EXAMINATION OF THE HOST RESPONSE IN BRAIN TISSUE DURING HERPES SIMPLEX VIRUS ENCEPHALITIS

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By

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ABSTRACT

**Background:** Herpes simplex virus encephalitis (HSVE) continues to be one of the most devastating infections of the central nervous system (CNS) despite effective antiviral treatment. The pathogenesis of the disease has not been completely studied, and little is known about the molecular mechanisms underlying cellular death and tissue damage. Better understanding of the HSVE pathogenesis is required to develop better treatment and reduce of patients’ morbidity and mortality.

**Method:** Post-mortem brain tissue from adult HSVE (n=3) and road traffic accident (RTA; n=5) cases were examined to characterise the neuropathological changes, immune activation and infiltration of the inflammatory cells, and changes in transcript abundance during herpes simplex virus type 1 (HSV-1) infection. Patients with HIV encephalitis (n=3) provided further control tissue.

**Results:** There was extensive neuropathological change and widespread necrosis in temporal and frontal regions of the brain among the HSVE cases. CNS cells showed characteristic signs of lytic damage. Infection was associated with microglial activation and infiltration by macrophages and lymphocytes. Genome-wide gene-expression micro-array analysis of the brain tissue demonstrated 287 host transcripts with significantly lower abundance in HSVE compared to RTA cases. Mitochondrial DNA encoded transcripts were significantly over-represented in the set of low abundant transcripts (p<0.0001), with 28 out of 33 mitochondrial genes represented on the array exhibiting lower abundance. Reflecting the array findings, immune staining for cytochrome c oxidase subunit 1, a mitochondrial encoded protein, was reduced in HSVE compared to RTA cases. Furthermore, there was a reciprocal pattern of staining among the HSVE patients, with reduced staining for cytochrome c oxidase subunit 1 in areas of high HSV1 staining.

**Conclusion:** The neuropathological findings confirm previous findings for HSVE. However, the transcript data demonstrates a new finding. There is a preferential loss of mitochondrial transcripts in brain tissue of HSVE patients. The immuno-histochemical results support the array findings. The tissue results also imply that the mitochondrial damage occurs in response to HSV infection. The findings suggest that adjunctive treatments which preserve mitochondrial function could be developed as a new therapeutic option in the treatment of HSVE.
DEDICATION

This work is dedicated to my family and in particular to my parents, my husband, my daughter and son.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisors Dr. Michael Griffiths, Prof. Tom Solomon and Prof. Anja Kipar, for all their invaluable support, assistance and guidance throughout my PhD. Their expertise and directions enabled me to complete the work presented in this thesis. My special thanks to Dr Michael Griffiths for a guidance and advice in molecular microarray experimental study. I also would like to take this opportunity to thank Dr Malgorzata Wnek for her invaluable assistance, encouragement, advice and helpful suggestions throughout my degree. I am also grateful for support, friendship and assistance in the lab offered by the other members of the Brain Infections Group and also to Ms. Shauna Mahoney for her invaluable administrative support.

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<td>AIDS</td>
<td>acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<td>cytomegalovirus</td>
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<td>Glasgow coma scale</td>
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<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
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<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
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xxvi
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>HSVE</td>
<td>herpes simplex virus encephalitis</td>
</tr>
<tr>
<td>HSK</td>
<td>herpes simplex keratitis</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>HVEM</td>
<td>herpes virus entry mediator</td>
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<tr>
<td>ICP0</td>
<td>infected cell protein 0</td>
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<tr>
<td>ICP4</td>
<td>infected cell protein 4</td>
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<td>infected cell protein 6</td>
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<td>infected cell protein 22</td>
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<td>ICP27</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>JEV</td>
<td>Japanese Encephalitis Virus</td>
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<tr>
<td>LATs</td>
<td>latency associated transcripts</td>
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<td>MRI</td>
<td>magnetic resonance imaging</td>
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<tr>
<td>mtDNA</td>
<td>mitochondria DNA</td>
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<td>mt mRNA</td>
<td>mitochondria messenger RNA</td>
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<tr>
<td>NLRs</td>
<td>nod like receptors</td>
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<tr>
<td>PAMPs</td>
<td>pathogen associated molecular patterns</td>
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<tr>
<td>PARP</td>
<td>polyADP ribose polymerase</td>
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<tr>
<td>Abbreviation</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PRRs</td>
<td>pattern recognition receptors</td>
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<tr>
<td>qPCR</td>
<td>quantitative real time polymerase chain reaction</td>
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<tr>
<td>RLRs</td>
<td>rig like receptors</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<td>RTA</td>
<td>road traffic accident</td>
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<td>toll like receptors</td>
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<td>toll like receptor 2</td>
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<tr>
<td>TLR9</td>
<td>toll like receptor 9</td>
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<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl tranferase mediated dUTP nick-end labelling</td>
</tr>
<tr>
<td>UL</td>
<td>unique long</td>
</tr>
<tr>
<td>US</td>
<td>unique short</td>
</tr>
<tr>
<td>VA</td>
<td>viral antigen</td>
</tr>
<tr>
<td>Vhs</td>
<td>virion host shutoff</td>
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<tr>
<td>VPs</td>
<td>viral proteins</td>
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CHAPTER 1

INTRODUCTION

1.1 Viral Encephalitis

Encephalitis is an acute inflammation of the brain parenchyma (Davis 2000, Solomon et al. 2007). The clinical presentations include fever, headache, altered consciousness and neurological deficits with focal or generalised seizures. Viral infection is the most common cause of encephalitis, although infection with other organisms can also lead to encephalitis. Encephalitis must be distinguished from encephalopathy which is a non-inflammatory brain dysfunction. This can be caused by metabolic disorders, intoxication and pathogens, such as Falciparum parasites, that do not invade the brain parenchyma but instead alter brain blood flow by sequestering in cerebral blood vessels (Nahmias et al. 1989, Chaudhuri and Kennedy 2002).

There are a broad range of viruses causing encephalitis with herpes viruses and arboviruses being especially important. Solomon et al. grouped common viruses that cause encephalitis as being geographically restricted and geographically non-restricted (Solomon et al. 2007). Geographically non-restricted group (also known as sporadic causes of viral encephalitis) includes herpes viruses, enteroviruses, paramyxoviruses and other rarer causes (Table 1.1). Geographically restricted causes of encephalitis are mostly spread through arthropod-borne vectors such as
Japanese Encephalitis Virus (JEV) in Asia and West Nile Virus (WNV) in Africa and America.

Due to high morbidity and mortality, viral encephalitis is a major public health concern. The incidence varies between studies and countries but is generally between 3.5 and 7.4 per 100,000 individuals per year (Granerod and Crowcroft 2007) and is found to be higher in children and elderly. In England, an annual incidence of 1.5 per 100,000 has been reported, although this has been regarded by others as an underestimate (Davison et al. 2003). Herpes simplex virus (HSV) infection of the central nervous system (CNS) is among the most severe of all viral infections of the human brain (Whitley 2006). The high prevalence of HSV, infecting between 40 and 80 percent of people worldwide, is most probably due to several evolutionary and epidemiological factors of the virus: such as its ability to manipulate various host cell surface receptors to obtain multiple routes of entry into the cell, establishment of life-long latency in its host, and a lack of an effective vaccine.

Antiviral drugs targeting the suspected causative virus, e.g. aciclovir for HSV, is typically given to most patients once a strong suspicion of viral encephalitis has been made based on clinical presentation, initial CSF and/or imaging findings (Solomon et al. 2007). Viral pathogen is confirmed via viral culture or, more commonly, pathogen sequence specific PCR of the CSF. Supportive treatments to relieve the symptoms associated with encephalitis can also be provided. Supportive therapies include intravenous fluid to help reduce dehydration, anti-epileptic
medication to control seizures, analgesia and anxiolytics to relieve pain and anxiety associated with the illness.

Table 1.1 Sporadic causes of viral encephalitis (non-geographically restricted)
(Solomon et al. 2007)

<table>
<thead>
<tr>
<th>Group</th>
<th>Viruses</th>
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<tbody>
<tr>
<td>Herpes Viruses</td>
<td>Herpes Simplex Virus, Varicella Zoster Virus, Epstein-Barr Virus, Cytomegalovirus, Human Herpes Virus 6 and 7</td>
</tr>
<tr>
<td>Enteroviruses</td>
<td>Coxsackie Virus, Echoviruses, Enterovirus 70 and 71, Parechovirus, Poliovirus</td>
</tr>
<tr>
<td>Paramyxoviruses</td>
<td>Measles, Mumps Virus</td>
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<tr>
<td>Others (Rare causes)</td>
<td>Influenza Virus, Adenovirus, Parvovirus, Lymphocytic Choriomeningitis Virus, Rubella Virus</td>
</tr>
</tbody>
</table>

1.2 **Herpes Simplex Virus (HSV)**

1.2.1 Structure of HSV

Herpes simplex virus is an alpha herpes virus under a family of *Herpesviridae*. There are two known serotypes of HSV, HSV-1 and HSV-2; both strains infect humans. HSV is a nuclear replicating enveloped virus with a spherical capsid, of an average diameter of 186-225nm (Fields et al. 2007). The virion consists of four
elements; i) a central core containing the viral deoxyribonucleic acid (DNA); ii) the icosahedral capsid surrounding the core; iii) the tegument layer surrounding the capsid; and iv) the envelope (Figure 1.1). The envelope is the outer-most layer, it is composed of a bilayer lipid that is embedded with glycoproteins.

The HSV genome consists of two unique sequences, UL (unique long) and US (unique short), each flanked by repeated sequences (Figure 1.2). Both components can be inverted relatively to one another to produce four different types of DNA molecule (Whitley and Roizman 2001).
The icosahedral capsid contains six different viral proteins (VPs) present on its surface. The tegument contains 22 VPs, and the envelope contains 11 glycoproteins. The HSV expresses at least 84 VPs during productive infection. Protein expression is regulated in an orderly fashion (Whitley et al. 1998). The proteins have multiple functions; including virus entry into the host cell, regulation of viral DNA replication and assembly of progeny virions (Arduino and Porter 2008).

Figure 1.2 Schematic HSV genome organised in 2 components, Long (L) and Short (S). Each component contains a unique region (U) flanked by inverted repeat regions and these are linked to generate a total genome size of approximately 150 000bp. Adapted from (Taylor et al. 2002).

The icosahedral capsid contains six different viral proteins (VPs) present on its surface. The tegument contains 22 VPs, and the envelope contains 11 glycoproteins. The HSV expresses at least 84 VPs during productive infection. Protein expression is regulated in an orderly fashion (Whitley et al. 1998). The proteins have multiple functions; including virus entry into the host cell, regulation of viral DNA replication and assembly of progeny virions (Arduino and Porter 2008).

1.2.2 HSV Life Cycle

The process of HSV entry into the cells involves four main steps; i) attachment and entry; ii) gene transcription and protein translation; iii) genome replication and virus assembly; and iv) exit of virions from the cell (Figure 1.3).

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The HSV primarily enter cells through fusion of the virus envelope with the host cells’ outer membrane. The attachment of the virus to the target cell is mediated by interaction and binding of the envelope proteins with cell surface membrane. The envelope proteins, glycoprotein B (gB) and glycoprotein C (gC) are required for the attachment to the cell surface glyco-aminoglycans, whereas glycoprotein D (gD) facilitates virus entry by binding to the cell’s main receptors such as nectins, herpes virus entry mediator and heparan sulphate (Herold et al. 1991, Herold et al. 1994). Glycoprotein B together with glycoprotein H (gH) and glycoprotein L (gL) also assist in the fusion between virion envelope and plasma membrane.


Figure 1.3 Diagram shows the replication cycle of HSV-1. The numbering describe; 1 attachment of the virus to the host cell, 2 entry into the host cell, 3 viral DNA transcription, 4 translation of viral RNAs, 5 genome replication, 6 virus assembly and 7 exit and releasing virions from infected cell into external environment. Image adapted from (Carter and Saunders 2007)
Following fusion, the tegument protein and nucleocapsid are released into the cytoplasm and are transported to the nucleus by microtubules. The capsid then binds to the nuclear pore and releases virus DNA into the nucleus. Once inside the nucleus, viral DNA transcription begins. Viral gene expression occurs in a tightly regulated cascade involving three phases of gene expression: immediate early (IE), early (E) and late phase (L). The expression of viral genes is important for the virus to inhibit the host from producing its own proteins and to facilitate generation of viral proteins.

Immediate early (IE)/α genes are expressed shortly after the infection to regulate viral replication. The IE genes are activated by the tegument protein, viral protein 16 (VP16) (Arnosti et al. 1993). The IE genes act by stimulating the transcription factors by recruiting the host RNA polymerase II. There are five IE genes (infected cell proteins ICP0, ICP4, ICP27, ICP22 and ICP47) that are known to be involved in some aspect of the early/β gene activation (Samaniego et al. 1995). The virus protein translation process is optimised by degradation of host mRNA by one of the tegument proteins known as virion host shutoff (vhs) (Smiley 2004).

Early/β genes promote viral DNA replication and stimulate expression of late/γ genes. The β genes are not produced in the absence of IE genes. The γ genes are expressed once the viral DNA synthesis has commenced. The γ proteins consist primarily of viral structural proteins and are involved in the assembly of progeny virions. They can be divided into early late (γ1), expressed early into infection, and leaky late (γ2) which are expressed late into infection. They encode virion
components such as VP16, Us3, gD, gJ and vhs proteins (Goodkin et al. 2004). The capsid is formed in the nucleus after the late phase of the viral infection.

The last stage of the productive infection involves capsid assembly and exit of virions from the cell. The nucleocapsid is constructed after the viral DNA is inserted into the procapsid (immature capsid). The nucleocapsid undergoes a budding process through the inner nuclear membrane of nuclear envelope into the perinuclear space giving a temporary envelope (Carter and Saunders 2007). The temporary envelope fuses with the outer membrane of the nuclear envelope and releases the nucleocapsid into the cytoplasm (Figure 1.3). In the cytoplasm the tegument and envelope proteins are added. The envelope proteins are derived from a Golgi complex (Carter and Saunders 2007). The final step involves fusion of the vesicle membrane/enveloped virus with the plasma membrane, releasing the virions from the infected cell into the external environment.

1.2.3 Epidemiology of HSV Infection

HSV infection occurs worldwide with no seasonal preferences and, although naturally only infects humans (Whitley and Roizman 2001), it can be induced experimentally in animals (Arduino and Porter 2008) and in vitro cell cultures. In humans, HSV-1 and HSV-2 are transmitted through different routes and usually affect different areas of the body.

However, much overlap is seen between the epidemiology and clinical manifestations of these two viruses. HSV-1 infection usually occurs following a
direct contact of mucosal surfaces of the oral cavity/lips or abraded skin with the virus, whereas HSV-2 infections are usually sexually transmitted. However, either type of virus can infect oral and/or genital areas. The estimated numbers of individuals infected with HSV-1 and HSV-2 are determined by the development of antibodies against the viruses (antibodies are only present if an individual is infected with the virus). This information provides population prevalence of HSV infection.

Geographic location, socioeconomic status, and age are the primary factors that influence the acquisition of HSV infection. In the United States, a sero-prevalence study for HSV infections was undertaken using sera obtained from randomised National Health and Nutrition Examination Surveys (NHANES). The NHANES are a series of cross-sectional national surveys conducted by the National Center for Health Statistics. The NHANES study in 1999-2002 reported the overall sero-prevalence for HSV-1 in children age 6 to 13 years old is 31.1 per cent (Xu et al. 2007). The sero-prevalence in HSV-1 increased with age, from 26.3 per cent in 6-7 years old to 36.1 per cent in 12-13 years old, and varied by race/ethnicity, birthplace and poverty level (Xu et al. 2007). The sero-prevalence of HSV-1 in 6-7 year old children were; 39.5 per cent in non-Hispanic blacks, 35.3 per cent in Mexican American and only 22.8 per cent in non-Hispanic whites. By the age of 12-13 year old, the sero-prevalence in non-Hispanic blacks was 57.4 per cent, 48.9 per cent in Mexican American and 25.9 per cent in non-Hispanic whites (Figure 1.4).
Sero-prevalence of HSV-1 infection increases gradually from childhood to adult life. NHANES study between 1999 and 2004 showed the overall seroprevalence of HSV-1 in Americans (aged 14-49 years) were 57.7 per cent, decreased from 62 per cent from a study in 1988 and 1994 (Xu et al. 2006).

Most of the genital HSV infections are caused by HSV-2; however, an increasing number of cases of HSV-1 genital infections have been reported in some developed countries (Scoular et al. 2002, Xu et al. 2006). The prevalence of HSV-2 is affected by sex, race, marital status, number of sexual partners and place of residence (Whitley and Roizman 2001). The sero-prevalence of HSV-2 in Americans (aged 14-49 years) between 1997 and 2004 was 17 per cent and between 2005 and 2008 was 16.2 per cent, with women showing a higher rate (20.9 per cent) than men (11.5


Figure 1.4 NHANES study 1999-2002 demonstrates HSV-1 seroprevalence by age and race/ethnicity. Images adapted from (Xu et al. 2007)
per cent) and were higher among blacks (39.2 per cent) than whites (12.3 per cent) (http://www.cdc.gov/std/herpes/herpes-NHANES-2010.htm).

1.2.4 Pathogenesis of HSV Infection

Primary and recurrent infections are important in the pathogenesis of HSV infection in CNS (Dudgeon 1969). Primary infection occurs in susceptible individuals (no previous immunity) following first time exposure to HSV. Most primary infections are acquired through a direct contact with a lesion or body fluids of an infected individual (Whitley 2006). A person who develops the infection could be symptomatic or asymptomatic. In asymptomatic infection, no vesicles are found but the shedding of virus is common.

Gingivo-stomatitis is one of the clinical conditions that occur during primary infection of HSV, always seen in children and young adults. After two to twenty days of an incubation period, nonspecific symptoms such as malaise and myalgia arise. This is followed by muco-cutaneous vesicular eruptions that affect the tongue, lips, gingivae, buccal mucosa, hard and soft palate. The oral mucosa lesions, in the form of vesicles, break down to form a shallow ulcer on erythematous base that gradually heals afterwards. The other accompanied symptoms include sore throat, pyrexia, difficulty swallowing, loss of appetite, hypersalivation and cervical lymphadenopathy.

Following primary infection the virus is often not completely eliminated from the host and can enter into a stage of latent infection (Dudgeon 1969). Latent infection
is defined as the presence of genome of pathogen in the host tissue without the production of infective particles. The virus enters peripheral sensory nerve endings and is transported by retrograde axonal transport to the nucleus where it may persist silently (Rozenberg et al. 2011). The trigeminal ganglia are usually the primary site for the latent HSV-1 and sacral ganglia for latent HSV-2. During latency some of the genome is switched off and only a few are transcribed such as latency associated transcripts (LATs). LATs play a role in inhibiting apoptosis in the trigeminal ganglion leading to the survival of neurons infected with latent HSV (Jin et al. 2003). Little is known regarding the exact mechanisms by which the virus established and maintained latency. However, latent infections are life-long with often multiple reactivations.

A symptomatic reactivation of the virus from the latency phase in trigeminal ganglia occasionally demonstrates lesions known as labial herpes or cold sores. HSV-1 can also cause ocular infection and even severe ocular disease manifestations such as ocular keratitis or corneal infection and stromal keratitis, which can lead to scarring, loss of vision and blindness. Figure 1.5 demonstrates sites for HSV infection and related diseases.
1.3 Herpes Simplex Virus Encephalitis (HSVE)

1.3.1 Epidemiology of HSVE

Herpes simplex virus encephalitis (HSVE) continues to be one of the most devastating infections of the CNS despite effective antiviral treatment. It is the most common cause of non-epidemic, sporadic, acute focal encephalitis in the Western world (Whitley 1990, Raschilas et al. 2002). About 90 per cent of all HSVE cases in adults and children are due to HSV-1 (Kennedy and Chaudhuri 2002) and another 10 per cent are due to HSV-2 (Solomon et al. 2007). HSV-2 is responsible for HSVE in neonatal and occasional adult cases, particularly immune-compromised individuals (Sauerbrei et al. 2000).
HSVE is estimated to account for 5 to 10 percent of all cases of acute encephalitis worldwide (Tunkel et al. 2008). It has no seasonal preferences, no geographic restrictions and occurs equally in men and women regardless of their age (Granerod et al. 2010). In the United States, the incidence has been estimated about 1 in 250,000 to 1 in 500,000 persons per year (Whitley 1990). Although it affects all age groups, it is most common and severe in children and elderly patients. Half of the patients are over the age of 50 and one-third are young adolescents less than 20 years old (Whitley et al. 1982, Sabah et al. 2012). Despite available effective antiviral drugs treatment, there is an extremely high mortality rate up to 70 per cent in untreated cases and 19 per cent in treated cases (Whitley et al. 1977). Furthermore, a significant morbidity, with a high incidence of severe and permanent neurological sequelae exists among treated cases.

1.3.2 Pathology of HSVE

The pathological changes have been extensively studied in post-mortem brain tissues. Patients with HSVE have a specific characteristic of neuropathological changes observed in the affected brain. Gross brain examination reveals oedema and haemorrhagic foci scattered throughout the brain (Figure 1.6 A and B). The meninges overlying the temporal lobes often appear clouded or congested, and frank necrosis and liquefaction is observed after two weeks of the infection (Arvin 2007). The pathological changes affect both sides of the brain but usually one cerebral hemisphere is more affected than the other (asymmetrical). The changes are more prominently observed in the temporal lobe, but an involvement of other
localised regions of the brain including the frontal, cingulate, insular and adjacent limbic areas are common.

The histopathological changes observed include vascular changes (blood vessels congestion and haemorrhage), necrosis and inflammation. The inflammation is characterised by inflammatory cells infiltrations around the perivascular blood vessels (Figure 1.6 C), proliferation of microglia, and the formation of microglia nodules (Booss and Kim 1984). Other common microscopic changes are necrotic neurons (Figure 1.6 D) and the presence of intranuclear inclusion bodies. The involvement of oligodendrocytes is also common, but these tend to affected very late in the disease.
Figure 1.6 Representative gross and histological changes in post-mortem brain tissue of HSVE patients. (A) Brain slice of a patient that died after 2 weeks of onset showed focally haemorrhagic at right temporal lobe. (B) Brain of a patient that died 3 months after the onset in which there is severe destruction of the temporal lobe (left more than right), insula and cingulate gyrus. (C) Perivascular infiltrations by lymphocytes. (D) Shrunken neurons (arrows) demonstrate ischaemic cell changes. Images adapted from (Adams and Miller 1973, Kennedy et al. 1988).


The pathogenesis of HSVE is largely unknown. One third of HSVE cases are due to primary infection and the other two third of cases are due to viral reactivation of latent infection. A patient with reactivation of the latent virus demonstrates pre-existing antibodies against HSV. Johnson et al. reported that HSV reaches the CNS following reactivation of the virus from the olfactory bulb and trigeminal ganglia (Johnson et al. 1968). Furthermore, it is uncertain how reactivation occurs, either spontaneously or by various triggering factors such as trauma, sunlight exposure, immune-suppression or X-ray irradiation.

The extensive cell damage including neuronal death is observed in post-mortem brain tissue of HSVE patients (Esiri 1982). Although the pattern of neuron death in HSVE correlates with necrosis, including changes such as karyorrhexis, pyknotic nuclei with shrunken cell bodies and eosinophilic cytoplasm, other studies report apoptotic changes of neurons and glial cells in post-mortem HSVE brain tissue (Athmanathan et al. 2001, DeBiasi et al. 2002, Aurelian 2005). This was supported with the evidence of apoptotic changes, such as membrane blebbing, cleavage of DNA and apoptotic bodies, in animal models and in vitro cell cultures of HSV infection (Athmanathan et al. 2001, Perkins et al. 2003).

