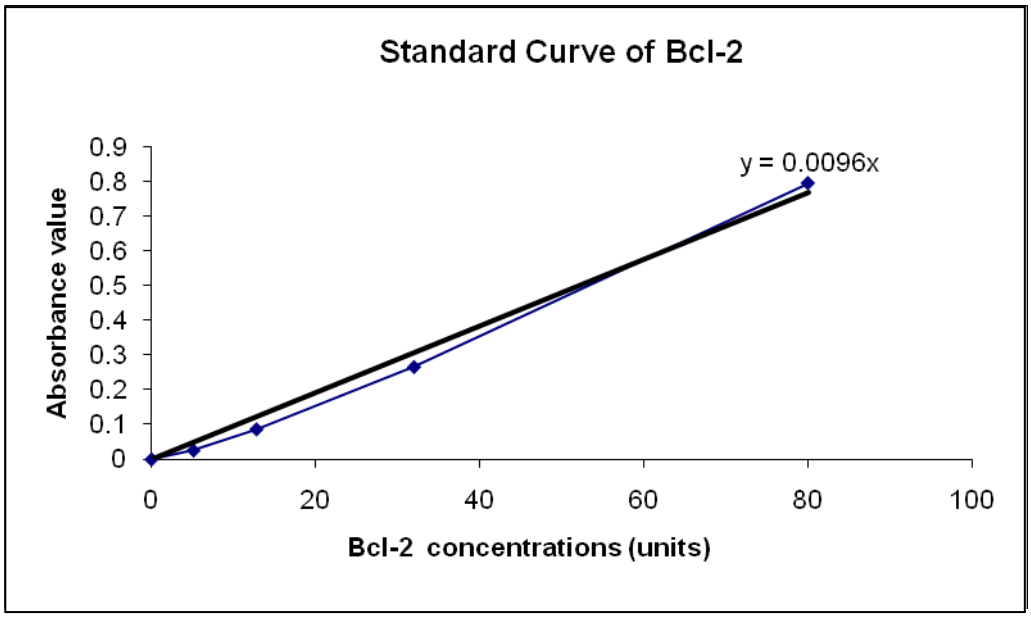
APPENDIX A

Standard Curve for Bcl-2



Calculation of Bcl-2 concentrations in healthy controls and patients:

The relationship between absorbance and concentration of Bcl-2 was obtained from the standard curve, which is

$y = 0.0096x$, where

y is the absorbance

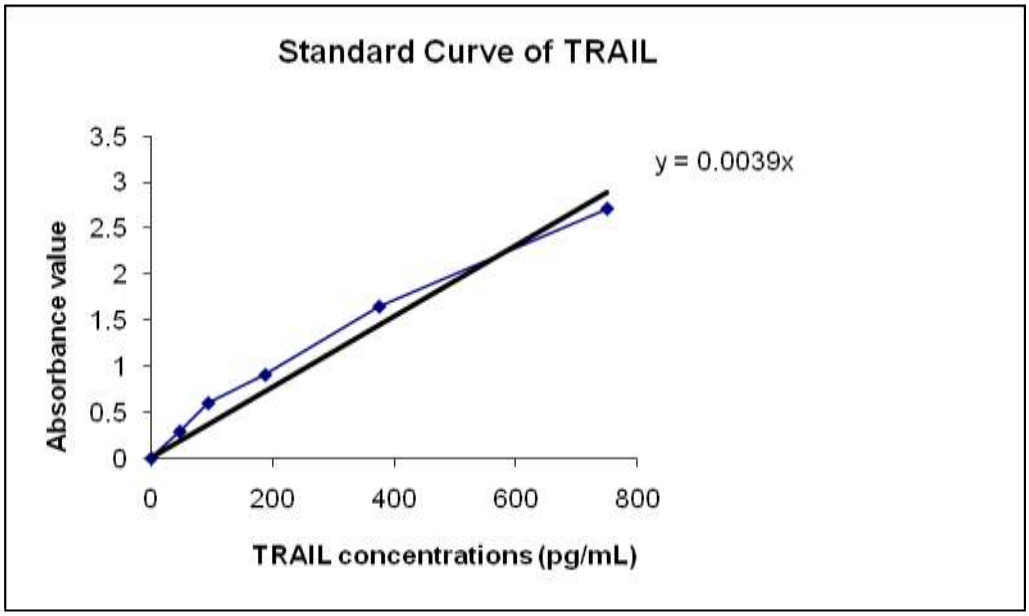
x is the concentration of Bcl-2

$\therefore$ The Bcl-2 concentrations in healthy controls and patients

= (absorbance / 0.0096)

APPENDIX B

Standard Curve for TRAIL



Calculation of TRAIL concentrations in healthy controls and patients:

The relationship between absorbance and concentration of TRAIL was obtained from the standard curve, which is

$y = 0.0039x$, where

y is the absorbance

x is the concentration of TRAIL

$\therefore$ The TRAIL concentrations in healthy controls and patients

= (absorbance / 0.0039)