HSV-1 is thought to trigger apoptosis during cell invasion and later block this process to maximise the production of viral progeny and promote viral spread to neighbouring cells. Viral immediate early protein, infected cell protein 0 (ICP 0) has been shown to possess pro-apoptosis activity (Sanfilippo and Blaho 2006). Other viral components such as ICP 4, ICP 22, ICP 27, Us3, glycoprotein D,
glycoprotein J and Latency associated transcripts (LATs) confer anti-apoptotic response during HSV infection (Miles et al. 2004, Sanfilippo and Blaho 2006).

Infiltrations and proliferation of inflammatory cells are also observed in post-mortem brain tissue of HSVE patients. These inflammatory cells produce cytokines to attract more inflammatory cells to contain the spread of infection. While controlling the infection, aberrant accumulation of inflammatory cells, particularly T lymphocytes, can cause a destructive inflammatory brain damage. Although the exact mechanism of how HSV induce cell damage is not completely understood, it is believed that both direct virus-mediated and indirect immune-mediated mechanisms contribute to the damage (Lokensgard et al. 2002).

1.3.4 Clinical Features of HSVE

The detailed assessment of HSVE requires a combination of the patient’s history, clinical examination, and laboratory investigations to determine the cause of encephalitis. The index of suspicion of HSVE should be high when a patient presents with the typical features of encephalitis. Among them are fever (90 per cent), headache (81 per cent), confusion, personality change and altered consciousness (Whitley et al. 1982, Kennedy and Chaudhuri 2002). Moreover, other focal neurological signs are usually associated with a constellation of fronto-temporal signs such as aphasia/mutism, personality change, and focal or generalised seizures (Chaudhuri and Kennedy 2002). The onset of HSVE is usually abrupt with the clinical course rapidly progressing over several days.
The characteristic clinical features of HSVE have been retrieved from studies that analysed the presenting signs and symptoms of patients diagnosed with HSVE. For example, Adams and Miller reported from 22 cases of HSVE; half of the patients presented with influenza-like illness and the remainder had sudden onset of headache and confusion. They also observed a signs of meningeal irritation and focal epilepsy during the course of the disease. In addition, 54 per cent showed an increase in intra-cranial pressure with papilloedema and/or increased lumbar CSF pressure on manometry (Adams and Miller 1973).

Kennedy reported in the retrospective clinical-pathological study of 46 cases of HSVE. The authors listed various symptoms on admission including; prodromal influenza-like illness (48 per cent), headache/confusion and altered consciousness (52 per cent), meningism (65 per cent), aphasia/ mutism (46 per cent), deep coma (35 per cent), increased intracranial pressure (33 per cent), focal neurological signs (89 per cent) and seizures (61 per cent) during the course of disease (Kennedy 1988). Other symptoms included personality changes which the authors reported may be subtle and easily missed.

1.3.5 Diagnosis and Laboratory Investigations

A combination of clinical data and laboratory examination contribute to the diagnosis of HSVE. The diagnosis is urgent because the virus rapidly multiplies and the prognosis depends on the stage at which the treatment is initiated. Laboratory investigations that aid diagnosis include the laboratory examination of cerebrospinal
fluid (CSF) and imaging, such as magnetic resonance imaging (MRI) or computed
tomography (CT) looking for focal (typically temporal) signs of inflammation and
an electroencephalogram looking for ‘lateralising’ changes in the rhythm.

The examination of CSF obtained from lumbar puncture is indicated in patients
with altered mental status provided there are no contradictions such as increased
intracranial pressure (Whitley 2006). The CSF examination includes both the
assessment of laboratory parameters and detection of viral specific DNA sequences
via PCR.

PCR is the diagnostic method of choice for detection of HSV DNA in CSF. It has
an excellent specificity (95 per cent) and sensitivity (98 per cent) (Whitley and
Kimberlin 2005). False negative results are extremely low, and typically occur if the
PCR is undertaken too early (within 24 to 48 hours of the onset) or too late in the
disease course (after 10 to 14 days of illness). Positive PCRs are typically detected
up to 12 days from the onset of neurological symptoms (Sauerbrei et al. 2000). The
PCR method is fast and can be completed quickly, typically within 24 hours. This
greatly assists the decision to start the treatment earlier and thus improves the
prognosis.

Laboratory CSF parameters for HSVE are non-diagnostic and are similar to the
other diseases that mimic it. The white blood cell count (predominantly
lymphocytes) and CSF proteins are elevated (Whitley et al. 1989). In addition the
glucose level is normal, and in some cases the presence of red blood cells is
observed (Kennedy and Chaudhuri 2002).
The isolation of HSV from tissues obtained at brain biopsy provides a definitive diagnosis. This procedure is highly sensitive (96 per cent) and specific (99 per cent) (Whitley et al. 1977). However, the procedure is invasive, patients can develop serious complications such as haemorrhages and a longer time is required for diagnosis. Currently, brain biopsy has a value in atypical clinical presentations, in which there remains serious doubt about the diagnosis (Kennedy and Chaudhuri 2002). Furthermore, the availability of PCR detection in the CSF has replaced tissue PCR for diagnostic purposes (Whitley and Kimberlin 2005, Tunkel et al. 2008).

Non-invasive investigations include MRI, CT scan and EEG. MRI is the most sensitive imaging for detection of early lesions such as focal oedema and also distribution of cerebral injury (Chaudhuri and Kennedy 2002). MRI is abnormal in 90 per cent of patients (Sabah et al. 2012). It may demonstrate unilateral or bilateral high signal in the medial temporal lobes, insular cortex and orbital surface of the frontal lobes. The T2-weighted images show hyperintensity signal corresponding to the oedematous changes in the affected lobes (Sabah et al. 2012).

In contrast, a CT scan shows only focal abnormalities in half of HSVE patients. EEG is invariably abnormal. EEG changes are characterised by non-specific slowing in early illness and high voltage periodic lateralising epileptiform discharges in the later stages (Whitley et al. 1977, Lai and Gragasin 1988). Occasionally patients present with atypical findings in imaging studies with normal CSF examination. However, the advancement of current diagnostic methods allows virus DNA detection via PCR in patients with atypical presentation.
1.3.6 Treatment and Prognosis

Aciclovir (Zovirax), an analogue of 2'-deoxyguanosine, is an effective antiviral agent for treatment of HSV infection. Aciclovir acts by selectively inhibiting viral replication and inactivates viral DNA polymerase (Steiner 2011). The standard dose for the treatment of adults with HSVE is 10mg/kg given intravenously three times daily for 14 days (Whitley and Kimberlin 2005). The treatment is sometimes extended up to 21 days particularly in immune-suppressed patients to prevent a relapse of disease. The adverse reactions associated with aciclovir are limited. However, the renal function needs to be monitored closely because of the risk of renal failure (Whitley 2002). Aciclovir also is a drug of choice in treatment of neonatal HSV infection. Because of the exceptional safety profile of aciclovir, and lower risk of renal complications in this age group, 20mg/kg/three times daily can be given intravenously to treat neonatal HSVE.

Many previous studies have consistently showed that aciclovir is the most effective treatment for HSVE. When given early in suspected cases of HSVE, it reduces both mortality and morbidity in treated patients. It was reported that 38 per cent of treated patients irrespective of their age regain normal neurological function and mortality reduces from 70 per cent to 19 per cent with intravenous acyclovir therapy (Whitley et al. 1986).

However, the treatment with anti-viral drugs does not always result in complete recovery (Nakano et al. 2003). Thus, in certain situations, anti-inflammatory corticosteroids are combined with acyclovir in a treatment regimen to improve a patient’s condition. Basically, the effect of corticosteroids treatment remains
controversial. Reluctance to use steroids in routine therapeutic treatment results from their immune-suppressive effect (Gold et al. 2001) which might increase viral replication, viral spread and alter the efficacy of aciclovir (Meyding-Lamadé et al. 2003).

There is limited evidence of use of steroids in human cases of HSVE. However, there are animal studies that support the use of steroids to treat HSVE. For example, Meyding-Lamade et al. studied the association between viral load and MRI changes in a mouse model of HSVE treated with monotherapy acyclovir or combined therapy with methylprednisolone (Meyding-Lamadé et al. 2003). They demonstrated that viral load did not differ significantly between both treatments suggesting that the addition of steroid on top of aciclovir does not increase viral burden. However, cranial MRI findings showed there was a significant reduction in severity of oedema in a combination of aciclovir/corticosteroid treated animal (Meyding-Lamadé et al. 2003).

In addition, Sergerie et al. demonstrated dexamethasone administered 3 days after infection in experimental mice with HSVE can lead to neuroprotection by controlling viral replication and restricting neuronal cell death (Sergerie et al. 2007). This study showed that a combination of dexamethasone and aciclovir therapy had a beneficial effect on the treatment of HSVE. Steroids have also been used as an adjunctive treatment for bacterial meningitis and tuberculous meningitis; however more studies are needed to clarify its role in HSVE (Fitch and van de Beek 2008).
The prognosis or the clinical outcome is determined by several factors such as age, duration of disease and level of consciousness or Glasgow coma scale (GCS) at the onset of treatment (Whitley 2006). Regardless of age or drug used, the GCS of 6 or less predicts a poor therapeutic outcome (Whitley et al. 1986). Better prognosis is usually observed if aciclovir treatment is initiated early, in younger patients of less than 30 years old with a higher level of consciousness. Patient with GCS of more than 10 and still in the initial phase of disease (less than 4 days of illness) appear to do well (Kennedy and Chaudhuri 2002, Whitley 2006).

However, most patients are still left with significant permanent neurological deficit despite having received aciclovir treatment. Thirty-eight percent of patients do not exhibit any neurological impairment or have minor impairment; nine percent have moderate neurological sequelae and fifty-three percent develop severe neurological sequelae (Whitley and Kimberlin 2005). Common complications associated with HSVE include cognitive and memory deficits. Untreated HSVE is progressive and often fatal within 7 to 14 days. Relapse with HSVE can occur, if so it typically occurs within the first three months of completing drug therapy. There are currently no effective HSV vaccines.

1.4 Immune Response against HSV

The virus’ ability to reach the central nervous system (CNS) depends on the host ability to mount a defence. Virus recognition by members of toll like receptors (TLRs) induces production of Type I IFN α and β (interferon alpha and beta) to control virus replication. TLRs are a class of pattern recognition receptors that
recognise a wide range of pathogens. Macrophages, astrocytes, microglia and neutrophils produce pro-inflammatory cytokines in a predominantly TLR2 dependent manner following HSV-1 infection (Wang et al. 2012). A previous study using primary microglial cells showed that TLR2 signalling is required for the production of pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, IL-6, IL-12, CCL7, CCL8, CCL9, CXCL1, CXCL2, CXCL4 and CXCL 5 (Aravalli et al. 2005). TLR2 also mediates apoptosis in microglial cells in response to HSV infection (Aravalli et al. 2007). TLR2 together with TLR9 stimulates innate antiviral activities for protection against HSV infection in the brain (Sørensen et al. 2008).

The activated microglia produced a considerable amount of cytokines including TNF-α, IL-1β, IP-10 and RANTES together with a smaller amount of IL-6, IL-8 and MIP-1α that attract cells of the somatic immune system such as T lymphocytes, monocytes and neutrophils into the brain to contain the spread of infection (Lokensgard et al. 2002). However, while controlling the infection, an aberrant accumulation of T lymphocytes induce destructive inflammatory brain damage. This is supported by animal studies, in which corneal inoculation of HSV resulted in fatal HSVE, with widespread damage caused by an inflammatory response initiated early in the disease process (Lundberg et al. 2008). Thus, the mechanism for brain damage following herpes simplex encephalitis, appears to involve both direct virus mediated damage and secondary damage by indirect immune mediated (Lokensgard et al. 2002).
1.5 Research Hypothesis and Aims

The pathogenesis of HSVE has been widely studied using animal models and examination of human post-mortem brain tissues. In this PhD thesis I used archived material from formalin-fixed paraffin-embedded post-mortem brain tissue of HSVE patients. The examination of post-mortem human brain tissue focus on the host responses and events that can lead to damage of host cells during the disease process. The main hypothesis of this study is the HSV infection of the brain induces novel host responses during infection, providing novel insight into HSVE pathogenesis.

To address the hypothesis, the aims which outlines the studies undertaken are further described. In this study, I examine histopathological changes that occur in the brain parenchyma of HSVE patients as a result of virus mediated infection and indirect immune mediated cell damage. In addition, the host inflammatory cells which are recruited to the sites of brain infection during the disease, such as macrophages, resident microglia cells, T lymphocytes and B lymphocytes, are characterised. The inflammatory cells’ infiltrations in HSVE are compared with HIV encephalitis and non-encephalitic road traffic accident (RTA) controls.

Examining the host whole-genome gene-expression patterns within brain tissue among HSVE patients compared to RTA cases, I aimed to identify novel changes in host response that occur during the disease. Comparison with brain tissue from RTA cases allowed control for changes that occur secondary to the agonal process or post-mortem tissue processing. Subsequent examination of gene ontological categories offered an opportunity to identify new host pathways involved in the
disease process. I thus identified transcripts significantly differentially expressed in HSVE compared to RTA cases, validated by RT-PCR and immuno-histochemical staining of the corresponding protein in the brain tissue. Examining the geographical relationship between host proteins and HSV within the brain tissue helped me determine whether host changes are linked to HSV infection.

Host pathways that are perturbed in HSVE thus provided new insight into HSVE pathogenesis. The findings may also offer fresh targets for future development of adjunctive treatments to treat HSVE patients.
CHAPTER 2

MATERIALS AND METHODS

2.1 Formalin Fixed Paraffin Embedded (FFPE) Tissue

2.1.1 Human Post-mortem Brain Tissue

Archived formalin fixed paraffin embedded (FFPE) post-mortem human brain tissue was obtained from the Thomas Willis Brain Bank at Oxford University. Permission for using the tissue was granted by the Thomas Willis Brain Bank Review board. Ethical permission to collect the original brain samples was obtained through the Oxford Research Ethics Committee. The post-mortem brain tissues are from patients with a confirmed diagnosis of herpes simplex virus encephalitis (HSVE) (n = 3) and human immunodeficiency virus encephalitis (HIVE) (n = 3). The brain tissue of road traffic accident (RTA) cases (n = 5) was included as negative controls in this study. The HIVE cases which is non-necrotising encephalitis were included in the study for the comparison with necrotising HSVE. The clinical data for patients and RTA controls were not provided. Thus the exact injuries and the associated pathologies that occur to RTA controls were not known.

The FFPE brain tissue was obtained as pre-cut sections of about 10µm in thickness. Slides submitted for histopathological examination were routinely stained with and eosin (H&E). Immunohistochemistry (immuno-histochemical) staining for Herpes simplex virus type 1 (HSV-1) and mitochondria encoded Cytochrome c oxidase 1
(CO1) was performed on serial sequential slides. The brain tissue was also obtained as pre-cut sections of 5mm$^2$ blocks of FFPE tissue for RNA extraction and microarray analysis. Table 2.1 summarises a list of patients and parts of brain regions examined in this research.

Table 2.1 Summary of cases and brain regions involved in this study

<table>
<thead>
<tr>
<th></th>
<th>Temporal</th>
<th>Frontal</th>
<th>Cingulate</th>
<th>Amygdala</th>
<th>Thalamus</th>
<th>Insular</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control 2</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control 3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Control 4</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Control 5</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HSVE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSVE 1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HSVE 2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>HSVE 3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HIVE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIVE 1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIVE 2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HIVE 3</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+, represents available samples and -, represents no samples provided.
2.2 Haematoxylin and Eosin (H&E) Staining

Pre-cut 10µm sections of FFPE post-mortem human brain tissue of HSVE, HIVE and RTA brain sections were stained for routine examination with haematoxylin and eosin (H&E). In general, this staining method involves application of two basic dye, haematoxylin and eosin. Haematoxylin stained the structures with blue/basophilic colour and eosin stained the structures with bright pink/eosinophilic colour. In a typical tissue, nuclei are stained blue whereas, the cytoplasm and extracellular matrix are stained pink (Fischer et al. 2008). The H&E staining have been used for at least a century because it works well with a variety of tissue and fixatives (Fischer et al. 2008).

The FFPE tissues were de-waxed in xylene and hydrated through descending grades of alcohol (100 %, 96 %, 85 %, 70 %) and finally with distilled water. The sections were then stained with haematoxylin for 5 min and washed under running water for 6 min. The sections were next stained with eosin for 2 min. The stained sections were hydrated 3 times with 96 % alcohol for 1 min, 3 times with 100 % alcohol (1 min) and 2 times in xylene (1 min). Finally the sections were mount with DPX and a coverslips were placed on top of the slides. DPX mountant is a mixture of distyrene, a plasticizer, and xylene used as a synthetic resin mounting media.

2.3 Immuno-histochemical staining for CO1 and HSV-1

Pre-cut 10µm sections of FFPE tissue were de-waxed in xylene and hydrated through graded alcohols. Serial microscopic slides were used so that consecutive
sections could be assessed for the presence of antigens and tissue damage. Immunohistochemical staining for CO1 and HSV-1 was performed on sequential slides.

For CO1 staining, the slide sections were incubated with 6ml H$_2$O$_2$ mixed with 360 ml methanol for 30 min to inhibit endogenous peroxidase activity and washed three times with distilled water. This was followed by incubations with normal horse serum for 10 min to block non-specific binding sites in the tissues. The sections were then incubated overnight at 4°C with primary antibody, mouse monoclonal antibody (1D6E1A8 Abcam) at dilution 1:100 as reported previously (Mahad et al. 2009). Positive control was in vitro human brain astrocytes. Horse anti-mouse IgG was employed as a secondary antibody (Vector Labs Inc). The reaction was highlighted by DAB EnVision brown chromogen (EnVision Kit®, Dako).

For HSV-1 staining, the tissues were de-waxed in xylene, hydrated through graded alcohols and further processed accordingly to a heat-induced epitope/antigen retrieval protocol. Antigen retrieval method is requires in formalin fixed tissues for unmasking the antigenic sites (Shi et al. 1991). This is because tissue exposure to formalin fixation is known to cause an extensive cross-linking between nucleic acids and proteins (Park et al. 1996, Lehmann and Kreipe 2001). The antigen retrieval method involves a treatment of FFPE tissue sections with heat and/or enzymatic digestion (Syrbu and Cohen 2011). In this study, an enzymatic procedure with the use of protease antigen retrieval gave most optimal staining in comparison with other antigen retrieval buffers such as EDTA and citrate. Previous study showed that the introduction of protease digestion of FFPE tissue unmasked antigenic sites hidden by cross-linked proteins (Battifora and Kopinski 1986).
The protease stock solution was prepared by dissolving 1g of protease in 20 ml of PBS (Phosphate buffered saline). Slides were incubated in warm 1x PBS, at 37°C for 5 min. Slides were then transferred into 35ml of warm 1% PBS containing 350µl of protease and incubated at 37°C for 5 min. Finally, slides were washed four times with ice cold TBST for 5 min.

Following antigen retrieval, the sections were treated with DAKO REAL™ peroxidase blocking solution for 10 min and washed three times with TBST (Tris-buffered saline Tween 20) buffer, followed by an overnight incubation at 4°C with primary antibody, mouse monoclonal antibody (ab49553 Abcam) at optimal dilution (1:50). Dako EnVision® anti-mouse IgG was employed as a secondary antibody. The reaction was highlighted by DAB EnVision brown chromogen (EnVision Kit®, Dako). For HSV-1 staining a positive control of cultured African green monkey kidney (Vero) cells infected with HSV-1 was also included.

2.3.1 Immuno-histochemical staining for Inflammatory Cells

The slides were also stained for inflammatory markers. The examination of inflammatory cell surface markers are using the cluster of differentiation (CD) nomenclature. The CD system is commonly used as cell markers in immunophenotyping, allowing cell to be defined based on what molecules are present on their surface. Table 2.2 summarises list of inflammatory markers examined in this study.
Table 2.2 Details information regarding inflammatory markers used in this study

<table>
<thead>
<tr>
<th>Inflammatory markers</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>The signaling component of T cell receptor complex</td>
</tr>
<tr>
<td>CD4</td>
<td>A co-receptor for MHC Class II. CD4 is expressed on the surface of peripheral T cells, thymocytes and also present on mononuclear phagocytes and some dendritic cells</td>
</tr>
<tr>
<td>CD8</td>
<td>A co-receptor for MHC Class I. CD8 is predominantly expressed on cytotoxic T cells, but also present on natural killer cells, thymocytes and dendritic cells</td>
</tr>
<tr>
<td>CD79a</td>
<td>A protein expressed on the surface of B lymphocytes</td>
</tr>
<tr>
<td>CD68</td>
<td>A lysosomal protein/molecule that are more abundant in macrophages (hence a phagocytic marker)</td>
</tr>
<tr>
<td>CD163</td>
<td>An endocytic scavenger receptor for haemoglobin-haptoglobin complexes. Detection of perivascular macrophages and macrophage-like microglia in areas of blood brain barrier breakdown</td>
</tr>
</tbody>
</table>
Table 2.3 summarises the information regarding those antibodies, its sources, dilutions and specificity. The antibodies and antigen retrieval method was optimized with the use of automated immunohistochemistry stainer (Bond Leica Microsystem) at Buxton laboratories at The Walton Centre for Neurology and Neurosurgery NHS Trust.

Table 2.3 Details information regarding inflammatory cells antibodies

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Source</th>
<th>Dilution</th>
<th>Specificity</th>
<th>Antigen Retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit Polyclonal anti-Human CD3</td>
<td>DakoCytomation</td>
<td>1:200</td>
<td>T cells</td>
<td>EDTA based pH 9</td>
</tr>
<tr>
<td>Mouse Monoclonal anti-Human CD4</td>
<td>Novocastra</td>
<td>1:50</td>
<td>T helper cells</td>
<td>EDTA based pH 9</td>
</tr>
<tr>
<td>Mouse Monoclonal anti-Human CD8</td>
<td>DakoCytomation</td>
<td>1:50</td>
<td>Cytotoxic T cells</td>
<td>EDTA based pH 9</td>
</tr>
<tr>
<td>(C8/144B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Monoclonal anti-Human CD68</td>
<td>DakoCytomation</td>
<td>1:1600</td>
<td>Macrophages, monocytes, microglial cells</td>
<td>Citrate based pH 6</td>
</tr>
<tr>
<td>(KP1)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Monoclonal anti-Human CD68</td>
<td>DakoCytomation</td>
<td>1:100</td>
<td>B cells</td>
<td>Citrate based pH 6</td>
</tr>
<tr>
<td>(JCB117)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse Monoclonal Antibody CD163</td>
<td>Leica Biosystem</td>
<td>1:400</td>
<td>Monocytes, macrophages</td>
<td>Citrate based pH 6</td>
</tr>
<tr>
<td></td>
<td>(Novocastra)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4 Ribonucleic Acid (RNA) Extraction

Animal brain tissue provided by School of Veterinary Science, University of Liverpool was used in the earlier stage of study to optimize the RNA extraction method from the FFPE tissue. The archived human brain tissue was subjected to RNA extraction only after the method was established.

The RNA was extracted using a commercial kit, Absolutely RNA FFPE Kit (Stratagene product division, Agilent technologies, US). The basis for selection of this kit was based on previous study by (Ribeiro-Silva et al. 2007). The protocol involved the extraction of 2 slices of 10 µm of tissue sections that underwent three main steps; deparaffinization and rehydration, proteinase K digestion and RNA isolation. Two slices of 10 µm of tissue were deparaffinised with 1 ml of d-limonene and incubated at room temperature (RT) for 10 min, followed by three washes with different concentrations of ethanol (100 %, 90 % and 70 %) to remove residual d-limonene.

After the final wash, the ethanol was aspirated, and the pellet was air dried for 5 min/RT. The pellet was re-suspended in 100 µl of proteinase K buffer and 10 µl of proteinase K enzyme (20 mg/ml) and incubated at 55 °C for 3 h, in order to solubilise the fixed tissue and release the nucleic acids into solution. This was followed by RNA isolation in which RNA binding buffer and β-Mercaptoethanol was added, followed by homogenisation of the sample with vigorous pipetting or vortexing.

Sample was then loaded onto RNA spin column, centrifuged and washed with low salt and high salt wash buffers. At the final step, 30 µl of pre-heated elution buffer
was added directly onto the fiber matrix of the column and centrifuged for 1 min at maximum speed. The recovered RNA was stored at -80 °C.

However, the above method produced low RNA yields. Different steps of the manufacturer’s method were thus modified accordingly until a satisfying RNA purity and yield was achieved.

2.4.1 Modification Steps and Trizol RNA Extraction Method

No changes were introduced to the deparaffinization and rehydration steps of the manufacturer’s protocol, however incubation with proteinase K enzyme was extended from 3 h to 18 h at 55°C. Extending the digestion time with Proteinase K has been reported to improve the amount of extracted RNA (Chung et al. 2006, Abramovitz et al. 2008, Bonin et al. 2010). In addition, the concentration of proteinase K was increased to 20 mg/ml.

Thorough homogenisation assists the release of RNA from the lysed sample. Thus, vortexing and pipetting were replaced by two steps of motor pestle homogenisation, performed after 1 h of proteinase K incubation and again after 18 h of incubation. This was followed by RNA isolation using Trizol RNA extraction method (Invitrogen, Life Technologies). Trizol has been widely used for the extraction of nucleic acid and has been shown to produce higher amount of RNA (Glenn et al. 2010).

For Trizol RNA extraction, the homogenised tissue was further homogenised by pipetting and vortexing with 1 ml of Trizol reagent and 1µl of β-mercaptoethanol,
and freeze-thawed. After thawing, sample then incubated at RT/5 min to allow a complete dissociation of the nucleoprotein complex. 200 µl of chloroform was added, sample was vortexed for 15 sec and incubated at RT/3 min. Sample was then centrifuged at maximum speed (14000 rpm-16000xg) for 30 min at 4°C. The upper aqueous phase containing RNA was transferred into a new tube and was mixed with 2 to 5 µl of glycogen carrier, 20 µl of 5M sodium chloride and 0.5 ml 100 % isopropanol, incubated at -80 ºC for 30 min and centrifuged at maximum speed for 30 min at 4°C. The resulting RNA pellet was washed with 1 ml of 75 % ethanol and centrifuged at maximum speed for another 30 min at 4°C. After two rounds of 75% ethanol wash, pellet was dried at room temperature for 10 min and RNA was re-suspended in 20 µl of RNAse-free water. Total RNA was measured with Nanodrop ND-2000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific, Wilmington, DE) and RNA was stored at -80 ºC.

2.4.2 Ethanol Precipitation

The tissue with absorbance ratio (A260nm/ A230nm) of less than 1 was subjected to further ethanol precipitation in order to increase the RNA purity (target ratio 2 or higher). 1 to 2 µl of glycogen carrier was added to the 20 µl of sample, followed by an addition of 0.1 volume of 5M sodium chloride (2 µl of 5M sodium chloride) and two to three volumes of 100 % ethanol. Sample was then incubated at -80°C for 1 h to overnight. Sample was then centrifuged at maximum speed (14000 rpm) for 30 min at 4°C. The supernatant was removed and the pellet was transfer into a new tube, washed
with 1 ml of 80% ethanol and centrifuged as before. After one additional round of 80% ethanol wash, the pellet was left dry on the bench or in heating block at 30 °C and RNA was re-suspended in 20 μl of nuclease free water.

2.4.3 DNAse Treatment with TURBO DNA-free™ Kit

TURBO DNase-free Kit (Ambion, Applied Biosystem) was used to remove traces of DNA from the RNA sample. 0.1 volume of 10x DNase buffer and 1 μl of TURBO DNase were mixed with RNA sample and incubated for 20 to 30 min at 37 °C. To inactivate DNase enzyme, 2 μl of inactivation reagent was added and the sample was incubated at room temperature for 5 min, with occasional mixing. Finally, the reagent was centrifuged at 10000xg for 1.5 min and supernatant was transferred into a fresh tube. The recovered RNA was measured using NanoDrops and stored at -80°C.

2.5 Microarray

2.5.1 Transplex® Complete whole Transcriptome Amplification Kit (WTA2)

The RNA amplification was conducted using Transplex® Whole Transcriptome Amplification Kit (Sigma Aldrich) following the specified protocol. The protocol allows for amplification of nanogram quantities of total RNA to microgram quantities of a product that is suitable for real time quantitative PCR (QPCR) and
microarray. Furthermore, WTA2 kit is optimised to amplify RNA from FFPE and other damage or degraded samples.

500 ng of the total RNA from cingulate was mixed with 2.5 µl of library synthesis solution and a nuclease free water to a total volume of 16.6 µl. The RNA was primed in thermocycler at 70 ºC for 5 min and hold at 18ºC. The cooled-primed RNA was immediately incubated with 2.5 µl of library synthesis buffer, 2 µl of library synthesis enzyme and 3.9 µl of nuclease free water for reverse transcription process to create a complimentary DNA (cDNA) library of target fragments with a universal end sequence. The reaction runs using the following parameters:

1. 18 ºC for 10 minutes
2. 25 ºC for 10 minutes
3. 37 ºC for 30 minutes
4. 42 ºC for 10 minutes
5. 70 ºC for 20 minutes
6. 4 ºC for ∞

The cDNA library was amplified using the universal primer to produce a whole transcriptome amplification product. The total of 25 µl of cDNA library mixed with the master mix for amplification reaction, which consists of 37.5 µl of amplification mix, 7.5 µl WTA dNTP mix, 3.75 µl of amplification enzyme and 301 µl of nuclease free water. The entire reaction was divided into five 75 µl reactions, and each PCR reaction was run as follows:

1. 94 ºC for 2 minutes
2. 94 ºC for 30 seconds
3. 70 ºC for 5 minutes
4. Step 2-3 for 17 cycles
5. 4 ºC for ∞

After the cycling was completed, the reactions were stored at -20 ºC until ready for purification.

2.5.2 QIAquick PCR Purification

It is essential for DNA to be free from contaminants. This purification step involves purification of 100-1000bp DNA fragments from primers, nucleotides, polymerase and salts. Five volumes of buffer PB was mixed with one volume of PCR sample before being transferred into a spin column and centrifuged at maximum speed (14800 rpm/16100xg) for 60 s. The flow through was removed, and the column was washed with 0.75 ml of buffer PE, and centrifuged twice for 60 s. Qiaquick column was placed in a 1.5 ml microcentrifuge tube and 30 µl of elution buffer was added to the centre of the column membrane, incubated for 1 min at room temperature and centrifuged at maximum speed for 1 min. The DNA was then measured by Nanodrop and stored at –80 ºC.

2.5.3 Universal Linkage System (ULS) Labelling

The labelling of cDNA with cyanine-3 was performed using ULS labelling kit as per manufacturer’s protocol. This protocol is specifically designed for nucleic acids
recovered from FFPE tissue. The cDNA was labelled using a non-enzymatic one-step labelling method named Universal Linkage system (ULS). ULS has proven to be a better method for labelling of DNA from archived FFPE tissue (Alers et al. 1999, van Gijlswijk et al. 2001).

For labelling, 1500 ng of DNA mixed with 1.5 µl of ULS-cyanine 3 dye and 2 µl of 10X labelling solution to make a total volume of 20 µl. The sample was incubated at 85 ºC for 30 minutes and the reaction stopped by transferring the tube into ice. The non-reacted dye was then removed using the Agilent KREApure columns.

2.5.4 KREApure Column

The Agilent KREApure column was mixed briefly by a vortex mixer. The cap was loosened quarterly and the bottom closure removed and centrifuged at maximum speed for 1 min. Flow through was removed and 300 µl of water was added to the column. The columns again centrifuged at maximum speed for 1 minute and flow through discarded. After 3 to 5 times of centrifugation and removal of flow through, the column was transferred into a clean 1.5 ml microcentrifuge tube and ULS labelled sample is transfer to the column, followed by short incubation at room temperature for 1 min. Finally, the column was centrifuged at maximum speed for 1 min and purified labelled sample collected in the collection tube. The labelled DNA was measured using Nanodrop to quantify for the degree of labelling as shown by the formula below:

\[
\text{Degree of labelling} = \frac{340 \times \text{pmol per } \mu\text{l dye}}{(\text{ng per } \mu\text{l cDNA} \times 1000)} \times 100 \%
\]
2.5.5 Array Hybridization

2.5.5.1 Pre-hybridization

100 X blocking agent was prepared by adding 50 µl of nuclease free water to a vial of lyophilized 10 X GE blocking agent. The mixture was vortexed briefly and incubated at room temperature for 60 min to reconstitute the sample.

2.5.5.2 Hybridization

The components and volume per reaction of master mix are shown in Table 2.4. 30 µl of the hybridisation master mix was added to 8.5 µl (1000 ng) of ULS-labelled DNA to make a total volume of 38.5 µl and incubated at 95 °C for 3 min. The sample was then placed on ice before mixing with 12.5 µl of Agilent-CGHblock to make a total volume of 51µl and dispensed on Agilent Sureprint G3 8 x 60 K (Design ID 030495) custom human-specific microarrays. The sample was hybridised at 65 °C for 40 h at 20 rpm before washing.
Table 2.4 Summary of the components and volume of master mix for array hybridization

<table>
<thead>
<tr>
<th>Components</th>
<th>Per reaction (µl)</th>
<th>Per slide (µl) (including excess) x 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>100X GE blocking agent</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>2x Hi-rpm GE Hyb Buffer</td>
<td>25</td>
<td>250</td>
</tr>
<tr>
<td>Final volume of Hyb Master mix</td>
<td>30</td>
<td>300</td>
</tr>
</tbody>
</table>

2.5.6 Washing Step

A wash buffer 1 and 2 were prepared by adding 2 ml 0.005% of Triton X-102. Triton X-102 eliminates background wash artefacts that can occur in gene expression microarray and has no adverse effects on the gene expression data itself. Gene expression wash buffer 2 was warmed to 37 °C before using.

After completed 40 h of hybridisation, the slides were disassembled and transferred into a dish containing wash buffer 1. The slide washed for 1 min with constant stirring and transferred to a second dish with pre-warmed wash buffer 2 and washed for 1 min.
2.5.7 Slide Scanning

Hybridised and washed arrays were scanned with Agilent microarray scanner (Agilent Technologies). The scanner measures fluorescence of labelled nucleic acids bound to microarray. Raw fluorescent intensity was measured and initial quality control assessment undertaken using an Agilent softwares.

2.6 Real Time Quantitative PCR (QPCR)

2.6.1 Reverse Transcriptase PCR

Complimentary DNA was generated from 1μg of RNA using RETROscript® Kit (Ambion, Applied Biosystem). Master mix which consisted of 2 μl of random hexamers, 2 μl of 10X RT buffer, 4 μl of dNTP mix, 1 μl of RNase inhibitor and 1μl of MMLV-reverse transcriptase per reaction was prepared. Random hexamers are perform better in synthesize of complementary DNA from degraded samples. The total 1 μg of RNA and nuclease free water was added to make up the final volume of 20 μl. The cycling conditions were as follows:

1. 44 °C for 1 hour
2. 92 °C for 10 minutes
3. 4 °C for ∞
2.6.2 QPCR

Quantitative real time PCR was performed using the ABI PRISM 7000 sequence detection system (Applied Biosystem, USA). The primer for mitochondrial gene, Cytochrome c oxidase 1 (CO1) (assay ID Hs02596864_g1) that was down regulated in microarray study were amplified. The QPCR was performed using TaqMan gene expression assay (Applied Biosystem). The PCR reaction was carried out as per manufacture’s protocol. Reaction mixture of 20 µl consisted of 10 µl of TaqMan gene expression master mix which contained AmpliTaq Gold® DNA Polymerase, UltraPure (UP), Uracil-DNA Glycosylase(UDG), deoxyribonucleotide triphosphates (dNTPs) with deoxyuridine triphosphate (dUTP), ROX™ Passive Reference, and buffer components optimized for sensitivity, precision, specificity and duplexing. 1 µl of TaqMan gene expression primer was added to the reaction, 50 to 100 ng of cDNA and nuclease free water to make final volume of 20 µl. The cycling conditions were as follows:

1. Incubate at 50 °C for 00:02:00
2. Incubate at 95 °C for 00:10:00
3. Incubate at 95 °C for 00:00:15
4. Incubate at 60 °C for 00:01:00
5. Plate read
6. Go to line 3 for 54 more times
7. Melting curve from 65 °C to 95 °C, read every 0.2 °C, hold 00:00:01
8. Incubate at 4 °C for 00:10:00
9. End.
CHAPTER 3

HISTOPATHOLOGICAL EXAMINATION OF BRAIN TISSUE FROM HSV ENCEPHALITIS PATIENTS AND CONTROLS

3.1 Introduction

The herpes simplex virus encephalitis (HSVE) is the inflammation of brain tissue associated with the herpes simplex virus (HSV) whereas the human immunodeficiency virus encephalitis (HIVE) is the inflammation of brain tissue associated with the human immunodeficiency virus (HIV). This chapter aims to examine the histopathological changes in post-mortem brain tissue of patients with HSVE and HIVE in comparison with road traffic accident (RTA) controls.

3.1.1 Road Traffic Accident (RTA) Controls

In RTA control brains, histopathological changes are expected to differ significantly to those observed during encephalitis, with minimal inflammation. Acute death of patients due to a road traffic accident may however result in damage of the brain, resulting from either direct trauma or a secondary injury. Secondary injury is a complex set of cellular processes and biochemical cascades that occur minutes to days following initial trauma. These include: release of factors that cause inflammation, free radicals overload, influx of sodium and calcium into neurons,
mitochondria dysfunction, insufficient blood flow to the brain/ischemia, insufficient oxygen/hypoxia, cerebral oedema and increased intracranial pressure.

Forensic autopsy study of patients dying acutely following automotive injuries, in which no history of hypoxic attack was reported, revealed a lack of morphological changes in neurons and no microglia activation (Kitamura 1994). However, some cases demonstrated a swelling of astrocyte cell bodies, which most probably occurred during early stages of hypoxic/ischemic injury or post-mortem autolysis.

In addition, a post-mortem examination of patients that survived up to five hours after hypoxic/ischemic injury revealed some ischemic neuronal changes, including shrinkage of cell bodies, eosinophilia of cytoplasm and pyknosis of the nuclei, but no apparent activation of glial cells was found. In cases with longer post-traumatic survival, even further neuronal loss is observed (Kitamura 1994, Kubo et al. 1998). In response to ischemic injury, activation and proliferation of microglia and astrocytes were found in brain regions that show severe neuronal necrosis (Petito et al. 1990, Panickar and Norenberg 2005).

Furthermore, infiltration of blood-derived inflammatory cells, particularly T lymphocytes and macrophages, was also observed in trauma patients. Animal studies show that T lymphocytes and neutrophils infiltrate into the brain parenchyma and reach their peak at 24 to 48 hours, while macrophages and microglia reach their peak at 7 days post injury (Clausen et al. 2007).
3.1.2 Herpes Simplex Viral Encephalitis (HSVE)

The histopathology of HSVE has been previously studied using post-mortem and brain biopsy materials. Herpes simplex virus was first isolated from post-mortem brain tissue of fatal encephalitis in the 1940s by researchers from The Army Institute of Pathology, Washington. Their histopathological findings have been extensively described in various papers, published separately by Zarafonetis and Smadel, Whitman et al. and Haymaker (Zarafonetis and Smadel 1944, Whitman et al. 1946, Haymaker 1949). Smith et al. first reported the pathologic findings that proved to be the result of herpes simplex encephalitis (Smith et al. 1941).

Pathologies of viral infections of the central nervous system (CNS) vary significantly depending on the: (1) type of the causative virus; (2) location of the infection and; (3) the nature/severity of the disease (i.e. acute or chronic). Although most of the viral infections demonstrate similar pathological reactions there are certain characteristics of neuropathological changes that are specific to HSVE.

Pathological examination of the autopsy of HSVE brains shows multiple haemorrhages, swelling and tissue necrosis in the temporal lobe. These changes affect both sides of the brain but usually one cerebral hemisphere is more affected than the other. HSVE particularly localises to the temporal lobe. However, previous studies also reported the involvement of other regions, such as a frontal lobe, cingulate, insular gyrus and adjacent limbic areas.

The characteristic diagnostic features of HSVE are death of neurons, and the presence of viral particles/intranuclear inclusion bodies. Death of neurons are
characterised by pyknotic nuclei with shrunken cell bodies and eosinophilia cytoplasm (Esiri 1982) (Figure 3.4A).

Intranuclear inclusion bodies represent newly synthesised virus within the host cell nucleus. The cell nucleus appears eosinophilic with marginalised chromatin. The intranuclear inclusion bodies are often observed in the first week of infection. Most of these inclusion bodies are found in glial cells, mainly astrocytes (Figure 3.5A). However, neurons can also exhibit inclusion bodies (Zarafonetis and Smadel 1944). Interestingly, viral inclusion bodies are only reported in 50 % of HSVE cases.

HSVE infected cells produce pro-inflammatory cytokines and chemokines that activate an extensive immune response against the infection (Lokensgard et al. 2002). The major inflammatory cells in HSVE are infiltrating lymphocytes that accumulate around the blood vessels and spread into the infected adjacent parenchyma. In the early stages of the infection, the polymorphonuclear leucocytes may also be present. In some cases, the inflammation can spread to the meninges. (Smith et al. 1941, Zarafonetis and Smadel 1944). The microglia also become activated, proliferates and form clusters around the dead and infected neurons, which are then phagocytosed.

Vascular blood vessels are also affected in HSVE. Some vessels appear engorged and dilated. The red blood cells remain confined within these vessels, and the vessels exhibit an intact endothelial lining (vascular congestion). In other brain regions multiple small to large haemorrhages can be seen. The vessel wall usually appears normal with some swelling/hypertrophy of the endothelial cells (Smith et al. 1941).
In the first week from HSVE onset, there are limited signs of inflammation in the brain tissue. Inflammation is mainly focused around the perivascular regions of the superficial cortex and meninges. Shrinkage and eosinophilia of neuronal cell cytoplasm is observed. There is also marked vascular congestion and capillary dilatation, with petechial haemorrhages and occasional intranuclear and intracytoplasmic inclusion bodies (Esiri 1982). After two weeks there is a marked oedema and necrosis, loss of cortical neurons, increased macrophage/microglial phagocytosis, and significant perivascular infiltration with lymphocytes and plasma cells. Lipids bodies accumulate inside phagocytes. Microglia appear clustered around dead neurons. Generally, necrosis and inflammation appears to reach peak intensity in the third week from the onset of the disease. Tissue from patients that die in their fourth week of illness show severe tissue destruction, fibrous glial scarring and a residual mononuclear cell infiltration in the parenchyma (Esiri 1982).

3.1.3 Human Immunodeficiency Viral Encephalitis (HIVE)

HIV encephalitis is the infection of the brain tissue by the human immunodeficiency virus (HIV). It usually occurs weeks or months after the individual first contracted the HIV infection. It is believed that the virus is carried into the brain by infected CD4 lymphocytes or macrophages. Within the CNS, HIV replicates mainly in cells of the monocyte/macrophage lineage, including microglia. They are believed to acquire the virus by phagocytosing infected lymphocytes. There is no productive infection of either neurons or glial cells, although a population of astrocytes has been shown to harbour the virus in vitro. Therefore, it
is believed that brain damage in HIVE is not a direct result of HIV-induced neuronal death, but is secondary to reactive inflammation through activated macrophages and microglia.

Histopathological examination of the post-mortem HIVE brain tissue reveals multinucleated giant cells. Giant cells are considered a specific hallmark of HIV encephalitis. However, they can be seen in other brain infections, such as TB, fungal infection, sarcoidosis and other granulomatous disease (Gelman 2007, Budka 2008). Multinucleated giant cells are a fusion of multiple macrophages infected with HIV-1 (Figure 3.7B). They are typically seen in the perivascular areas of white matter. However, some cases of HIVE do not display multinucleated giant cells.

Other histological changes during HIVE, but not specific to this type of encephalitis, are multiple microglia nodules (clusters of activated microglial); loss of white matter staining; and reactive astrocytes (Leite et al. 2005). A reactive astrocytes is morphologically characterised by a distinct cytoplasm and larger nuclei that is eccentric in position inside the cell. Reactive astrocytes, also known as gemistocytic astrocytes, can form in response to neuronal injury or an inflammatory cascade. Tissue from HIVE patients can also exhibit more diffuse microglial activation (not in clusters) and perivascular lymphocyte infiltration. In severe cases, structural disorganization and vacuolization of the neuropil or grey matter (poliodystrophy) may also be seen.
3.2 Materials and Methods

Pre-cut 10µm sections of formalin fixed paraffin embedded human brain tissue of HSVE, HIVE and road traffic accident brain sections were stained for routine examination with haematoxylin and eosin (H&E) staining. Only a section from the frontal region were provided for HIVE patients, compared to HSVE and RTA for which temporal, frontal, cingulate, amygdala and insular regions were analysed (Figure 3.1). H&E stains particular components within the tissue and allows researchers to visualise the nucleus of the cells and their morphological state. Details regarding the methods of haematoxylin and eosin staining are described in Chapter 2.
Figure 3.1 Image represent the brain regions examined in this study. (A) The lateral surface of the brain shows the frontal lobe (composed of: a, orbital gyri; b, middle and inferior frontal gyri; c, superior frontal gyrus) and temporal lobe (composed of: d, temporal pole; e, superior temporal gyrus and f, middle and inferior temporal gyrus). (B) The medial view shows cingulate gyrus (g), thalamus (h) and hypothalamus (i). (C) The amygdala (j) is part of limbic system. (D) Insular cortex (k) is part of cerebral cortex located beneath the frontal, parietal and temporal gyri. Images A to C are from http://www.columbia.edu/itc/hs/medical/neuroanatomy/neuroanat/ and F from http://www.scholarpedia.org/
3.3 Results

The results of the histopathological examination are presented separately for each individual patient involved in this study. Thus, three cases of HSVE, three cases of HIV positive encephalitis and five road traffic accident cases were analysed and presented.

3.3.1 Road Traffic Accident (RTA) Controls

3.3.1.1 RTA Control 1

Six brain regions, including temporal, frontal, cingulate, amygdala, insular and thalamus were analysed for the evaluation of histopathological changes in all five road traffic accident controls. RTA 1 demonstrated pronounced but localised reactive changes. These changes were seen within the deep cortical matter of the temporal lobe. The remaining brain regions showed limited abnormalities. These abnormalities were restricted to mild perivascular infiltrations by lymphocytes and diffuse vascular congestion. The tissue contained normal looking neurons, each characterised by a relatively large cell nucleus with a single prominent nucleoli and prominent Nissl substance (Figure 3.2A). Normal looking pyramidal neurons were also observed in the grey matter of the temporal cortex (Figure 3.2B).
Figure 3.2 Normal looking neurons in the cingulate (A) and temporal (B) region of RTA Control 1. Neurons exhibit large nuclei with a prominent nucleoli and Nissl substance (arrow).
Within the deep cortical grey matter of the temporal cortex, focal reactive changes were observed. There was depletion of some of the cortical neurons. These neurones were replaced by macrophages with foamy cytoplasm (gitter cells), microglial and reactive astrocytes (Figure 3.3A). Reactive astrocytes were seen and were again characterised morphologically by distinct cytoplasm and large nuclei that were in an asymmetric position inside the cells. There was diffuse vacuolisation of the white matter. Some of the meningeal vessels were congested with multi-focal marginalisation of neutrophils (Figure 3.3B). Congested vessels again appeared engorged and dilated with red cells confined within the vessels.
Figure 3.3 Representative histopathological changes in the temporal region of RTA 1. (A) Tissue shows focal necrosis of the grey matter, which has become populated by numerous gutter cells, microglia and reactive astrocytes (arrow). (B) Presence of inflammatory cells within the blood vessels of the cerebral meninges. Magnification A = x10 and B = x4.
3.3.1.2 RTA Control 2

No significant findings were observed in the histopathological examination of the brain sections of RTA Control 2. Mild perivascular infiltration was observed in the temporal region. In addition, a mild vascular congestion within the meninges and parenchyma were also observed. The neurons appeared normal.

3.3.1.3 RTA Control 3

The temporal, frontal, cingulate, amygdala and thalamus brain regions of RTA Control 3 demonstrated only mild histopathological changes. In general, only mild diffuse vascular congestion, occasional perivascular infiltrations and normal looking neurons were observed.

Focal but significant pathologic changes were observed within the insular region. These changes involved extensive cavitation of the white matter, with massive infiltration of macrophages with foamy cytoplasm (gitter cells w). The gitter cells diffusely replaced large areas of the white matter. Furthermore, a significant depletion of neurons within the grey matter was noted, again with replacement by gitter and other inflammatory cells.
3.3.1.4 RTA Control 4

Mild histopathological changes were observed for this control. No significant abnormalities were observed. Grey and white matter appeared normal. Mild reactive changes were seen in the temporal cortex, with clusters of neutrophils within the lumen of some of the meningeal vessels.

3.3.1.5 RTA Control 5

No evidence of morphological abnormalities was detected within most of the brain sections examined in this control. However, there were occasional multifocal small haemorrhages, mild microglia proliferation and a few reactive astrocytes within the parenchyma of the amygdala.
3.3.2 Herpes Simplex Virus Encephalitis (HSVE)

3.3.2.1 HSVE Patient 1

Histopathological examination of the brain sections of HSVE Patient 1 revealed numerous necrotic neurons in the hippocampus and the temporal lobe. Figure 3.4A shows the typical features of necrotic neurons: shrunken neurons with hyper-eosinophilic cytoplasm. Necrotic neurons were also observed in the frontal, cingulate, amygdala and insular regions. Numerous intra-nuclear inclusion bodies were observed within astrocytes. These intra-astrocytic inclusion bodies were scattered throughout the parenchyma of the temporal region. In contrast, inclusion bodies were rarely seen within the other brain regions.

Microglia, the brain’s tissue resident macrophages, were diffusely scattered throughout the parenchyma of the temporal region. Some microglia accumulated around necrotic neurons. These microglia were probably phagocytosing dying neurons. In other areas, dead neurones were replaced by microglial nodule (Figure 3.4B). Marked inflammatory cell infiltrations were observed throughout most of the brain regions examined for the case. Infiltrations were particularly prominent within the perivascular spaces and the adjacent parenchyma (Figure 3.4C). These inflammatory infiltrations appeared to be predominantly composed of lymphocytes.

Numerous gitter cells occupied large necrotic areas of superficial cortex within the temporal and frontal regions. Gitter cells were also observed in areas of focal necrosis in the cingulate, amygdala and insular regions.
Multiple large haemorrhages were observed in the parenchyma of the temporal (Figure 3.4D) and frontal lobe. The other brain regions showed occasional small haemorrhages.
Figure 3.4 Representative histopathological changes in the brain tissue of HSVE patient 1. Images A, B and D are from the temporal region. Image C is from the frontal region. (A) Numerous shrunken and hypereosinophilic necrotic or dead neurons. (B) A microglia nodule remains following removal of necrotic neurons. (C) A lymphocyte-dominated perivascular infiltration composed of 1 to 3 layers of cells. (D) Multiple large haemorrhages within the parenchyma of the temporal region. Magnification A, B and C = x20, and D = x4.
3.3.2.2 HSVE Patient 2

Milder histopathological changes were observed in HSVE Patient 2 compared to the Patient 1. A large proportion of the pyramidal neurons of the hippocampus and cerebral cortex of the temporal lobe exhibited necrotic changes. Necrosis was also observed among scattered neurons of the cerebral cortex in the cingulate and the amygdala regions. Intranuclear inclusion bodies were encountered more frequently in or near the necrotic neurons. For example, numerous astrocytes with large intranuclear inclusion bodies were found within the temporal cortex in association with necrotic pyramidal neurons. The intranuclear inclusion bodies were also present within scattered astrocytes of the frontal region and the amygdala (Figure 3.5A).

In the temporal region, the meninges were heavily infiltrated with inflammatory cells. However, only mild perivascular infiltrations were observed within the parenchyma of the temporal (Figure 3.5B) and other brain regions. In addition, mild to moderate microglia proliferation was also observed in all brain regions in this patient.

Multiple large haemorrhages were seen in the parenchyma of the temporal cortex. Occasional small haemorrhages were also observed in the amygdala and cingulate brain region. Blood vessels in the other brain regions appeared engorged and dilated with red cells confined within the vessels.
Figure 3.5 Representative histopathological changes in the amygdala of HSVE Patient 2. (A) An eosinophilic intranuclear inclusion body within the astrocytes. (B) Mild perivascular infiltrations with proliferation of microglial in the tissue parenchyma near the vessel (arrow). Magnification A = x40 and B = x10.
3.3.2.3 HSVE Patient 3

Histopathological examination of Patient 3 revealed severe necrotizing encephalitic changes in all of the brain regions examined. The majority of neurons were depleted and necrosis was extensive throughout the grey matter. The necrosis also extended into the superficial and deep white matter. Severe liquefactive necrosis was observed in the frontal cortex with a complete loss of tissue structure. The entire cerebral cortex was replaced by gitter cells (Figure 3.6A). There was diffuse proliferation of reactive astrocytes and microglia. There were also numerous inflammatory cell infiltrations in the perivascular spaces. Perivascular infiltrations appeared to be dominated by lymphocytes. The endothelial lining of the vessels also appeared swollen and hypertrophied (activated endothelial cells) (Figure 3.6B).

Other brain regions, such as cingulate, insular and amygdala regions, also showed liquefactive necrosis. However, in these regions necrosis was focal. This was presumably because the lesions were restricted by the laminar arrangements of the external cerebral cortex. The severity of necrosis appeared reduced in the white matter of these regions. The meninges were inflamed throughout, with infiltration mainly by lymphocytes. A few plasma cells were also observed in all brain regions. The cerebral cortex also exhibited numerous reactive astrocytes (gemistocytic astrocytes). These astrocytes extended into the superficial and deep white matter of the frontal brain region.
Figure 3.6 Representative histopathological changes in the brain tissue of HSVE Patient 3. Both images are from the frontal region. (A) Severe necrosis of the cerebral cortex. Neurons are replaced by an abundant accumulation of gitter cells (inset image) and lymphocytes. (B) The perivascular infiltrating cells are predominately composed of lymphocytes. The endothelial cells (arrow) are swollen. A massive accumulation of gitter cells is also seen in the adjacent parenchyma. Magnification A = x4, and B = x20.
3.3.3 Human Immunodeficiency Virus Encephalitis (HIVE)

3.3.3.1 HIVE Patient 1

Histopathological changes for the HIVE patients are described for the frontal brain region (the only region available). There was a diffuse decrease in myelin staining with resultant pallor of the white matter in patient 1. Numerous reactive astrocytes were disseminated throughout the white matter (Figure 3.7A). Occasionally, multinucleated giant cells were also observed within the white matter (Figure 3.7B). Multinucleated giant cells are typically composed of fused mononuclear phagocytes that are infected with HIV-1. Blood vessels showed mild vasogenic oedema with mild perivascular infiltrations of inflammatory cells. The subpial grey matter exhibited a small cavity with scattered inflammatory cells and reactive astrocytes.

3.3.3.2 HIVE Patient 2

Histopathological changes for this patient were similar to the previous case. In general, there was a decrease in myelin staining, with a few reactive astrocytes scattered within the white matter. Swollen fragmented axons were also observed within the white matter. Occasionally, inflammatory cells were observed in close proximity with the fragmented axons. Inflammatory cell infiltration of the perivascular spaces was essentially absent.
3.3.3.3 HIVE Patient 3

Examination revealed a mild vascular congestion with focal area of a decreased myelin staining in the white matter. Reactive astrocytes were occasionally seen in the white matter. In addition, there was a diffuse sub-meningeal oedema associated with a mild proliferation of microglia.
Figure 3.7 Representative histopathological changes in the brain tissue of HIV-1 patient 1. (A) Multiple ‘plump’ reactive astrocytes with abundant cytoplasm and asymmetrically positioned nuclei (gemistocytic astrocytes) scattered within the white matter. (B) A typical HIV infected multinucleated cell indicated by the arrow. Magnification A = x10, B = x20
3.4  Discussion

In this retrospective study, histopathological examination of the post-mortem brain tissue, using of routine HE staining proved to be a simple and very effective method of appreciating the distinct characteristics of brain injury associated with RTA, HSVE and HIVE.

3.4.1  Summary of RTA findings

In sudden death following a road traffic accident, I would expect minimal pathological changes in the brain tissue, because there is limited time for the body to react to the injury. In line with this expectation, three RTA subjects demonstrated mild or insignificant neuropathological features, suggesting early demise (within 24 hours) following their accidents. In contrast, subjects 1 and 3 did show inflammatory changes. These changes were mild and restricted to a focal area within a single brain region. The inflammatory cell recruitment into the brain parenchyma suggests that both deaths were delayed following their road traffic accidents, compared to the other three subjects.
3.4.2 Summary of HSVE

The histopathological changes seen in all three HSVE cases are in keeping with the original findings reported by Haymaker (Haymaker 1949).

In all three cases the temporal lobe was affected. All three cases showed necrotic neurons in the grey matter. All cases also exhibited characteristic astrocytic intranuclear inclusion bodies within the parenchyma, occasionally in close proximity to necrotic neurons. Reactive astrocytes and activated microglia were also seen in the parenchyma. All cases also exhibited infiltration of immune cells into the perivascular spaces, with congested vessels and variable degrees of haemorrhage. In areas of marked neuronal loss, often large numbers of activated microglia, and fat laden macrophages, gitter cells, were observed.

However, there were also differences in the extent of necrosis and inflammatory cell activation between the three cases. Patient two showed damage predominantly in the temporal lobe, although relatively little damage was observed throughout all brain regions examined. Patient one showed marked damage in the temporal lobe. Patient three showed very marked tissue destruction and inflammatory cell infiltration throughout all brain regions.

Based on previous histopathological descriptions provided by Esiri (1982), the differences between the three post-mortem findings probably reflect different lengths of illness prior to death and / or differing severity of disease.

Patient 2 probably died within the first week following acute infection. This is supported by the mild inflammatory cell infiltrations in the perivascular spaces, presence of necrotic neurons with limited accompanying neuronal loss, vascular...
congestion and a few smaller haemorrhages. Patient 1 most probably died within two weeks of the disease. This patient showed loss of cortical neurons, and replacement of the affected cortex with inflammatory cells. There was also a more intense perivascular infiltration, and an increased proliferation of microglia around the necrotic neurons. Patient 3 demonstrated widespread necrosis and inflammation with severe tissue destruction. Such findings are usually reported in patients dying two-three weeks from the onset of illness.

3.4.3 Summary of HIVE

The histopathological changes seen in all three HIVE were again consistent with previous autopsy examinations of HIVE (Petito 1988, Leite et al. 2005).

All three cases were characterised by neuropathological changes almost entirely restricted to the white matter (leukoencephalitis). All showed loss of myelin staining in the white matter with proliferation of reactive microglia.

A common feature among HIVE cases is decrease staining of myelin. This is reported to primarily occur in areas of white matter adjacent to blood vessels. The loss of myelin staining is typically accompanied by an increased inflammatory infiltrate in the corresponding perivascular spaces. A more wide-spread loss of myelin within the white-matter is reported in advanced cases of HIVE infection.

Another common finding in HIVE, again observed in this study, is the presence of reactive astrocytes within the affected white matter. The changes in astrocytes may represent a non-specific reaction to the white matter damage or could be a
component of in a multi-cellular inflammatory cascade in response to a non-productive and limited HIV-1 infection within the CNS (Gelman 2007).

Multinucleated giant cells are the hallmark for HIVE. They are observed in about 25% of post-mortem cases (Petty 1994). In this study, multinucleated giant cells were only seen in patient 1.

3.4.4 HSVE versus HIVE

The histopathology of HSVE was very different to HIVE cases. The main histopathological changes for HSVE were observed within the cortical grey matter (polioencephalitis), whereas HIVE involved mainly the white matter (leukoencephalitis).

The result demonstrate that the predilection of viruses to target different cells influences the pathology of the diseases. HSV is a neurotrophic virus that directly infects neurons. This infection is associated with neuronal damage and death. Infected and damaged neurons and astrocytes are reported to stimulate recruitment and activation of inflammatory cells. These cells are initially observed to accumulate in the perivascular spaces and are later seen to spread to the adjacent brain parenchyma. In-vitro studies have shown that HSV directly damages CNS cells. However, researchers have also suggested that inflammatory cell infiltrates may also cause further neuronal cell damage.

HIV is known to infect cells in the monocyte/macrophage linage, including microglia. There are no reports that the virus infect neurons (Leite et al. 2005).
Thus any tissue damage that occurs in HIVE is believed to occur as a result of exposure to the inflammatory molecules, excitotoxins, and proteases that are released by activated microglia and other inflammatory cells, rather than by direct viral damage.

In line with previous reports, the tissue damage observed among HSVE was more severe compared to HIVE cases. This increased damage probably reflects HSV’s ability to directly infect and damage cells within the brain tissue.

3.4.5 HSVE versus RTA

As anticipated, HSVE exhibited more marked tissue damage and neuronal necrosis compared to RTA subjects. Associated with the prominent brain cell damage, HSVE also exhibited more extensive inflammatory infiltrates, particularly in areas of profound cell loss.

Interestingly, RTA cases, particularly subject 3, also demonstrated focal areas of significant neuronal loss. The finding indicates that multiple mechanisms can lead to neuronal death. This comparison highlights that, although HSV infection can directly cause cell death, other factors such as local interruption of blood supply or increased inflammatory infiltrates may also be involved.
3.5 Conclusion

Having confirmed in this chapter that the HSVE brains had histopathological changes consistent with those described previously, and compared the findings with those observed in HIVE and RTA brain controls, in the next chapter I focused on the inflammatory cells, which I postulated could potentially play a key role in brain cell damage.
CHAPTER 4

CHARACTERISATION OF THE CELLULAR INFLAMMATORY INFILTRATE IN BRAIN TISSUE FROM HSV ENCEPHALITIS PATIENTS AND CONTROLS

4.1 Introduction

Infection with a neurotrophic virus, such as herpes simplex virus (HSV), can result in acute encephalitis which leads to severe morbidity and mortality. The pathology of the disease is thought in part to be due to the recruitment and activation of inflammatory cells into the brain tissue. This chapter aims to study the recruitment of inflammatory cells in viral encephalitis, particularly HSVE and HIVE, in comparison with non-encephalitic RTA controls.

4.1.1 Microglia versus Macrophages

Microglia are the immune cells of the brain. They are reported to be involved in neuroinflammatory and neurodegenerative processes. They account for approximately 12% of the cells in CNS. Interestingly, the cellular origin of microglia has been the subject of debate for many years. Among the proposals for their origin are that (i) like the other tissue resident macrophages, they are derived from circulating blood monocytes that originate from bone marrow and penetrate
the CNS at birth and postnatally or (ii) they are derived prenatally from mesenchymal/mesodermal (myeloid) progenitor cells (Chan et al. 2007).

Currently, many people agree with the latter proposal. This is supported by the fact that microglia express the myeloid-specific transcription factor PU.1 (Walton et al. 2000) and stem cell and progenitor cell marker CD34 (Ladeby et al. 2005a). Furthermore, it was reported that microglia share certain characteristics with immature dendritic cells; some of the dendritic cell population are also derived from myeloid progenitors (Rezaie et al. 2005). The cellular origin of microglia has regained importance from a therapeutic perspective, particularly with the increased use of agents directed against specific leukocyte sub-types when treating CNS disorders.

In a normal brain, resting microglia have a characteristic morphology with highly ramified branching processes (Rock et al. 2004). Resting microglia are evenly distributed throughout the CNS, they exhibited limited phagocytic activity, and display little variations in phenotype within a mature healthy brain. However, during pathological events, these cells become activated and transform into phagocytic cells. The activated microglia exhibit ameboid morphology with enlarged, round and thickened stumpy processes following a graded activation processes. The graded activation response involves proliferation, migration and growth around the site of damage (Streit et al. 1999). Images showing transformation of resting to activated microglia were demonstrated by Figure 4.1. Similar differences between resting and activated microglia have been reported in several papers (Streit et al. 1999, Fiske and Brunjes 2000, Roberts et al. 2004).
Besides cellular morphological changes, activated microglia demonstrate up-regulation of distinct cell surface/phenotypic markers. Table 4.1 describes the cell surface markers of activated microglia, regardless of species (humans and animals).

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Figure 4.1 Morphological transformation of microglia from resting to being activated in response to different types of neural injury. The immunohistochemical staining for CD11b of the frontal section obtained from a normal adult rat and from a rat subjected to global cerebral ischaemia or intracerebroventricular injection of the excitotoxin kainic acid. Images adapted (Ladeby et al. 2005b)
Table 4.1 Cell surface markers of activated microglia (regardless of species)

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<tr>
<th>Markers</th>
<th>Activated Microglia</th>
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<td>CD 4</td>
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- Not expressed; +/- low or controversial expression; + expressed; ++ strong expression; +++ highly expressed. Table modified and adapted from (Guillemin and Brew 2004)

Under pathological conditions, mononuclear phagocytes infiltrate into the brain parenchyma. These monocytes, which have migrated out from blood vessels, differentiate into macrophages once in the brain. Distinguishing between activated microglia and infiltrating macrophages is challenging because they share several surface antigens, such as CD11b and CD68 (Guillemin and Brew 2004). Thus, it is necessary to examine several markers together to be able to accurately differentiate macrophages from microglia (Guillemin and Brew 2004). A review by Davous et al. and by Guillemin and Brew describes a comprehensive collection of markers for
activated microglia in comparison with blood-derived macrophages (Guillemin and Brew 2004, Davoust et al. 2008).

Microglia can also be discriminated from macrophages by using scanning electronic microscopy (SEM). Guilian et al. demonstrated that the cell surface of microglia are covered with distinctive spines. In contrast, tissue macrophages exhibit a ruffled cell surface (Figure 4.2) (Giulian et al. 1995). However, SEM is complicated and not applicable for many in vitro, ex vivo and in vivo studies (Guillemin and Brew 2004).

Rezaie et al. demonstrated the expression of CD163 antigen (scavenger receptor for haptoglobin-haemoglobin complex) on mononuclear phagocytes, was also associated with CNS macrophages, including macrophages found in the perivascular spaces and the choroid plexus. The antigen was not express by microglia residing in the parenchyma of healthy adult human brains (Rezaie and Male 2003). The authors postulated that these differences in CD expression were secondary to phenotypic variations or fundamental differences in the ontogeny of these cell populations. CD163 is a useful marker for monitoring the activities of populations of mononuclear phagocytes and to discriminate between newly arrived infiltrating macrophages and resident microglia (Rezaie and Male 2003). Animal studies demonstrate CD163 on the cell surface of monocytes/macrophages in normal and acute infected animals. They were not seen on microglia. However, in chronically infected animals, CD163 expression has been detected among activated microglia (Borda et al. 2008). However, according to Boche et al although microglial activation exhibit changes in morphology, the morphological changes
cannot predict accurately the function being undertaken by microglial cells (Boche, D et al. 2013). This is because microglia exhibit widely different functions both under physiological and pathological condition.

Different activation states of macrophages are extrapolate and anticipated to apply also to the response of microglia to an insult, injury or disease occurring in the CNS. The activation of macrophages are termed M1 and M2. M1 is also known as ‘classical activation’ in which the activated macrophages produced high level of pro-inflammatory cytokines that not only killing the pathogen but may cause collateral damage to host cells. Whereas M2 is known as ‘alternative activation’ in which the activated macrophages express a tolerant profile (Mosser and Edward 2008). M2 category is further divided into function relating to tissue repair and wound healing and second to a state of acquired deactivation. It was suggested that the same macrophage may adopt to M1 or M2 profiles based on either the type of stimulus or on the initial cell status as already activated or not before the stimulus (Mosser and Edward 2008).
Figure 4.2 Morphology of microglia and macrophages from SEM that were isolated from different tissues of newborn rats. (A) Microglia show long processes and spines that radiate out from the cell body. (B) Peritoneal macrophages with ameboid shape, ruffled surfaces, few spines, and short stubby projections. (C) Round, smooth of bone marrow mononuclear phagocytes. (D) Spleen-derived macrophages with short stubby processes and ruffled surfaces. Adapted from (Giulian et al. 1995).

4.1.2 Inflammatory Cells in RTA Controls

The central nervous system (CNS) has traditionally been considered as immunologically privileged, with few immune cells being detected in a healthy brain. Under normal conditions, microglia, although relatively inactive and perform immune surveillance to monitor the microenvironment in the brain. Immune cells from the peripheral circulation, such as T lymphocytes (particularly CD4+ T lymphocytes), are also believed to migrate into the brain parenchyma to perform immune surveillance, in order to monitor for signs of infection or inflammation (Hickey et al. 1991). B lymphocytes have been shown to enter the parenchyma of a normal human brain, although their functional role under normal conditions is unclear (Anthony et al. 2003).

Activation of resident microglia and infiltration and accumulation of inflammatory cells has been observed in patients that suffer a delayed death following traumatic injury. The examination of brain tissue from biopsies obtained from patients with primary trauma, such as brain contusion, revealed infiltrations of polymorphonuclear leukocytes, lymphocytes and monocytes/macrophages within intravascular and perivascular spaces of the blood vessels in the acute phase. After 3 to 5 days of trauma, a massive inflammatory response was reported that consisted of large infiltrations of monocytes/macrophages, activated microglia and CD4+ and CD8+ T cells in the parenchymal tissue (Holmin et al. 1998).
4.1.3 Inflammatory Cells in HSVE

The type and distribution of immune cells in CNS during HSVE has previously been described by Sobel et al. (1986). The authors examined brain biopsies from 19 patients with HSVE using immuno-histochemistry. Length of history for those cases was unknown, but it was presumed to be no more than a few weeks, since viral antigen was detected in 18 of the 19 biopsies (viral antigen is reported not to be detected in HSVE patients 3 weeks after the onset of disease (Esiri 1982). Sobel et al. reported that the parenchyma and meningeal inflammatory infiltrates were composed of T cells with few natural killer and B cells. They also observed a variable number of macrophages and T helper cells were equal in number to T cytotoxic cells. T cells were present in both the perivascular spaces and scattered throughout the parenchyma (Sobel et al. 1986).

There have been relatively few other studies of inflammatory cell response in viral encephalitis. A post-mortem examination of meningo-encephalitic brain tissue with varied aetiology i.e subacute sclerosing panencephalitis, allergic encephalitis, suspected enterovirus and undetermined origin by Esiri et al. reported that in the acute phase of inflammation, macrophages and T lymphocytes predominate in the parenchyma. T helper and cytotoxic cells were equal in number in the perivascular spaces acutely, whereas T cytotoxic cells dominated in chronic encephalitis (Esiri et al. 1989). B cells were relatively sparse in the CNS infiltrates in the conditions studied.

Macrophages trafficking from the peripheral blood vessels into the CNS have been observed following inflammation. Townsend demonstrated that in a mice model
with HSVE, macrophages were recruited early, starting from the third day of infection (Townsend 1985). In animal models with intranasal inoculation of HSV-1, rounded positive stained macrophages were detected on day 4 post-inoculation (Esiri et al. 1995). The round-bodied macrophages, stained with Ricinus communis agglutinin 1 (RCA-1), were confined to the areas in which the virus could be detected and persisted for several weeks, even after the acute infection had finished (as determined by absence of viral antigens). In contrast, activated microglial reactions were more widespread and were also present at sites remote from where virus antigen was detected (Esiri et al. 1995).

4.1.4 Inflammatory Response in HIVE

Multiple studies have reported that macrophages and microglia are the main cell types that are productively infected with the virus in HIVE. The tropism of HIV was observed in T cells and monocytes/macrophages that express the CD4 molecule. These cells, primarily monocytes/macrophages, are thought to bring the virus into the CNS. The activated microglia produced immunomodulatory molecules such as cytokines and chemokines, and potential neurotoxins including nitric oxide, protease, arachidonic acid derivatives, and quinolinic acid (Streit et al. 1999, Roberts et al. 2004, Yadav and Collman 2009) that can cause damage to the neurons and CNS.

There are only a few studies that have examined immune activation in the brain of HIV-positive in comparison to HIV-negative individuals. Primarily, macrophages, some T lymphocytes including CD4+ and CD8+ cells, and occasional B cells were
found in perivascular spaces and the brain parenchyma of HIV-positive patients (Tyor et al. 1992). Petito et al. demonstrated that CD4+ and CD8+ T lymphocytes participated in the local inflammatory response of HIVE in end-stage AIDS patients (Petito et al. 2003). A study of the brain tissue of an asymptomatic HIV-positive adult who died accidentally of an unnatural cause also revealed inflammatory changes in CNS. The inflammatory response included extensive mononuclear cell infiltrates composed primarily of T cells and monocytes/macrophages. The tissue also showed vasculitis, leptomeningitis, activation of microglial cells, up regulation of major histocompatibility complex II, and local production of cytokines (Gray et al. 1996). In HIV-positive children (in the absence of opportunistic infection or malignancy) the perivascular and mononuclear cell infiltrates again consist primarily of T cells (CD3+, CD8+ cells), and to a lesser extent T helper CD45RO+ variant cells (Katsetos et al. 1999).

In a necropsy study of macaques infected with Simian immunodeficiency virus (SIV) with and without encephalitis, the monkeys with encephalitis, showed a greater amount of T cell infiltration than those without encephalitis (Bissel et al. 2008). However, T cell infiltration was more modest than that observed in classical acute HSVE. CD4+ T cells were relatively rare in brains of macaques with SIV encephalitis. In contrast, the number of CD8+ T cells was significantly higher in brains with SIV encephalitis compared to other CNS viral infections.
4.2 Material and Methods

Pre-cut 10µm sections of formalin fixed paraffin embedded in human brain tissue of HSVE, HIVE patients and RTA controls were subjected to immuno-histochemical staining to determine the type of inflammatory cells recruited during the disease. Only selected sections were subjected to the staining: the temporal and frontal region for RTA controls, temporal for HSVE, and frontal for HIVE patients. Inflammatory cells examined include T cells, B cells, monocytes/macrophages, and microglial cells. An automated immunohistochemistry stainer at Buxton laboratories at The Walton Centre of Neurology and Neurosurgery NHS Trust was used.

The inflammatory markers that were used to detect different inflammatory cells include: CD3 marker for determination of T cells, with CD4 and CD8 marker for detection of T helper and T cytotoxic sub-sets respectively; CD79a marker for detection of B cells; and CD68 marker. Anti-CD68 antibody binds lysosomes. Lysosomes are frequently abundant in macrophages and microglia. Thus this marker helps to detect macrophages and microglia in the brain parenchyma. CD163, the scavenger receptor for the haptoglobin-haemoglobin complex was also used in this study to identify infiltrating monocytes/macrophages and to help discriminate macrophages from resident microglia. CD163 has been reported to demonstrate specificity for monocytes/macrophages.
4.3 Results

4.3.1 Inflammatory Cells in RTA Controls

4.3.1.1 RTA Control 1

As described in the previous chapter, RTA Control 1 showed focal inflammatory changes in the temporal lobe, evidenced by the infiltration of numerous macrophages, microglia and lymphocytes in the affected brain parenchyma. Immuno-histochemical examination of the inflammatory markers further confirmed these findings. Staining for the CD68 marker revealed numerous CD68+ microglia/macrophages within multifocal areas of grey matter and focal white matter areas in the temporal lobe (Figure 4.3A). A few scattered CD68+ cells were also present within the subarachnoid space. Similar findings were observed with the CD163 marker, with numerous CD163+ staining microglia/macrophages present within the same parenchymal region (Figure 4.3B).

Only small infiltrates of CD3 positive cells (CD3+) were observed in the perivascular space in the temporal (Figure 4.3C). They were also observed scattered through the parenchyma of the frontal lobe. Occasional CD3+ cells were also observed within the subarachnoid space of the temporal lobe. CD8 positive (CD8+) cells constituted the main subset of CD3+ cells within the perivascular space (Figure 4.3D). Occasional CD8+ cells were also observed in the grey matter of the frontal cortex, the perivascular spaces and diffusely distributed within the subarachnoid space.

Both the temporal and frontal regions demonstrated sparse CD4 positive cells (CD4+) within the white matter, perivascular and subarachnoid space. Occasional
CD79a+ B cells were also observed within the perivascular space of the temporal cortex. Within areas of tissue necrosis in the temporal cortex, numerous CD3+ cells were observed (Figure 4.3E), as well as CD8+ cells (Figure 4.3F).
Figure 4.3 Characterisation of inflammatory cells within the temporal region of RTA Control 1. (A) Intense infiltration of CD68+ cells within the affected parenchyma (B) Numerous CD163+ cells are also widely scattered throughout the parenchyma. (C) CD3+ cells within the perivascular space of a small vessel in the white matter. (D) Few CD8+ cells are also observed within the perivascular cuff and scattered near the adjacent parenchyma. (E) Numerous CD3+ cells infiltrate the area of necrosis, and (F) CD8+ cells constitute the majority of cells from CD3+ cells. Magnification A, B, E and F = 4x, C, D = 10x.
4.3.1.2 RTA Control 2

CD68+ cells with ramified morphology were scattered particularly in the parenchyma of white matter for both temporal (Figure 4.4A) and frontal regions. Scattered CD68+ cells are also observed within the subarachnoid space. CD163+ cells, consistent with the morphology of perivascular macrophages, were observed lining the external surface of the blood vessels (Figure 4.4B). These CD163+ cells were also sparsely distributed within the subarachnoid space.

Occasional CD3+ and CD4+ cells were observed within the perivascular spaces of both the temporal and frontal regions. A few CD8+ cells were also seen in the perivascular space in the white matter and the subarachnoid space. Rarely CD79a+ B cells were found within the perivascular spaces.

4.3.3.3 RTA Control 3

Numerous CD68+ cells with ramified morphology were diffusely scattered throughout the parenchyma, predominantly in the white matter of the temporal (Figure 4.4C) and frontal lobes. However, there were only small numbers of CD68+ cells observed in the perivascular spaces. Staining for CD163 marker showed few CD163+ perivascular macrophages lining the external surface of the blood vessels (Figure 4.4D). Occasional CD3+ T cells were present within the perivascular spaces (Figure 4.4E). The T cells were mainly CD8+ cells (Figure 4.4F). There was an absence of CD4+ T cells and CD79a+ B cells. In figures 4.5, panels A and B highlight the more ramified morphology of CD68+ cells compared to CD163+ cells in RTA Control 3.
Figure 4.4 Characterisation of inflammatory cells within the temporal region of RTA Control 2 (A and B) and RTA Control 3 (C to F). (A) CD68+ cells which elongated to ramified morphology are scattered within the parenchyma of white matter. (B) CD163+ macrophages are present in the perivascular space beside one of the small vessels (arrow). (C) CD68+ cells in parenchyma of RTA Control 3. (D) Sequential image of (C) for CD163 staining revealed only positive perivascular cells. (E) A few small vessels are occupied with occasional CD3+ cells and (F) Occasional CD8+ cells were also observed within the multiple vessels of the white matter. Magnification A to D = 10x and E and F =4x.
Figure 4.5 Inflammatory cells in the temporal region of RTA Control 3 with larger-scale images. (A) Slightly ramified feature of CD68+ cells scattered in the white matter. (B) CD163+ cells only observed in the perivascular of the blood vessels. Magnification A and B = 10x.
4.3.1.4 RTA Control 4

Predominantly within the white matter of the temporal cortex, there were a few diffusely scattered CD68+ ramified cells. Rarely CD68+ cells were also observed within the perivascular and subarachnoid spaces, whereas CD163+ cells mainly lined the external aspect of the blood vessels.

Similar findings were observed within the frontal region. There was an absence of CD3+, CD4+ T cells and B cells in the temporal region. Only occasional CD3+ and CD8+ cells were found within the perivascular spaces of the frontal cortex.

4.3.1.5 RTA Control 5

A few CD68+ ramified cells were diffusely scattered throughout the brain parenchyma, particularly in the white matter of the temporal and frontal lobes, with occasional positive cells observed in the perivascular space.

The staining for CD163 demonstrated a few CD163+ perivascular macrophages lining the external surface of vessels of the temporal region. A few CD3+ cells were observed within the perivascular spaces of the frontal brain region, but none were found in the temporal region. Absence of CD4+ cells was observed in both the temporal and frontal regions. Rarely occasional B cells were observed within the perivascular space of both brain regions.
4.3.2 Inflammatory Cells in HSVE

4.3.2.1 HSVE Patient 1

Histopathological examination of this patient (described in Chapter 3) showed an intense inflammatory infiltration, particularly of macrophages and microglia, lymphocytes and also few plasma cells. We performed immuno-histochemical staining to confirm the lineage of the recruited inflammatory cells during HSVE. Numerous CD68+ cells were disseminated throughout the entire tissue section of the temporal region. Most of the CD68+ cells exhibited a round morphology and some exhibited a slightly ramified appearance (Figure 4.6A). Similar findings were observed with the CD163 marker (Figure 4.6B), in which numerous CD163+ macrophages/microglia were diffusely disseminated in the brain tissue.

Lymphocytes were mainly seen in the perivascular spaces. Some were also scattered within the parenchyma, particularly in the grey matter of the temporal cortex (Figure 4.6C). The CD8+ cells constituted the main subset (Figure 4.6D). Only a few CD4+ cells were detected within the perivascular spaces. A few CD4+ cells were again also scattered within the parenchyma (Figure 4.6E). Rarely CD79a+ B cells were observed within the perivascular spaces (Figure 4.6F).
Figure 4.6 Characterisation of the inflammatory cells in the temporal region of HSVE patient 1. (A) Staining for CD68 identifies abundant macrophages/microglia cells within the perivascular infiltrates and parenchyma. (B) Abundant CD163+ macrophages/microglia within the parenchyma and perivascular space. (C) CD3+ T cells are common in the perivascular infiltrates and are also seen in the adjacent parenchyma. (D) CD8+ T cells are also common in the perivascular cuffs and scattered within the adjacent parenchyma. (E) CD4+ T cells and (F) CD79a+ B cells were sparse within the perivascular spaces. Magnification A to F = x10.
4.3.2.2 HSVE Patient 2

As described in Chapter 3, HSVE Patient 2 exhibited a milder pathological changes compared with the other two patients. Nevertheless, there was still a significant inflammatory cell response in most of the brain sections, particularly in the temporal cortex. Abundant CD68+ cells with morphological characteristic of macrophages or activated microglia were disseminated within grey and white matter (Figure 4.7A). A similar observation was found for CD163 staining: abundant CD163+ macrophages /microglia were widely distributed predominantly within the grey but also white matter (Figure 4.7B). Some perivascular macrophages were also identified among CD163+ cells.

Unfortunately, the CD3 and CD4 staining was not distinguishable enough to reliably interpret the results. CD8+ cells were the main type of lymphocytes within the perivascular space. They were also scattered in grey and subcortical white matter. Rarely CD79a+ B cells were observed in the perivascular spaces. They were not seen within the parenchyma.
Figure 4.7 Distribution of macrophages and microglia within the temporal region of HSVE Patient 2. (A) CD68+ macrophages/microglial cells were scattered throughout the parenchyma. (B) CD163+ cells were comparable to the CD68+ cells observed in the sequential image from white matter. Magnification A, B = 10x.
4.3.2.3 HSVE Patient 3

The histopathological examination (Chapter 3) of this patient revealed that severe inflammation and necrosis affected most of the brain regions. The CD68+ macrophages/activated microglia were numerous and widely disseminated throughout most of the tissue examined (Figure 4.8A). Similar findings were observed with CD163 marker in which CD163+ cells were diffusely disseminated throughout the perivascular spaces and parenchyma of the affected temporal region (Figure 4.8B).

CD3+ cells predominantly occupied the perivascular spaces, but were also scattered within the parenchyma (Figure 4.8C). Numerous CD3+ cells also accumulated within the meninges. CD8+ cells constituted the main subset of CD3+ cells. Again they were mainly seen in the perivascular spaces. A few scattered in the parenchyma (Figure 4.8D). In contrast, CD4+ cells were sparse within the perivascular spaces and rarely observed in the parenchyma (Figure 4.8E). Smaller infiltrates of CD79a+ B cells were observed among the perivascular spaces, and few were scattered within the parenchyma (Figure 4.8F).
Figure 4.8 Characterisation of the inflammatory response in the temporal region of HSVE patient 3. (A) Staining for CD68 identifies abundant macrophages/microglia cells within the perivascular infiltrates and parenchyma. (B) An intense infiltrate of CD163+ cells within the parenchyma and perivascular space. (C) CD3+ T cells dominate the perivascular infiltrate and are also seen in the adjacent parenchyma. (D) CD8+ T cells were predominant in the perivascular cuffs and scattered within the adjacent parenchyma. (E) CD4+ T cells were sparse within the perivascular space, and (F) Smaller infiltrate of CD79a+ B cells within the perivascular space and parenchyma. Magnification A to F = 10x.
4.3.3 Inflammatory Cells in HIVE

4.3.3.1 Inflammatory Cells in HIVE Patients

Overall, the inflammatory cell infiltrations in HIVE patients were mild. CD68+ microglia and macrophages were diffusely scattered within the white matter of the frontal lobe, with smaller infiltrates observed within the perivascular spaces (Figure 4.9A). Similar findings were observed for CD68 staining in HIVE Patients 2 and 3.

CD163 staining showed a very comparable result to the findings for CD68, with positive cells present predominantly within the white matter and with smaller infiltrates observed within the perivascular spaces (Figure 4.9B). Other CD163+ cells that were observed within the white matter included gemistocytic astrocytes, multinucleated giant cells and perivascular macrophages. Comparable findings were found for HIVE Patients 2 and 3.

Smaller infiltrates of CD3+ cells were observed within the perivascular space (Figure 4.9C). No discernible CD4+ cells were observed among all three HIVE cases. The CD8+ cells were scattered within white matter and a few were also seen within the perivascular space (Figure 4.9D). No B cells were observed in any of the HIVE cases.
Figure 4.9 Expression of inflammatory cells of frontal region of HIVE Patient 1. (A) CD68+ microglia and/or macrophages scattered within the parenchyma, with smaller numbers seen in the perivascular spaces. (B) CD163+ cells are also scattered within the white matter, with a smaller infiltrate within the perivascular spaces. (C) A small infiltrate of CD3+ cells is seen within the perivascular spaces, and (D) CD8+ cells are also seen within the perivascular spaces. Magnification A, B = 4x and C, D = 20x.
4.4 Discussion

4.4.1 Inflammatory Cells in RTA Controls

In the present study, we confirmed that the brain tissue of the RTA controls (temporal and frontal regions) exhibited little or no inflammation, except for Control 1 which showed a focal inflammation in the temporal cortex. The focal inflammation in RTA Control 1 was characterised by infiltrations of numerous macrophages/activated microglia within the affected parenchyma, with smaller infiltrates of lymphocytes, particularly in the perivascular space. These changes could be secondary to ischemic processes or other conditions that were related to the brain injury following the accident. Furthermore, these focal inflammatory changes suggest that the RTA Control 1 patient did not die acutely following the trauma.

Examination of the frontal and temporal cortex of RTA Controls 2, 3, 4 and 5 demonstrates CD68+ cells with ramified morphology scattered in the brain parenchyma. Anti-CD68 is considered one of the markers used for detection of microglia (Roberts et al. 2004). CD68+ cells with ramified morphology are characteristic of microglia. Examination of a more extensive panel of surface markers would confirm the cells are microglia. Since the activation of microglia is known to be a graded process, a more detailed examination of the cells morphological appearances could also help define the cells relative state of activation.

In healthy human brain tissue, the CD163 marker can be used to discriminate between monocyte-derived macrophages and resident brain microglia. Rezaie et al.
reported that CD163 marker is largely undetectable on parenchymal microglia (Rezaie and Male 2003, Borda et al. 2008). However, Roberts et al. reported that in SIVE and HIVE, the CD163 marker was capable of identifying a population of semi-activated, yet ramified, microglia in the parenchyma. In these chronic encephalitis infections, the CD163 expression pattern was indistinguishable from that of other microglial markers (Roberts et al. 2004).

Out results for the examination of CD163 in RTA controls tend to agree with the findings by Rezaie et al (Rezaie and Male 2003, Borda et al. 2008). In RTA Controls 2, 3, 4 and 5, perivascular macrophages were stained with CD163. In contrast, the parenchymal microglia did not stain for CD163 (see Figure 4.4). However, RTA Control 1 was an exception. In this case, both parenchymal and perivascular inflammatory cells stained CD163+, suggesting that as the inflammatory process progressed, either microglia become more active and express CD163, or more macrophages migrate into the parenchyma. In line with the findings by Robert et al., the result may suggest that patient 1 experienced a prolonged agonal process prior to death.

The infiltration RTA brain tissue with peripheral blood lymphocytes was sparse. Limited numbers of T cytotoxic and T helper cells were observed in the perivascular spaces. B cells were either absent or rarely seen.
4.4.2 Inflammatory Cells in HSVE

4.4.2.1 Macrophages in HSVE

In this study, we found a striking response involving macrophages/activated microglia during HSVE, particularly in Patients 1 and 3. Numerous macrophages/activated microglia were observed in the perivascular spaces and disseminated within the parenchyma of grey and adjacent white matter of the temporal lobe. A similar marked reaction has previously been described in HSVE. (Esiri et al. 1989).

Interestingly, staining for CD163, regarded by some researchers as a relatively selective monocyte/macrophage marker, gave comparable results to CD68 staining. There were numerous CD68+ and CD163+ cells throughout the parenchyma. Their cell morphology was consistent for either activated microglia or macrophages. These results are in keeping with a previous study that demonstrated both macrophages and microglia stained positively for CD163 in inflamed brain tissue (Fabriek et al. 2005). This finding is further supported by in vitro and in vivo studies of Simian Immunodeficiency Virus Encephalitis (SIVE) of macaques, which showed that in chronic encephalitis, CD163 was not only detected in the perivascular macrophages but also in activated microglia (Borda et al. 2008).

Although there are many reports on CD163 being a selective marker for macrophages, few studies have examined CD163 in the context of human
encephalitis. To my knowledge, this is the first report of the distribution of CD163+ cells in HSVE.

Overall, I found it was difficult to differentiate between the infiltrating macrophages and activated microglia in HSVE cases using only CD68 and CD163. Taking these findings forward, I would recommend using a panel of several markers to attempt to differentiate between macrophages and microglia during HSVE.

4.4.2.2 T Lymphocytes in HSVE

There have been relatively few studies to determine the involvement of T lymphocytes in viral encephalitis. Among them is the examination of brain biopsies to characterise the inflammatory cells in HSVE by Sobel, Collins et al. and in a study of encephalitis with varied aetiology by Esiri, Reading et al. (Sobel et al. 1986, Esiri et al. 1989).

In the present study, immuno-histochemical staining revealed that T lymphocytes are present in the meninges, perivascular spaces, and parenchyma of brain tissue. I observed smaller infiltrates of helper CD4+ cells compared to cytotoxic CD8+ T cells in the perivascular spaces and parenchyma. It is known that T lymphocytes play a key role in the host response to encephalitis. Cytotoxic T cells are reported to destroy infected host cells that express class I MHC molecules. T helper cells are also reported to produce cytokines that activate T cytotoxic cells and phagocytic cells to help to clear cells infected with the pathogen. My study of the distribution
of T cells in HSVE is in keeping with the previous studies by Sobel et al and Esiri et al. (Sobel et al. 1986, Esiri et al. 1989).

4.4.2.3 B Cells in HSVE

Sobel, et al. and Esiri, et al. demonstrated that B cells are relatively sparse and almost entirely confined to the perivascular space in HSVE and in encephalitis cases of different aetiologies (Esiri et al. 1989). B cells mature in the perivascular spaces before migrating to surrounding parenchyma as plasma cells, and play a key role in promoting antibody production. The findings for B cells in HSVE from this study are entirely in keeping with those of Esiri et al. and Sobel et al. (Sobel et al. 1986, Esiri et al. 1989).

4.4.2.4 Inflammatory Cells in HIVE

Although only mild inflammatory infiltrates in HIVE patients were observed in this study, there was a consistent pattern of an inflammatory cell response in areas linked to myelin loss. This pattern was characterised by large infiltrations of macrophages and microglia within the parenchyma of white matter. Small infiltrates of T cells in the perivascular space of the blood vessels and no discernible CD4+ cells and B cells. T cell response is mild in HIVE due to phagocytosis of HIV infected lymphocytes by macrophages/microglia. Interestingly, the giant cells positively stained with CD163 marker.
These findings correlate well with the pathogenesis of HIVE, in which the HIV productively infects microglia and macrophages. Following the entry to CNS, the main target cells for the virus infection are macrophages and microglia. This explains the presence of numerous microglia and macrophages within the main affected areas for HIVE, particularly in the white matter. Furthermore, the multinucleated giant cells that represented a fusion of mononuclear phagocytes have also been actively infected with HIV. HIVE demonstrates similar observation to HSVE in that both infiltrating macrophages and activated microglia were stained with CD163. Once again, I was unable to discriminate between the cells. Overall, the inflammatory infiltrate, reflecting the pathological damage, was much less severe in HIVE compared to HSVE.

4.5 Conclusion

In this chapter I characterised the inflammatory cell infiltrates in HSVE, comparing with HIVE patients and RTA controls. This was important because host inflammatory cells may contribute to brain cell damage during encephalitis. Given HIV is not known to infect neurones or glial cells. The findings from the HIVE patients suggest inflammatory cells drive myelin loss. In HSVE more inflammatory cells were present in areas of severe tissue damage. I cannot confirm, from these observations, whether inflammatory cells drive neuronal loss or respond to brain cell damage. Nevertheless, given their abundance, inflammatory cells could certainly contribute to the damage. In the current study, M1 and M2 pathway was not considered but in the future it would be interesting to examine macrophage
activation products that determine M1 and M2 pathway in the pathogenesis of HSVE.

In the next chapter I begin to examine the host response to HSV infection using a genome-wide perspective gene-expression microarrays.
CHAPTER 5

COMPARISON OF HOST TRANSCRIPTIONAL RESPONSES IN BRAIN TISSUE FROM HSV ENCEPHALITIS PATIENTS AND CONTROLS

5.1 Introduction

Formalin fixed paraffin embedded (FFPE) post-mortem brain tissue is widely available and routinely archived in many hospitals. In this chapter the FFPE brain tissue from HSVE and non-encephalitic RTA cases were subjected to RNA extraction following their histopathological examination. I aimed to establish the optimum RNA extraction method for FFPE tissue. Then using this method I recovered RNA from FFPE post-mortem human brain tissue from HSVE and RTA patients. Subsequently, I compared genome-wide gene-expression between the HSVE and RTA cases using a microarray approach.

5.1.1 Formalin Fixed Paraffin Embedded (FFPE) Tissue

Fresh frozen tissue is one of the best sources of tissues for nucleic acid and protein recovery (Klopfleisch et al. 2011). Fresh tissue contains good quality RNA for gene expression studies, however fresh frozen samples are not readily available. Following post-mortem, tissue is fixed and archived as FFPE. One of the most commonly used fixatives is formalin, also known as formaldehyde. Formalin is
cheap, easy to handle, and preserve tissues efficiently by fixation of structural and cytoskeletal proteins. Formalin also prevents tissue autolysis and act as anti-microbial by changing highly infectious tissue into non-infectious material (Klopfleisch et al. 2011). FFPE tissue has the following advantages; such tissue is routinely archived, it is widely available from most hospitals, it preserves the morphology and integrity of the tissue for histological examination and for long term storage.

Nevertheless, there are challenges to using FFPE for molecular studies. The amount and quality of nucleic acid recovered from FFPE is less optimal compared to fresh tissue because of extensive cross linking between proteins and nucleic acid following formaldehyde fixation (Park et al. 1996, Farragher et al. 2008). Fixation also causes fragmentation of DNA and RNA in the affected tissue (van Deerlin et al. 2002).

Fragmented RNA is the main barrier to use of FFPE tissue in gene expression studies. The extent of fragmentation varies depending on tissue type and fixative used, but the typical length of fragments for RNA in FFPE is around 200 base pairs (bp) (Lehmann and Kreipe 2001). It is therefore suggested that the amplification of fragments longer than 200 bp should not be attempted due to failure of amplification process in molecular experimental studies (Lehmann and Kreipe 2001, Farragher et al. 2008).

Beside the fixative, there are other parameters that influence the quality and quantity of RNA or DNA retrieved from FFPE tissue. These parameters includes length of time from necropsy or surgical excision to fixation (pre-fixation time),
fixation conditions, prolonged tissue hypoxia and temperature (Lehmann and Kreipe 2001). Prolonged tissue hypoxia reduces pH in tissue leading to further degradation of nucleic acids (Srinivasan et al. 2002).

5.1.2 Development of RNA Extraction from FFPE

Reflecting the fact extracting RNA from FFPE tissue is challenging, there are significant efforts to improve the extraction of RNA. Researchers have introduced various modifications to the extraction steps. These include de-paraffinisation and proteinase K digestion. Incomplete de-paraffinisation leads to lower quantity and quality of extracted RNA (Chung et al. 2006).

Tissue digestion with proteinase K enzyme is crucial for a maximum amount of RNA. Extending the digestion time with proteinase K has been reported to improve the total amount of RNA extracted (Chung et al. 2006, Abramovitz et al. 2008, Bonin et al. 2010, Glenn et al. 2010). By extending the digestion time, proteinase K is thought to release the protein cross-linked nucleic acids more efficiently. In contrast, other dissolving agents such as chaotrophic substance such as guanidium thiocynate failed to do so (Farragher et al. 2008, Klopflisch et al. 2011).

Many successful extractions of RNA from FFPE tissue have been reported previously (Ribeiro-Silva et al. 2007, Bonin et al. 2010, Glenn et al. 2010, Okello et al. 2010).
5.1.3 Selection of RNA Extraction Kit in Current Study

The basis for the selection of initial kit for RNA extraction in my study is based on paper by Ribeiro Silva. They examined four RNA extraction protocols for extracting nucleic acid from FFPE breast tissue samples. Samples had been archived from 3 months to 10 years. The authors reported that all four protocols were able to extract RNA from 10 year old FFPE samples with acceptable quality of RNA (Ribeiro-Silva et al. 2007).

The overall comparison of the four RNA extraction protocols based on quantity (in pg/µl) and quality (by RNA integrity number, RIN) of RNA in ten years old FFPE samples is demonstrated in Table 5.1. RIN is one of the methods used for RNA quality assessment and is measured using Agilent 2001 bioanalyzer. The Agilent 2001 bioanalyzer is an automated device that used microfluidics technology that provides electrophoretic separation of RNA by molecular weight (Ribeiro-Silva et al. 2007). The result is visualized as an electropherogram where the amount of measured fluorescence correlates with the amount of RNA of a given size (Schroeder et al. 2006). RIN values range from 10 (intact) to 1 (totally degraded).

Based on the findings represented in the table 5.1, the kit from protocol 3 was selected for RNA extraction in my study. Protocol 3 demonstrates a higher percentage of successful extractions from a low amount of tissue input and producing a reasonable amount of RNA. Protocol 1 did not perform as well as other protocols. Although Protocol 4 produced the highest quantity of RNA, it required higher input of tissue which may be not a good choice when the amount of available
tissue is scarce. Protocol 2 used the lowest amount of tissue input, produced high quantity of RNA but the proportion of successful extractions was low.

Table 5.1 Comparison of the four RNA extraction protocols in ten year old FFPE samples (Ribeiro-Silva et al. 2007)

<table>
<thead>
<tr>
<th></th>
<th>Protocol 1</th>
<th>Protocol 2</th>
<th>Protocol 3</th>
<th>Protocol 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>FFPE tissue input</td>
<td>80µm</td>
<td>10µm</td>
<td>20µm</td>
<td>50µm</td>
</tr>
<tr>
<td>Successful extractions</td>
<td>36/42 (86%)</td>
<td>26/42 (62%)</td>
<td>38/42 (90%)</td>
<td>30/41 (71%)</td>
</tr>
<tr>
<td>RNA (pg/µl)</td>
<td>15,964</td>
<td>51,985</td>
<td>29,661</td>
<td>107,042</td>
</tr>
<tr>
<td>RIN</td>
<td>1.8</td>
<td>2.5</td>
<td>2.3</td>
<td>2.4</td>
</tr>
</tbody>
</table>

5.1.4 Gene Expression from FFPE tissue

Genome-wide gene expression analysis allows the identification of transcripts that are have changes in abundance during a disease process. The expression of transcripts permits inferences to be made about the gene’s involvement in the disease process and facilitates the identification of candidate genes for more detailed investigation (Cummings and Relman 2000). The development of microarray technology assists the global analyses of the expression of thousands of genes in a single experiment. In cancer research, gene expression profiling has successfully identified genes that are differentially expressed between cancer and normal cells. This assists to the identification of novel molecular tumor subtypes and several molecular signatures that predict prognosis and response to treatment (Penland et al. 2007).

Previous studies demonstrated RNA extracted from FFPE samples produced successful results for gene expression measurement using real time quantitative PCR (QPCR) (Specht et al. 2001, Cronin et al. 2004). Cronin et al. demonstrated RNA extracted from FFPE breast cancer tissue dating from 1985 to 2001 yielded analyzable data for 92 genes assay in all tested specimens (Cronin et al. 2004). However, the use of FFPE tissue for gene expression studies is controversial. Some researchers have demonstrated the feasibility of gene expression studies, whereas others concluded that it is a poor substrate for such approaches.

Penland et al. reported that informative gene expression analysis can be performed on FFPE samples with the caveats that there is loss of information in gene signatures in some of the samples (Penland et al. 2007). While the results of some
studies are discouraging, many other researchers have successfully used FFPE tissue to identify prognostic and diagnostic gene signatures for numerous diseases including in various carcinoma cases. Fedorowicz et al. compared the performance of FFPE with matched fresh frozen samples and demonstrated FFPE samples could be successfully used to identify differentially expressed genes characteristic of ovarian carcinoma (Fedorowicz et al. 2009). They performed genome-wide gene expression on matched fresh frozen and FFPE tissue from an ovarian adenocarcinoma. Gene expression demonstrated high correlations between fresh frozen and FFPE samples. However, a lower number of differentially expressed genes were identified in FFPE compared to fresh frozen samples. (Fedorowicz et al. 2009).

Linton and colleagues examined gene expression from FFPE samples of soft tissue sarcoma using Affymetric microarray. They reported that reliable microarray data could be generated from FFPE tissue and this could be used for identification of prognostic genes (Linton et al. 2008). High correlation was obtained between fresh frozen and FFPE samples. However, the number of transcripts detected was again lower within FFPE tissue.

**5.1.5 Analysis of Gene Expression Study**

Genome-wide gene expression provides an opportunity to assess global gene function. Determining the conditions under which a gene is transcriptionally active allows inferences about its function. This is because a gene is only transcribed when and where its function is required. Assembling genes into groups with similar
expression patterns also allows insight into common functional activities that are occurring during that biological condition.

5.1.5.1 Genes Clustering

Clustering is an approach of grouping together genes with similar expression patterns. Two main families of clustering are partitional and hierarchical clustering. Hierarchical clustering is used for experiments that do not require the genes to be clearly partitioned into separate groups. Hierarchical clustering gives the degree of relative similarity in expression between all genes examined as a dendrogram. A dendrogram demonstrates gene-expression similarity as a tree shaped data structure. The shorter the branch length between two genes (the length being shorter at the terminal branches) the more closely related their expression.

Clustering algorithms can be defined as supervised and unsupervised. Unsupervised algorithms do not used any pre-defined guidelines on the object characteristics, whereas supervised initially classifies the object with respect to known reference data.

5.1.5.2 Statistical Analysis

Appropriate statistical tests are required to identify differentially expressed gene in microarray. The power of the test should be high to compensate for the lower number of samples used and no strong assumptions about the distributions of sample should be made. Applying $p$-value (probability of null hypothesis is correct)
to microarray data is complicated by the large number of multiple comparisons involved.

Multiple comparisons are required for controlling the Type 1 error rate (false positive result). The approach that is typically employed for the correction of multiple comparisons is the Bonferroni adjustment. However, this method creates a criterion that is too strict, and may discard high numbers of truly significantly expressed genes. The control of multiple comparisons using the family wise type 1 error rate leads to unduly conservative estimates of significance (Benjamini and Hochberg 1995).

It has been proposed that researchers should tolerate a small number of Type 1 errors compared to the overall number of rejected null hypothesis. These considerations led to the development of the false discovery rate (FDR) which is a metric that allows the investigator to control the expected proportion of Type 1 errors among rejected null hypotheses (Dr Michael J. Griffiths, PhD thesis).

In my study, the significance analysis for microarray (SAM) program was used to analyse the differentially expressed genes. SAM identifies statistically significant genes by carrying out gene specific t tests. Each gene is assigned a score on the basis of its change in gene expression relative to the standard deviation of repeated permutations (Tusher et al. 2001). SAM measures the strength of the relationship between gene expression and a response variable and also gives the estimates of FDR. FDR is used in the SAM package to controls for multiple comparisons. SAM using permutations based procedures and measuring FDR has been shown to be an appropriate test for identification of truly differentially expressed genes.
5.2 Materials and Methods

Post-mortem FFPE brain tissue from three patients with confirmed HSVE and five RTA cases were examined in this study. The tissue had been anonymised, consequently information regarding patients’ history, clinical examination and investigations were not available.

The method for total RNA extraction from FFPE tissue was based on a protocol for the commercial kit Absolutely RNA FFPE Kit (Stratagene product division, Agilent technologies, US). The kit was selected for the reasons that described in section 5.1.2. Due to limited human brain tissue samples provided by brain bank, the initial RNA extraction method used animal brain tissue samples from Veterinary School, University of Liverpool. Nevertheless, there are challenges encountered during the extraction of RNA using FFPE post-mortem tissue. The challenges include low quantities of RNA (Table 5.3) following initial attempts of RNA extraction from FFPE animal brain tissue using the commercial kit. This was followed by an attempt to optimise several steps/methods of existing protocols from the commercial kit.

The optimization steps taken in RNA extraction method were increased the incubation time with the proteinase K enzyme from 3 hours to 18 hours, increased the concentration of proteinase K enzyme (20 µl, 20 mg/ml) , homogenisation of tissue using motorised pestle and RNA isolation using Trizol RNA extraction method (Invitrogen, Life Technologies). Details regarding optimization steps are also described in sections 2.4.1, 5.3.2 and 5.3.3. After the establishment of optimised RNA extraction method with animal brain tissue, I then proceeded with
the RNA extraction from human brain sample. NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) was used according to manufacturers’ guidelines to measure total RNA.

The RNA from cingulate region was amplified and labelled for microarray examination using a non-enzymatic one step labelling method named Universal Linkage system (ULS). ULS has proven to be a better method for labelling of DNA from archived FFPE tissue. The method for microarray gene expression was described in details in Chapter 2. Labelled cDNA was hybridised on Agilent SurePrint G3 GE 8 x 60K (Design ID 030495) custom-human specific microarrays following the manufactures instruction (Agilent Technologies). Agilent’s SurePrint G3 GE 8 x 60K microarrays provides a comprehensive coverage of genes and transcripts of mRNA target. The glass slide contains eight identical arrays and each array containing 62,976 features.

Arrays were scanned using Agilent DNA microarray scanner [G2505C] (Agilent Technologies). Raw fluorescent intensity was measured and initial quality control assessment undertaken using Agilent Feature Extraction software (FE 10.5.11). Transcripts were analysed as previously described (Griffiths et al. 2005). Fluorescent intensity for each gene probe was initially median centred. Those that exhibited > 0.8 standard deviations variation in abundance across the array set (n=8) were then selected using Cluster (http://www.eisenlab.org). This gene-set (n=2882) was visualised using Treeview (http://www.eisenlab.org). Genes exhibiting a significant change in abundance (< 5% False Detection Rate) between HSVE and RTA samples among the set of differentially expressed genes were identified using
significance analysis for microarrays (SAM) version 4.0 (http://www-stat.stanford.edu).

SAM identified 287 significantly differentially expressed genes (<5% False Detection Rate [FDR]) from the filtered data set. Pathway over-representation analysis (ORA) was undertaken among the set of significantly differentially expressed genes using the ORA program within InnateDB (www.innatedb.com). Significance was calculated using a hypergeometric algorithm and adjusted for multiple comparisons using the Benjamini Hochberg correction (Benjamini and Hochberg 1995).

Details for quantitative PCR (QPCR) experiment were described in Chapter 2. QPCR was performed with 50-100ng of cDNA using TaqMan Gene Expression Assays (Applied Biosystem). Changes in mitochondria encoded Cytochrome c oxidase I (CO1) abundance was expressed as the PCR fractional cycle number at which threshold fluorescence was achieved (Ct). Primer reference for CO1 encoded transcripts was demonstrated by Table 5.2. Only sufficient RNA (>100ng) from amygdala and temporal region for HSVE Patient 1 and 3 was available for QPCR. In RTA cases, only amygdala from two controls and temporal region from four controls was available. The samples were run in duplicate and the relative abundance results for HSVE and RTA patients were grouped accordingly. The Ct values were statistically evaluated using the Student’s t-test ($p < 0.05$).
Table 5.2 Demonstrate CO1 primer assay reference in this experiment

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name</th>
<th>TaqMan Assay ID</th>
<th>Amplicon length</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO1</td>
<td>Cytochrome oxidase 1</td>
<td>Hs02596864_g1</td>
<td>94</td>
</tr>
</tbody>
</table>

5.3 Results

5.3.1 RNA Extraction from Animal Brain Tissue using Absolutely RNA FFPE Kit (Stratagene, Agilent Technologies)

The Absolutely RNA FFPE Kit (Stratagene, Agilent Technologies, US) protocol involved three main steps; deparaffinization, proteinase K digestion and RNA isolation. Initial attempts to extract RNA from FFPE animal brain tissue using the Absolutely RNA FFPE Kit produced low quantities of RNA (Table 5.3).
Table 5.3 The RNA from FFPE animal brain tissue using the Absolutely RNA FFPE kit (Agilent Technologies)

<table>
<thead>
<tr>
<th>Tissue replicates</th>
<th>Sample input</th>
<th>RNA (µg/30µl)</th>
<th>A260/280</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 µm</td>
<td>0.38</td>
<td>1.70</td>
</tr>
<tr>
<td>2</td>
<td>10 µm</td>
<td>0.47</td>
<td>1.67</td>
</tr>
<tr>
<td>3</td>
<td>10 µm</td>
<td>0.32</td>
<td>1.70</td>
</tr>
<tr>
<td>4</td>
<td>10 µm</td>
<td>0.22</td>
<td>1.41</td>
</tr>
<tr>
<td>5</td>
<td>10 µm</td>
<td>0.38</td>
<td>1.62</td>
</tr>
<tr>
<td>6</td>
<td>10 µm</td>
<td>0.17</td>
<td>1.61</td>
</tr>
<tr>
<td>7</td>
<td>10 µm</td>
<td>0.52</td>
<td>1.98</td>
</tr>
<tr>
<td>8</td>
<td>10 µm</td>
<td>0.35</td>
<td>1.86</td>
</tr>
</tbody>
</table>

5.3.2 Optimised Protocols for RNA Extraction from Animal Brain Tissue

Modification of the existing protocols based on the initial results from FFPE animal brain tissue preceded the use of post-mortem human brain tissue to optimise recovery of RNA. There were four modification steps used in my study; 1) increased the incubation time with the proteinase K enzyme from 3 hours to 18 hours, 2) increased the concentration of proteinase K enzyme used, 3) homogenization of tissue using motorised pestle and 4) RNA isolation using Trizol RNA extraction method (Invitrogen, Life Technologies). These modification steps
resulted in the recovery of higher amounts of RNA. Table 5.4 summarises the modification steps.

Table 5.4 General comparison of commercial Absolutely FFPE Kit extraction method versus modification protocols

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Commercial Kit protocols</th>
<th>Modification protocols</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample input</td>
<td>2 slices 10 µm FFPE</td>
<td>2 slices 10 µm FFPE</td>
</tr>
<tr>
<td>Lyses condition</td>
<td>55 °C/ 3 hours</td>
<td>55°C/ 18 hours</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>10 µl (20 mg/ml)</td>
<td>20 µl (20 mg/ml)</td>
</tr>
<tr>
<td>RNA recovery</td>
<td>Silica based fiber matrix</td>
<td>Trizol RNA extraction method</td>
</tr>
<tr>
<td>Homogenisation</td>
<td>pipetting</td>
<td>Motor pestle</td>
</tr>
</tbody>
</table>

5.3.3 Comparison of RNA Amounts Following Optimisation Steps

Combination of motorised tissue homogenisation, 18 hours incubation, double concentration of proteinase K and using a trizol extraction method in the FFPE animal samples produced higher quantity of RNA compared by using only single or
double concentration of proteinase K or trizol extraction method alone (Table 5.5 and Figure 5.1)

Table 5.5 The RNA from FFPE animal brain tissue using the optimised methods

<table>
<thead>
<tr>
<th>Tissue replicates</th>
<th>Modification steps</th>
<th>Sample input</th>
<th>RNA (µg/20µl)</th>
<th>A260/280</th>
<th>A260/230</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3 h Proteinase K, 10µl (20mg/ml)</td>
<td>10µm</td>
<td>0.34</td>
<td>1.98</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>0.23</td>
<td>1.86</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>18 h Proteinase K, 10µl (20mg/ml)</td>
<td>10µm</td>
<td>0.74</td>
<td>2.07</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>0.66</td>
<td>2.09</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>18 h Proteinase K, 20µl (20mg/ml)</td>
<td>10µm</td>
<td>1.89</td>
<td>2.08</td>
<td>1.07</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>1.66</td>
<td>2.05</td>
<td>1.22</td>
</tr>
<tr>
<td>7</td>
<td>18 h Proteinase K, 10µl (20mg/ml), Trizol</td>
<td>10µm</td>
<td>2.52</td>
<td>1.98</td>
<td>0.26</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td>2.41</td>
<td>2.02</td>
<td>0.23</td>
</tr>
<tr>
<td>9</td>
<td>18 h Proteinase K, 20µl (20mg/ml), Trizol</td>
<td>10µm</td>
<td>3.68</td>
<td>1.97</td>
<td>0.33</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>3.15</td>
<td>1.98</td>
<td>0.31</td>
</tr>
</tbody>
</table>
Figure 5.1 Modification of existing protocol using animal tissue showed that; the mean RNA concentration is 0.3µg following a single proteinase K digestion 10 µl (20 mg/ml) with 3 hours incubation. Increasing the incubation time of proteinase K to 18 hours, yielded more RNA and a combination of a double amount of proteinase K, 20 µl (20mg/ml) and using a trizol extraction method produced the highest yield of RNA. pK = proteinase K, pK 1+ = single concentration /10 µl (20 mg/ml), pK 2+ = double concentration /20 µl (20 mg/ml). Each modified protocol was tested on four replicates.
The optimised steps that gave the highest amounts of RNA in animal tissue were tested on two replicates from human brain tissue (Table 5.6).

Table 5.6 The RNA from human brain tissue using the optimised methods

<table>
<thead>
<tr>
<th>Tissue replicates</th>
<th>Modification steps</th>
<th>Sample input</th>
<th>RNA (µg/20µl)</th>
<th>A260/280</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18 h Proteinase K, 20µl (20mg/ml), Trizol</td>
<td>10µm</td>
<td>6.85</td>
<td>1.78</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>1.87</td>
<td>1.72</td>
</tr>
</tbody>
</table>

Total RNA was subjected to ethanol precipitation to increase the RNA purity and treated with TURBO-DNase (Ambion, Applied Biosystems) to remove traces of DNA in the RNA sample. As described by Glen et al., Trizol RNA isolation method produced higher amount of RNA but requires additional DNase treatment due to contaminating genomic DNA (Glenn et al. 2010). The optimised methods were then used for the RNA extraction of post-mortem human brain tissue samples from all the brain regions examined in this study.
5.3.4 RNA Amount from Human Brain Tissue

Despite the difficulty in extraction, sufficient quantity of RNA from cingulate region for three HSVE patients and five RTA cases was achieved. The lowest amount of RNA was observed in HSVE Patient 1 which is 2.14 µg and the highest is 6.95 µg in RTA Control 2 (Table 5.7).

Table 5.7 The RNA from cingulate region of post-mortem human brain tissue

<table>
<thead>
<tr>
<th>Cases</th>
<th>Brain Region</th>
<th>RNA (µg/20µl)</th>
<th>A260/A280</th>
<th>A260/230</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSVE Patient 1</td>
<td>Cingulate</td>
<td>2.14</td>
<td>1.7</td>
<td>2.6</td>
</tr>
<tr>
<td>HSVE Patient 2</td>
<td>Cingulate</td>
<td>3.21</td>
<td>1.6</td>
<td>2.6</td>
</tr>
<tr>
<td>HSVE Patient 3</td>
<td>Cingulate</td>
<td>4.36</td>
<td>1.7</td>
<td>2.7</td>
</tr>
<tr>
<td>RTA Control 1</td>
<td>Cingulate</td>
<td>5.38</td>
<td>1.7</td>
<td>2.5</td>
</tr>
<tr>
<td>RTA Control 2</td>
<td>Cingulate</td>
<td>6.95</td>
<td>1.7</td>
<td>2.3</td>
</tr>
<tr>
<td>RTA Control 3</td>
<td>Cingulate</td>
<td>4.96</td>
<td>1.7</td>
<td>2.3</td>
</tr>
<tr>
<td>RTA Control 4</td>
<td>Cingulate</td>
<td>3.69</td>
<td>1.6</td>
<td>2.5</td>
</tr>
<tr>
<td>RTA Control 5</td>
<td>Cingulate</td>
<td>5.04</td>
<td>1.8</td>
<td>2.4</td>
</tr>
</tbody>
</table>
5.3.5 RNA Amplification

The RNA from cingulate was amplified using Transplex® Complete Whole Transcriptome Amplification Kit, WTA2 (Sigma Aldrich). This kit converted the RNA to cDNA and amplified the amount of cDNA. The amount of amplified cDNA after the initial 500ng of RNA was subjected to the amplification process range from 3.3µg to 5.2µg.

5.3.6 Microarray

The analysis of microarray data is described in details in material and method section. Before normalization, a few steps are taken to ensure that the data is of high quality and suitable for analysis. These steps include removing flagged (or poor quality) features, background subtraction and logarithmic transformation.

5.3.7 Cluster Analysis and Treeview

Fluorescent intensity for each gene probe was initially normalised and median centred. Fluorescence ratios were log transformed (base2) and normalization was applied to each array. Normalization is necessary to remove unwanted systematic variability and bias from microarray data. Data was centred to ensure that the distributions are equal and median parameter has the advantage of being more robust to outliers.
Those that exhibited > 0.8 standard deviations variation in abundance across the array set (n=8) were then selected using Cluster. This step is essential to filter the data that are unlikely to represent genes that display true differential expression and reduce the number of comparisons in the final data set. This process facilitates the identification of genes displaying truly significant differential expression.

Clustering is an approach to classify data into groups of genes with similar expression pattern. The fold changes of 2882 transcripts were visualised using Treeview (Figure 5.2) which presents the genes as matrix of squares labelled by gene on the x-axis and sample on the y-axis. Fold change is visually demonstrated based on the squares colour. Relatively abundant genes are represented by red and relatively low abundant genes by green. Black represent no change in abundance compared to median for the data set. The dendrogram on the left hand side of the image shows their similarities in gene-expression across the set of eight samples.
Figure 5.2 One way unsupervised hierarchical clustering of cingulate RNA from 3 HSVE patients and 5 RTA controls. Each row represents the relative level of expression for a single gene and each column shows the expression level for a single sample. The red and green colours indicate high and low expression respectively.
5.3.8 Significance Analysis of Microarray (SAM)

Genes that exhibiting a significant change in abundance (<5% of FDR) between HSVE and RTA samples were identified using SAM. At FDR of < 5%, 287 genes exhibited significantly lower abundance in HSVE compared to RTA tissue. Mitochondrial DNA (mtDNA) encoded transcript represented only 398/62977 (0.6%) of the total number of transcripts on the array. Nevertheless mtDNA transcripts were significantly over-represented in the low abundant transcript in HSVE cases. From 287 transcripts, 219 (76%) over represented in the set (p<0.0001). List of mitochondrial and nuclear genes that were down regulated in the differentially expressed set are presented in Table 5.8. Transcripts that linked to a series of mitochondrial related ontological gene categories were also significantly over-represented in the low abundance transcript set (Table 5.9). No transcripts exhibited significantly higher abundance in HSVE compared to RTA tissue.
### Table 5.8 Mitochondrial and nuclear genes down-regulated in HSVE versus RTA cases

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mitochondrial genes (n = 28)</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>Nuclear genes (n = 37)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT-TP</td>
<td>-214.3</td>
<td>MT-ATP6</td>
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Table 5.9 Mitochondria related categories over-represented with transcripts significantly less abundant in HSVE compared to RTA cases

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5.3.9 Quantitative Real Time PCR (QPCR)

Quantitative real time PCR was undertaken to confirm that the CO1, encoding for Cytochrome c oxidase 1; a key mitochondrial enzyme subunit, was less abundant in HSVE compared to RTA tissue as shown by the microarray data. Limited remaining amounts of RNA prevented a comprehensive analysis. Figure 5.3 demonstrates a higher median Ct (indicating a lower transcript abundance) in HSVE samples compared with RTA controls (p=0.1).

Figure 5.3 Threshold cycle, Ct for control group and HSVE was pictured in this bar graph. The median Ct for RTA is 33.5 and for HSVE is 36.2, p= 0.1, the difference in median is not significant. The boxes represent 25th-75th percentile (interquartile range).
5.4 Discussion

Initially, the aim of this chapter was to determine the human host responses that were perturbed during HSVE by examining genome-wide host transcript abundance. The modification and optimization of manufacturer’s protocols produced sufficient amounts of RNA for molecular analysis using FFPE. The transcript abundances in brain tissue from RTA was examined together with HSVE cases to control for changes secondary to death and the post-mortem process.

5.4.1 RNA Extraction

Although the RNA extraction from FFPE tissue is challenging, sufficient amount of RNA was obtainable in my study. The optimisation of existing protocols starting with a commercial kit produced greater amounts of RNA. The optimisation steps such as motorised pestle homogenisation, 18 hours incubation with proteinase K enzyme, double concentration of proteinase K and use of Trizol RNA isolation resulted in sufficient amount of RNA for genome-wide gene expression study.

Proteinase K acted by releasing RNA from RNA-protein cross-linking as a consequence of formation of methylol groups during formalin fixation (Masuda et al. 1999). The amount of RNA varied between one patient to another (data not shown). According to Penland et al. these variation are expected as the degradation in FFPE samples is unpredictable. The resultant RNA quantity did not strictly correlate with the size or amount of sample or the age of tissue block (Penland et al. 2007). To reduce for variation, only tissue from cingulate region was taken forward.
for gene expression study. Sufficient RNA for all HSVE and RTA cases was found in this region.

To limit the use of contaminated RNA samples, the RNA purity was assessed using the 260/280 nm absorbance ratio. The RNA absorbance ratio 260/280 was generally acceptable (above 1.7).

The RNA was subjected to amplification using special amplification kit, Transplex® Complete Whole Transcriptome amplification, WTA2 (Sigma Aldrich). This kit was developed for amplification of RNA from FFPE tissue and was optimised for damage and degraded samples. The RNA is reverse transcribed with quasi-random 3’ end and universal 5’ end primers. These primers eliminate 3’ bias in the amplification process. The final amplification product consists of 100 to 1000 base fragments flanked by universal end sequence.

5.4.2 Microarray

The microarray gene expression study identified a list of genes differentially expressed in HSVE compared to RTA cases. This is the first examination of genome-wide host transcripts abundance in brain tissue of HSV patients. One of the critical steps for successful hybridisation during microarray experiment is to obtain sufficient fluorescent dye labelling of the cDNA. In my study, a non-enzymatic one step labelling method, named Universal Linkage system was used (ULS). The main advantage of ULS is that labelling is independent of target fragment length and the 3’ bias often seen in enzymatic labelling approaches is
negated. Consequently, the reproducibility of labelling is increased (van Gijlswijk et al. 2001).

Gene expression analysis in this study revealed significant reduction in mitochondria-related transcript abundance in HSVE compared to RTA cases. These findings were unlikely to be secondary to agonal or post-mortem processes because the reduction in mitochondria transcript were not observed in RTA controls. Furthermore, the result shows that the selectively loss of mitochondrial transcripts is occur due to biological process rather than by chance. From 287 of low abundant transcripts, 219 are over represented in the set (p<0.001) (section 5.3.8). These findings are in agreement with earlier in vitro studies reporting loss of mtDNA following HSV-2 infection of hamster kidney cells and HSV-1 infection of Hela cells (Latchman 1988, Saffran et al. 2006). However, the main limitation of this microarray examination is particularly the fact that very few samples used and the formalin fixed paraffin embedded tissue are always associated with lower quality, lower amount and fragmented RNA compared to fresh frozen samples.

5.4.3 Quantitative Real Time PCR

The mtDNA encoded protein, cytochrome c oxidase subunit 1 (CO1) was selected for QPCR examination to confirm the array findings. CO1 was identified to be less abundant in the HSVE samples in this study. CO1 has also previously been used to determine the depletion of mtDNA during in vitro studies of HSV infection (Latchman 1988).
In theory, threshold cycle or Ct is defined as number of cycles required for the fluorescent signals to cross the threshold. The greater the abundance of nucleic acid in the sample, the lower the Ct is. In this experiment, positive reactions of moderate amounts of target nucleic acid were observed with Ct ranges from 30 to 37 cycles. However, RTA cases showed higher abundance of CO1 genes with Ct median of 33.5 compared to 36.2 in HSVE cases. Student’s t test ($p=0.1$) was not significant. This was probably due to the very low number of replicates used in the QPCR experiment.

The QPCR result used a limited number of tissue samples and measured CO1 in different brain regions (amygdala and temporal) to those measured in the microarray experiments. Nevertheless the QPCR findings demonstrated a similar trend to the microarray data, with a lower abundance for CO1 transcript in HSVE patients compared to RTA controls. This finding supports the gene expression data. Further gene testing would have been ideal, but no further RNA or tissue was available.

5.5 Conclusion

Gene expression analysis showed HSVE patients exhibited a highly selective loss of mitochondrial encoded transcripts in their brain tissue. This findings was supported by QPCR analysis. Next, I planned to look at mitochondrial protein to verify the microarray and QPCR findings.
CHAPTER 6

PATTERNS OF MITOCHONDRIAL PROTEIN ABUNDANCE IN BRAIN TISSUE FROM HSV ENCEPHALITIS PATIENTS AND CONTROLS

6.1 Introduction

Mitochondria are important multifunctional organelles that are widely distributed throughout the cytoplasm of eukaryotic cells. They play an essential role in the production of ATP, cell division, growth and death. The number of mitochondria varies among cells; it could be hundreds or even thousands in each cell depending on their metabolic activities (Alberts 2000).

The genome-wide gene expression study demonstrated a marked and highly significant reduction in the relative abundance of transcripts that encode for mitochondrial genes in HSVE patients compared to RTA controls. Damage to the mitochondria might significantly contribute to the pathogenesis of HSVE. Thus, this chapter aims to confirm the microarray findings by immuno-histochemical examination of protein abundance of CO1 (a mitochondrially encoded protein). Furthermore, the geographical relationships and distribution in relation to the HSV-1 antigen were also determined from sequential slides immuno-stained with anti-HSV-1 and anti-CO1 antibodies.
6.1.1 Mitochondria

The human mitochondrial genome consists of double-stranded DNA, which is 16569 bp long and encodes for 13 structural proteins of the components of oxidative phosphorylation, 2 ribosomal RNAs and 22 transfer RNAs that make a total of 37 genes (TANJI and Bonilla 2008). One of the main functions of mitochondria is ATP production, which is produced through an oxidative phosphorylation (OXPHOS) mechanism. Oxidative phosphorylation requires a concerted action of five multiprotein complexes, namely complex I, II, III, IV and V, that are encoded by both mitochondria and nuclear DNA (nDNA). These multiprotein complexes are located in the inner mitochondria membrane. Complex IV consists of 13 proteins, of which three are encoded by mitochondria DNA (mtDNA) (Wallace 1999, Manczak et al. 2004). Those three genes that encode for mtDNA are: Cytochrome c oxidase subunit 1 (CO1), subunit 2 (CO2) and subunit 3 (CO3). They confer the catalytic and proton pumping activities to the complex.

6.1.2 The Effect of HSV Infection on Mitochondria

Previous studies have identified many viruses that can affect the mitochondrial function following the infection. HSV has been shown to interact with the mitochondria and mediate the mitochondrial changes in infected cells. Murata et al. reported changes in their location during infection. Mitochondria changed from being widely scattered in the cytoplasm, to clustering around the cell nucleus. They also reported an accumulation of viral tegument protein in the cell culture infected with HSV-2 (Murata et al. 2000). The microtubules play a role in the migration of
mitochondria and the accumulation of viral tegument protein to the periphery. They proposed that the gathering of mitochondria around the nucleus may support replication of HSV.

Murata et al. also examined the expression of Cytochrome c oxidase subunit 2 (CO2). They demonstrated no changes in the expression at 6 hours post infection and only a slight reduction at 12 hours post infection (Murata et al. 2000). In contrast, the in vitro study by Latchman first reported the depletion of the mitochondrially encoded transcript in HSV-2 infected cells. A considerable decrease in the level of CO1 and CO2 was observed. Further study shows that the decline is brought by components of viral proteins (Latchman 1988). Depletion and complete degradation of mtDNA during productive infection was reported by Saffran et al. following the in vitro study of mammalian cells infected with HSV-1 (Saffran et al. 2006). The UL12.5 gene which is localised predominantly in mitochondria, triggers mtDNA depletion in absence of other HSV gene products (Saffran et al. 2006).

Previous studies also demonstrated that viral proteins gather in mitochondria following virus infection. For example, Calton et al. reported that in pseudorabies virus β protein, Uα3 is known to traffic into mitochondria in infected cells (Calton et al. 2004). The Uα3 gene is encoded for serine/threonine kinase and is conserved among all alpha herpesviruses including HSV-1 and HSV-2. It plays a role in anti-apoptosis, de-envelopment of primary envelope capsids from the perinuclear space into the cytoplasm and the spread of virus infection between cells. Other than the listed functions, the role of Uα3 was further examined to look at its involvement and
interaction with mitochondria in infected cell cultures with HSV-1. They reported that U3 mediate the suppression of mitochondrial respiration following HSV-1 infection (Derakhshan et al. 2006).

6.1.3 HSV-1 Viral Antigen

In human autopsy studies of HSVE cases, the antigen-antibody reaction was observed in neurons and glial cells in an infected lesion where both cytoplasm and nuclei were stained (Kumanishi and Hirano 1978). In neurons, a viral antigen is prominent in the perikarya/cytoplasm and processes, whereas both nuclei and cytoplasm are stained in glial cells and macrophages (Budka and Popow-Kraupp 1981, Schmidbauer et al. 1988). However, in patients with a longer duration of disease, which is more than one month, viral antigen is often not detected.

The viral antigen has been shown to be present in all HSVE post-mortem samples recovered within 3 weeks of onset (acute and subacute) but none thereafter (Esiri 1982). Other than neurons, the viral antigen has been found in all types of glial cells including astrocytes, oligodendrocytes and occasional ependymal cells. Furthermore, the study showed that necrosis and inflammation reached the highest peak when the viral antigen was less detectable (Esiri 1982). Thus, the viral antigen did not associate well with the inflammatory changes; the areas with severe destructive and inflammatory changes are not necessarily the areas that are most likely to contain the virus.
6.1.4 Quantitative Immunohistochemistry

Immunohistochemistry (immuno-histochemical) staining is an important technique. It is often used to validate biomarkers that were discovered through genomic methods (Rizzardi et al. 2012). Semi-quantitative immuno-histochemical uses the experience of the pathologist to score the immuno-stained tissue sections by visual scoring. Usually the data was scored on a scale of 0 to 3, with 0 indicating no positive reactions, and 3 with numerous or frequent positive cells. However, the scoring method is subjective as it can be biased and greatly influenced by inter- and intra-observer variations.

With the current advance in digital technologies, quantification can also be done by the digital image analysis. Digital image analysis has distinct advantages over other semi-quantitative methods, predominantly by removing the variations between the observers. However, there are many problems in using these types of software. For example, some are not freely available to the public and can be very expensive, they may require dedicated equipment or software installation, and some have licence restrictions that allow the program to be loaded only to a single computer (Papadopulos et al. 2007, Tuominen et al. 2010).

ImageJ is free software and can be downloaded from the website http://rsbweb.nih.gov/ij/ to as many computers as required. ImageJ software was originally developed by Wayne Rasband at the Research Services Branch of the National Institute of Mental Health (Bethesda, MD, USA). The software is supported by hundreds of ‘plugins’ that assist in expanding the range of functions available in ImageJ. Furthermore, this software has been used by many published

6.2 Materials and Methods

6.2.1 Immunohistochemistry Staining

Pre-cut 10μm sections of formalin fixed paraffin embedded human brain tissue of HSVE and RTA controls were subjected for immuno-histochemical examination. The immuno-histochemical staining for CO1 and HSV-1 was performed on sequential slides. The slides were incubated with mouse monoclonal anti-CO1 (Abcam) and mouse monoclonal anti-HSV-1 antibody (Abcam), respectively. This anti-CO1 targets the CO1 protein that is embedded in the mitochondrial inner membrane. Details regarding the immuno-histochemical staining protocols were described in Chapter 2.

6.2.2 Quantitative Immunohistochemistry

The area of interest was randomly chosen for image analysis; however, it was necessary to avoid areas that comprised massive necrosis or haemorrhages. The images were captured from the same area from a consecutive slide that stained for CO1 and HSV-1 using a photomicroscope that was attached to the camera Nikon eclipse 80i (NIS-Element BR). The images were collected in TIFF format with a resolution of 1280 x 960 pixels and saved within the portable hard disk.
The images were captured from the frontal, temporal, cingulate and amygdala regions of HSVE Patients 1, Patient 2, and RTA Controls 1 to 4. However, no images were captured from HSVE Patient 3 because of massive necrosis throughout the brain regions of this patient. No images were captured from RTA Control 5 because of weak staining from the slides of this case.

The image was captured under x20 magnification. Five images were photographed for each region and captured from the consecutive slides that were stained for CO1 and HSV-1. The analysis was performed using ImageJ.

First, the image was opened in the ImageJ program. Then, the background was substracted using the default factor that was set up by the program, which is 50. For an image with a dark background, the factor used varied from 10 to 50. A plugin for ImageJ called ImmunoRatio was then selected for the generation of pseudocolor images and the separation of positive and negative stained cells (Tuominen et al. 2010).

A pseudocolor/RGB image was split into three 8-bit images containing the red, green and blue components. The 8-bit blue image represented the positive stained cells and the red image represented the negative stained cells. These images were then thresholded. There was an automatic threshold that was provided by the program. The next step was the counting of the cells using a ‘particle tool’. A minimum value of 5µm was selected for the cell size settings. A value of 5µm was selected after measuring the diameter of the smallest nucleus in the image. Furthermore, this was supported by the fact that the size of the nucleus in most of cells averaged about 5µm. Finally, the outlines and summary for the number of
counted cells was produced. The percentage of positive stained cells was calculated by dividing the number of positive cells with the sum total of the cells (negative + positive cells) and multiplying by 100.

Details regarding the quantitation steps were demonstrated in Figures 6.1 and 6.2. Figure 6.3 generally showed the proportion of the captured image under x20 relative to x4 magnification for the quantitation method. This image was provided to give a general idea of the size of the area involved in the quantitation.
Open ImageJ

Background subtraction

The factor varies between 10 and 50

Plugin ImmunoRatio for separating positive and negative cell

Crop the region of interest

Split Channel

Blue

Positive cell

Threshold

Analyse particle tool for acquiring the cell counting

Red

Negative cell

Threshold

Analyse particle tool for acquiring the cell counting

Figure 6.1 Flowcharts of the applications of the computer-based analysis of immunohistochemistry.
Step 1 → Step 2 → Step 3
Step 4
   Blue
   Green
   Red
Step 5
   Blue
Step 6 → Step 7
Figure 6.2 A flowchart outlining the analysis for particle cell counting. Step 1: An RGB original image was opened in ImageJ. Step 2: The first step of the analysis involved the background substraction. A default factor of 50 that was set up by the program was used for substraction. However, the factor varies between 10 and 50 for an image with a dark background. Step 3: An image analysed in the ImmunoRatio plugin. Step 4: An RGB pseudocolor image that was produced by Immunoratio was cropped, and the channel was split to produce three 8-bit images (red, blue and green). Step 5: The 8-bit blue image represents the positive stained cells. Step 6: The image was thresholded and the particle cells were counted. Step 7: The number of positive cells and the outlines of measured cells were provided at the end of the analysis. Step 8: The 8-bit red image represents the unstained cells. Step 9: The image was thresholded and the particle cells were counted. Step 10: The number of unstained cells and the outlines of measured cells were provided at the end of the analysis
Figure 6.3 The images A and B were captured exactly from the same part of hippocampus of HSVE Patient 1 but under different magnification. Magnification A = x4 and B = x20
6.3 Results

The results in this chapter are divided into two parts. The first part is the description for immuno-histochemical staining for CO1 and HSV-1, and the second part is quantification based on the computer analysis program.

6.3.1 Mitochondria in RTA Controls

6.3.1.1 RTA Controls 1

In neurons and other cells that were positively stained with the mitochondria marker, the immunoreactivity against the CO1 was identified diffusely in the cytoplasm. The mitochondria were represented by granular structures with brown signal intensity present within the cells’ cytoplasm. In the temporal region of this control, the granular brown signal represented the mitochondria and was observed within the cytoplasm of neurons, particularly of the third to the sixth lamina of the cerebral cortex (Figure 6.4A).

In the area of focal necrotic lesions, among the positive stained cells were the macrophages, endothelial cells, activated microglia and reactive astrocytes. Similarly, the smooth muscle cells in the meningeal vessels and ependimocytes were also positively stained with CO1.

Numerous neurons in the frontal cortex also demonstrated a positive brown cytoplasmic signal. The cingulate and amygdala also demonstrated similar findings in which the immunoreactivity was detected within the cytoplasm of cortical neurons from the third to the sixth lamina of the cerebral cortex (Figure 6.4B).
Figure 6.4 Representation of the immuno-histochemical staining for mitochondria in RTA Control 1. (A) Higher number of cortical neurons of temporal cortex positively stained with CO1. (B) The immunoreactive granular brown signals in the cytoplasm of numerous neurons within the amygdala region. Magnification A = x4 and B = x20
6.3.1.2 RTA Control 2

In the temporal region, the mitochondria were detected within the cytoplasm of the neurons, particularly of the third to the sixth lamina of the cerebral cortex. Sparse large pyramidal neurons exhibited a positive immunoreactivity signal that obscured the cytoplasmic details. Other cells that positively stained for the mitochondria marker were endothelial cells, smooth muscle cells in meningeal vessels and ependimocytes.

The frontal cortex, cingulate and amygdala all exhibited similar findings to those of the temporal cortex.

6.3.1.3 RTA Control 3

The positive signal was observed within the cytoplasm of cortical neurons, especially the pyramidal neurons of the internal and external pyramidal layer. Other positive cells were endothelial cells and scattered astrocytes. Similar findings were observed within the frontal, cingulate and amygdala brain regions.

6.3.1.4 RTA Control 4

The temporal, frontal, cingulate and amygdala all showed similar findings for mitochondria staining in which the majority of pyramidal neurons exhibited a positive granular brown signal in the cells’ cytoplasm.
6.3.1.5 RTA Control 5

The mitochondria staining of this control was the same as the other controls in this study.

6.3.2 Mitochondria in HSVE

6.3.2.1 HSVE Patient 1

The pattern of immunostaining for mitochondria inside cells was essentially the same for controls and HSVE patients. The mitochondria are represented by diffuse immunoreactive granular structures with a brown signal intensity present within the cytoplasm of the cells. The examination of the hippocampus of the temporal lobe revealed that brown signal intensity was detected within the cytoplasm of the neurons of dentate gyrus (Figure 6.5A) and scattered pyramidal neurons of the cornus ammonis.

Furthermore, the positive brown signal is also observed within the cytoplasm of the pyramidal neurons and scattered large neurons (Bett’s neuron) of the cortical laminae of the temporal cortex. Generally, the number of positive stained neurons within the temporal cortex appears low, as compared to the other brain regions, indicating less mitochondria detected in the neurons as a result of massive virus infection. Among other positively stained cells were endothelial cells, reactive astrocytes and round cells, that is consistent with infiltrating inflammatory cells found in the perivascular spaces and parenchyma of the grey and white matter.
The frontal cortex exhibited similar findings as the temporal cortex, in which scattered pyramidal neurons and activated glial cells, including astrocytes (Figure 6.5B), were positive stained. The brown signal representing the mitochondria was also observed within the cytoplasm of inflammatory cells particularly within the perivascular space of the blood vessels (Figure 6.5C). Furthermore, similar findings to the frontal region were observed in the cingulate region.

The mitochondria staining for amygdala showed a positive signal intensity detected within the cytoplasm of numerous intermediate to large neurons. The cytoplasm of the pyramidal neurons, particularly of the III and V laminae, was full of densely packed immunoreactive granular structures that represent the mitochondria. The fine granular immunoreactivity also delineated the axons and larger cell projection (dendrites) of the neurons (Figure 6.5D). Furthermore, the number of positive stained neurons appears to be elevated as compared to the temporal cortex. The mitochondria were also detected within the cytoplasm of the endothelial cells, astrocytes, macrophages and lymphocytes.
Figure 6.5 Immuno-histochemical staining for CO1 marker of HSVE Patient 1. (A) Positive stained neurons scattered in the dentate gyrus of the temporal region. (B) Positive stained astrocytes scattered in the white matter. (C) The infiltrating inflammatory cells strongly stained in the perivascular space of the frontal. (D) The immunoreactive granular structures that represent mitochondria present in the perikarya of the neuron and delineate the axon and dendrites as well (arrow). Magnification A= x10, B, C, D =x20.
6.3.2.2 HSVE Patient 2

A smaller number of positive stained neurons were observed in the temporal region compared to the other brain regions in this patient. Within the temporal cortex, a faint immunoreactive brown signal was present within the scant cytoplasm of neurons of the dentate gyrus (Figure 6.6A), pyramidal neurons and large neurons (Bett’s neurons) within the cortex.

In contrast, the frontal, cingulate and amygdala all demonstrated a relatively higher number of positive stained neurons (Figures 6.6B and 6.6C). A positive brown signal intensity was detected within the cytoplasm of the majority of the pyramidal neurons, mainly of the III and V laminae of the cerebral cortex. The cytoplasm was dense with mitochondria, including in the axon and dendrites. Large numbers of mitochondria were also present within the endothelial cells, the astrocytes (Figure 6.6D) and inflammatory cells, including macrophages and lymphocytes. The latter were present within the subarachnoid and vascular lumens.
Figure 6.6 Representative images of the immuno-histochemical staining for CO1 in HSVE Patient 2. (A) Faint CO1 staining within the scant cytoplasm of neurons of the dentate gyrus are observed in the temporal region. (B) A relatively higher number of positively stained pyramidal neurons are present in the frontal cortex. (C) Relative to temporal lobe, elevated numbers of positive stained neurons are also present in the amygdala. (D) The mitochondria staining is also present within the cytoplasm of scattered astrocytes in the white matter. Magnification A, D = x10 and B, C = x20.
6.3.2.3 HSVE Patient 3

In this patient, there was no discernable mitochondrial signal generated by neurons in the temporal region. However, positive mitochondrial signals were observed in macrophages, reactive astrocytes and other inflammatory cells present in the perivascular spaces and diffusely scattered within the parenchyma. In the white matter, the inflammatory cells again were the main cells that exhibited a positive signal.

Examination of the frontal region showed similar findings to those of temporal region, with limited mitochondrial signal generated by neurons. However, occasional positive stained pyramidal neurons were observed within the cerebral cortex of cingulate region. Positive signals were again observed in the cytoplasm of the inflammatory cells present in the subarachnoid spaces, perivascular spaces and cortical grey matter.
6.3.3 HSV-1 Viral Antigen

6.3.3.1 HSVE Patient 1

The viral antigen-antibody reaction was demonstrated by a granular brown signal that was observed within the nucleus and cytoplasm of the cells. The viral antigen was observed in all the brain regions examined in this patient. Majority of the large pyramidal neurons of the cerebral cortex (Figure 6.7A), and scattered neurons of the cornus ammonis in the temporal region, were positive for the viral antigen. Furthermore, the viral antigen was also present within the cytoplasm of dorsal and ventral blades of the dentate gyrus (Figure 6.7B) as well as the astrocytes scattered in the white matter.

The HSV-1 antigen expression of the frontal cortex was similar to that of the temporal, in which numerous positive stained neurons were observed in the cerebral cortex (Figure 6.7C). A cytoplasmic granular brown material that is consistent with the presence of HSV-1 was also observed within the cytoplasm of macrophages in the perivascular spaces. In the cingulate and amygdala regions, the positive signal was again observed in the cytoplasm of the majority of neurons (Figure 6.7D).
Figure 6.7 Immuno-histochemical staining for viral antigen in HSVE Patient 1. (A) Immunopositive neurons within the temporal cortex. (B) Neurons in the dentate gyrus stain intensely for anti-HSV-1 antibody. (C) Brown staining consistent with the presence of the virus within numerous neurons of the frontal cortex. (D) The positive signal is also present within the cytoplasm and nucleus of neurons in the amygdala region. Magnification A, B, C = x10 and D = x20.
6.3.3.2 HSVE Patient 2

The viral antigen was demonstrated in the cytoplasm of most of the cells present within the grey matter of the temporal cortex. Oligodendrocytes were not stained. Similar findings were observed in the frontal cortex of this patient. The viral antigen was present in numerous cells within the grey matter. Among these cells were the cortical neurons and numerous widely distributed astrocytes. In the neurons, the viral antigen presented as a diffuse brown stain within the cytoplasm. In astrocytes, the viral antigen was observed in both the nucleus and the cytoplasm. Closely adjacent to the vessels, macrophages were also positively stained.

In the amygdala and frontal regions, cortical neurons and numerous astrocytes were positive for viral antigen. In the cingulate region, the changes were the same as in the amygdala, but the signal intensity and number of positive neurons was relatively decreased.

6.3.3.3 HSVE Patient 3

There were relatively few neurons seen within the temporal region, so positive HSV immuno-staining was mainly observed in other cells within the tissue. Viral antigen was observed within the cytoplasm of reactive astrocytes and scattered infiltrating inflammatory cells. Occasional pyramidal neurons also exhibited positive staining for viral antigen.

Staining for HSV-1 antigen within the frontal cortex was similar to that of the temporal, except the number of positive astrocytes appeared to be elevated,
particularly within the superficial grey matter. Scattered positive perivascular macrophages were also observed. The staining for viral antigen in the cingulate and amygdala regions were similar to that of the frontal region. Again astrocytes and perivascular macrophages were the main cell types positively stained for viral antigen.

6.3.4 Mitochondria versus Viral Antigen

A comparison between mitochondria and viral antigen staining was undertaken using sequential images from consecutive tissue sections. The sequential images demonstrated a dramatic reduction in CO1 signal intensity within the cytoplasm of neurons and astrocytes in areas where HSV-1 antigen immuno-stained strongly. A reciprocal pattern between the intensity of CO1 and HSV-1 signals was observed across all brain regions examined.

6.3.5 Quantitation Results

The proportion of cells that stain positively for CO1 and HSV was measured in two HSVE patients. Patient 3 was not used for this analysis. The extensive necrosis across all brain regions in this patient, meant there was a very limited number of intact cells, particularly neurons, that could be accurately identified in the tissue. For each of the two patients, quantitation was undertaken in four brain regions (temporal, frontal, cingulate and amygdala), with five tissue areas examined in each
brain region. Comparison of CO1 and HSV staining used sequential tissue sections recovered from the same tissue area.

HSV-1 antigen was present in all brain regions examined: the temporal, frontal, cingulate and amygdala regions of Patient 1 (Figure 6.8A) and Patient 2 (Figure 6.8B). All the brain regions examined in Patient 1 demonstrated high average percentages for cells immuno-stained with HSV-1; this was in contrast to lower percentages of cells immuno-stained for CO1 (Figure 6.8A). Across the regions examined, the proportion of cells positively stained for CO1 was persistently less (3-12%) compared with proportion of cells positively stained for viral antigen.

The temporal region of Patient 2 demonstrated a higher average percentage of cells immuno-stained for HSV-1 (over 60%) compared to cells stained with CO1 (26%). In the cingulate and amygdala regions, the proportion of cells stained for CO1 was again less than for HSV-1, although the differences between the proportion of cells staining for HSV-1 and CO1 were less. Interestingly, the frontal region showed an inverse finding. In this region, the proportion of cells stained for CO1 was higher than that for HSV-1. However, the difference was relatively small.

The results for the quantitation method for two HSVE patients are summarised in the proceeding graphs (Figure 6.8). The blue columns represent the mean proportion (%) of cells that stain positively for HSV-1. The red columns represent the mean proportion (%) of cells that stain positively for CO1. For each patient, results are presented for four brain regions (temporal, frontal, cingulate and amygdala). Five tissue areas were examined for each brain region.
Figure 6.8 Quantitative analysis from sequential images of HSVE Patient 1 (panel A) and Patient 2 (Panel B). (A) Higher percentages of positive cells immunostained with HSV-1, compared with cells stained for CO1, were observed in all brain regions in Patient 1. (B) Patient 2 demonstrated similar findings. However, the frontal region showed an inverse finding. Mean and 95% confidence intervals are presented (5 tissue areas per brain region).
To assess the proportion of ‘normal’ cells that immuno-stain for CO1 using the same quantitative method, the RTA tissue was also examined. Table 6.1 shows the result for the mean percentage of positive stained cells for CO1 in RTA controls. Figure 6.9 shows a graph of the mean percentage of positive stained cells for CO1 in RTA compared with HSVE cases. The percentage of cells that stained for CO1 was consistently higher in RTA compared to HSVE cases across all brain regions. Examples of the measurement process for CO1 positive stained cells in RTA control tissues are shown in Figures 6.10 and 6.11.

Table 6.1 Raw data for the average percentage of positive cells stained for CO1 in RTA controls

<table>
<thead>
<tr>
<th>Brain Part</th>
<th>RTA 1 (% Positive Cell)</th>
<th>RTA 2 (% Positive Cell)</th>
<th>RTA 3 (% Positive Cell)</th>
<th>RTA 4 (% Positive Cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temporal</td>
<td>52</td>
<td>54</td>
<td>43</td>
<td>49</td>
</tr>
<tr>
<td>Frontal</td>
<td>45</td>
<td>48</td>
<td>54</td>
<td>42</td>
</tr>
<tr>
<td>Cingulate</td>
<td>52</td>
<td>54</td>
<td>47</td>
<td>49</td>
</tr>
<tr>
<td>Amygdala</td>
<td>66</td>
<td>58</td>
<td>51</td>
<td>61</td>
</tr>
</tbody>
</table>
Figure 6.9 Comparison of the mean percentages of immuno-stained cells for CO1 in RTA controls and HSVE patients across four brain regions.
Figure 6.10 An image captured from a temporal region of tissue from RTA 1. The tissue was stained for CO1. The image was subjected to the quantitation method of particle cell counting using positive mitochondrial immuno-stained cells. The number of positive cells is 54, number of negative cells is 22; and the sum total of cells is 76. The proportion of positive CO1 stained cells present in the image is 71% (54/76).
Figure 6.11 An image captured from a temporal region of tissue from RTA 2. The tissue was stained for CO1. Again, the image was subjected to the quantitation method of particle cell counting using positive mitochondrial immuno-stained cells. The number of positive cells is 42; number of negative cells is 55; and the sum total of cells is 97. The proportion of positive CO1 stained cells present in the image is 43%.
A reciprocal pattern of HSV-1 and CO1 tissue staining from HSVE patient 2 is demonstrated in Figure 6.12. Images were captured using sequential tissue sections stained separately for HSV-1 and CO1. Examples of the quantitation process for measuring CO1 and HSV-1 positive stained cells in these HSVE tissue sections are shown in Figures 6.13 and 6.14.

Figure 6.12 Images of sequential image from the amygdala region of HSVE Patient 2. (A) HSV-1 staining, and (B) CO1 staining. Image A and B = x20 magnification.
Figure 6.13 Image of a tissue section stained for the HSV-1 antigen from the amygdala region of HSVE Patient 2. The image was subjected to the quantitation method for particle cell counting and the end result was: the number of positive cells is 49, negative cells is 43 and the sum total of cells is 92. The proportion of HSV-1 positive stained cells is 53% (49/92).
Figure 6.14 Image of the corresponding sequential tissue section stained for CO1 antigen from the amygdala region of HSVE Patient 2. The image was subjected to the quantitation method for particle cell counting and the end result was: the number of positive cells is 16, negative cells is 56, and the sum total of cells is 72. The proportion of positive CO1 stained cells is 22% (16/72).
6.4 Discussion

The immuno-histochemical study shows that mitochondria within CNS cells are damaged during HSVE infection, with reduced staining for CO1 in HSVE cases compared to RTA controls. Among HSVE cases, brain regions that exhibited low CO1 staining tended to have high HSV-1 staining, or vice versa.

6.4.1 The Effect of HSV-1 on Mitochondria

As mentioned previously, mitochondria are dynamic organelles that have many essential functions including energy production, maintaining calcium homeostasis, regulating innate immune signalling, and apoptosis. In non-encephalitic RTA controls, abundant mitochondria were present, particularly in the cytoplasm of neurons, astrocytes and other cells including the endothelial cells, smooth muscle cells and ependymocytes. Furthermore, mitochondria were also present in the cytoplasm of the inflammatory cells: lymphocytes, macrophages and microglia.

The immuno-histochemical examination of the sequential images stained for CO1 and HSV-1 antigen revealed that cells with strong immune-staining for HSV-1 tended to have poor immuno-staining for CO1, and vice versa. The reciprocal pattern of staining between HSV-1 and CO1 supports the decline in mitochondrial function being virus mediated. In contrast, in uninfected neurons, abundant mitochondria stained strongly for CO1.

Previous in vitro studies using cell lines of non-CNS origin have shown HSV damages mitochondria. My study confirms that these previous in vitro observations reflect events that occur during natural HSV infection in human brain tissue.
Previous in vitro studies have demonstrated that HSV infection is linked to modulation of mitochondrial function. Viral infection alters the location of mitochondria within the cell. During HSV infection mitochondria move from being scattered in the cytoplasm, to being massed in the perinuclear region. HSV infection has also been shown to impair mitochondrial ATP production (Murata et al. 2000). Further in vitro studies have demonstrated HSV infection is associated with a depletion of mitochondrial DNA encoded proteins. These proteins are involved in oxidative phosphorylation. Researchers have proposed that this impairment in ATP production may contribute to cell damage and ultimately cell death (Latchman 1988, Saffran et al. 2006). The viral protein UL12.5 is one of the proteins that triggers mitochondria DNA depletion in HSV-infected cells (Saffran et al. 2006).

Kramer and Enquist reported one of the first studies of mitochondrial dynamics in alpha herpes virus infected in murine neurons (Kramer and Enquist 2012). They showed that pseudorabies virus and HSV-1 infection of primary rodent neurons disrupts mitochondrial motility and morphology. The disruption of mitochondria transport, particularly in pseudorabies, was postulated to support the efficient growth and spread of the virus in the neurons.

This work raises several interesting and important questions worthy of future investigation. First, as the study already shows that the mitochondria were disrupted in HSVE, the exact pathway of how the HSV-1 causes mitochondria disruption in HSVE should be characterised. Furthermore, double-labelling immunohistochemical approaches and in vitro cell culture might be among the appropriate methods for further confirmation of the current results and could be implemented in future research.
Why the virus interacts with mitochondria is unclear. Previous researchers have proposed that mitochondrial disruption may enhance viral replication and spread within the tissue. However, since mitochondrial damage is likely to worsen HSV-1 associated cell damage, and precipitate host cell death, this interaction may alternatively impede HSV spread.

6.4.2 Quantitative Immunohistochemistry

The automated quantification method for particle cell counting is in agreement with my visual description of the tissue. Using both approaches, I found a reduction in CO1 signal intensity in brain regions that immuno-stained strongly for HSV-1 antigens (Figure 6.12), and vice versa. By using the computer analysis software, the quantitation method confirmed that areas that appeared to have poor immuno-staining for CO1 on visual inspection, also demonstrated a decreased percentage of CO1 immuno-stained cells. These results further support the theory that the decline in mitochondrial function is virus mediated.

This quantification method, based on digital image analysis using the public domain software package ImageJ, provides an objective and freely available alternative to the traditional manual semi-quantitative methods of analysing immuno-histochemical sections. Furthermore, ImageJ is a reproducible, cost-effective and time-saving method of automated image based immuno-histochemical evaluation (Drury et al. 2011). However, stable staining protocol/optimal immuno-staining conditions and optimised image capturing conditions are needed to allow for a balanced and accurate automated analysis (Väyrynen et al. 2012).
Severe background intensity can affect the analysis. The presence of heavy background staining in images can make the analysis difficult. However, utilising a background substraction software protocols, did help to significantly reduce variation in background staining. In the future, the reproducibility of the particle cell counting method should be re-assessed by comparing this method with manual cell counting using immuno-staining sections of the same area, but with different levels of background staining. Furthermore, the measurements should be repeated using more than one operator/investigator testing each approach, in order to reduce observer bias.

6.4.3 Viral Antigen

In this study, the HSV-1 antigen was found in the cytoplasm, cellular processes and nuclei of most cells, including neurons, astrocytes and inflammatory cells, in affected brain region. The distribution of positive viral antigen staining among the cells within the brain tissue from HSVE patients is in accordance with the previous report (Esiri 1982, Schmidbauer et al. 1988).

All three cases of HSVE showed viral antigen widely distributed throughout the brain tissue. Viral antigen was not only found in the temporal lobe but also other affected brain regions, including the frontal lobe, amygdala, cingulate and thalamus. Patient 3 exhibited massive necrosis and depletion of virtually all the neurons, across all brain regions examined. This resulted in a relatively modest staining for HSV antigen, derived mainly from a few positively staining astrocytes and inflammatory cells. This finding suggests that areas exhibiting severe tissue
destruction and replacement with inflammatory infiltrates are not necessarily the areas most likely to contain the virus. Similar observations have previously been reported by Esiri (Esiri 1982).

6.5 Conclusion

The immuno-histochemical study has shown that mitochondria within CNS cells are damaged during of HSVE infection, with reduced staining for CO1 in HSVE cases compared to RTA controls. Mitochondrial protein was reduced in both neurons and astrocytes, across multiple brain regions among the HSVE cases. I observed a reciprocal pattern of immunostaining for CO1 and HSV-1 within the encephalitis patients. Brain regions that exhibited low CO1 staining tended to have high HSV-1 staining, or vice versa. Furthermore, I have observed this finding, using both molecular (Chapter 5) and immuno-histochemical techniques.

Previous in vitro studies using cell lines of non-CNS origin have shown HSV damages mitochondria. However, this is the first study to report mitochondrial damage in brain tissue of HSVE patients.
Herpes simplex virus encephalitis (HSVE) is one of the most severe clinical manifestations of HSV infection. Multiple studies have examined the pathophysiology of HSVE; from in vivo animal models to in vitro cell culture studies. However, the pathogenesis of HSVE is still far from fully understood. Relatively little is known about the host molecular mechanism underlying cellular death and tissue damage during HSVE. Such studies are important because they may point the way towards new therapeutic options.

This study has confirmed a novel pathway of host responses in HSVE that acting through the mitochondria, thus confirming existing in vitro studies. In this thesis I examined the histopathological changes, inflammatory cell activation and changes in transcript abundance during HSV infection using post-mortem brain tissue from HSVE compared with RTA cases.

I observed widespread neuropathological damage in brains of HSVE patients. This damage is likely due to direct lytic effects of HSV infection, which lead to cellular damage and death. Accelerated viral replication in the infected cells, shutdown of cellular RNA and protein expression and build-up of toxic viral products, can all contribute to this lytic event. Indeed, in my histopathological investigation, multiple viral inclusion bodies were found in astrocytes and neurons, which exhibited
staining characteristic of necrosis; shrunken with hypereosinophilic cytoplasm (red neurons).

Furthermore, in vitro studies carried out by other members of the Liverpool Brain Infections Group, have now confirmed my histopathological observations of post-mortem tissue. Transmission electron microscopy has shown that primary human astrocytes infected with HSV-1 in vitro do not show any characteristics signs of apoptosis; such as chromatin condensation, cell shrinkage, membrane blebbing or formation of apoptotic bodies. Instead, the cells exhibited signs of necrotic death (Wnęk et al. to be published).

The inflammatory cells appear to play a major role in the pathogenesis of HSVE. Activation of inflammatory cells, including macrophages, microglia and lymphocytes, is crucial to restrict viral replication and spread. However, HSVE has been found to be associated with a considerable tissue destruction that often persists in the tissue even after the virus has been cleared. It has been postulated that this damage could arise as secondary to immune response and accumulation of inflammatory cells in the brain.

This is the first study that reported a significant loss of mitochondrial encoded transcripts in brain tissue of patients with HSV encephalitis. In addition, I observed a reciprocal pattern of the expression mitochondrial protein CO1 and HSV-1 antigens. My findings suggest that mitochondria are damaged during HSV-1 infection. This mitochondrial damage, evidenced by a loss of mitochondria gene and protein expression, is likely to be a direct result of a HSV pathogenesis.
This thesis aimed to investigate key mechanisms of cellular damage and host response in herpes simplex encephalitis to improve current understanding of the pathogenesis of HSV-1 infection.

1. The results in Chapter 3 confirm previous findings on the neuropathological changes that are observed in post-mortem brain tissue of patients with HSVE. It was important to do this, to ensure the broader applicability of my new findings on mitochondrial damage; ie I wanted to show that the HSVE brain material I was studying was representative of typical HSVE brains. The diffuse inflammation predominantly involved the grey matter (polioencephalitis) with vast majority of neurons exhibited typical signs of necrosis accompanied by pronounced microglia activation and astrogliosis. The immuno-histochemical examination revealed that the amount and distribution of the HSV-1 antigen correlated with the observed distribution of the neuropathological changes. Neuropathological changes were mainly found in temporal lobe but the other regions including frontal, amygdala, cingulate and thalamus were also affected.

HIV encephalitis, a sub-acute encephalitis, demonstrated different characteristics of neuropathological changes compared to HSVE. The pathological changes in HIVE were mostly observed in the white matter (leucoencephalitis), in the presence of multinucleated giant cells which is considered specific hallmark for HIVE. Neuropathological changes in HIVE differ significantly to HSVE due to predilection of viruses (HIV and HSV-1) to target different cells.
Apart from focal inflammation in the temporal lobe of RTA Control 1, no relevant morphological abnormalities were observed in the post-mortem brain tissue of other RTA controls.

2. The results in Chapter 4 show the characteristic of inflammatory cells that are found in HSVE and HIVE in comparison with non-encephalitic RTA cases. There have been relatively few studies examining the inflammatory cells response in HSVE. Macrophages and activated microglia were the predominant inflammatory cells, along with T lymphocytes, found in HSVE. The CD68 and CD163 markers immuno-stained both the activated microglia and the infiltrating macrophages in HSVE and HIVE cases. All of these cells are capable of producing pro-inflammatory cytokines which potential contribute to mitochondrial damage. In RTA cases, CD163 tended to stain macrophages present in the perivascular space but not microglia in the brain parenchyma. This findings support earlier report of CD163 marker being selective for mononuclear phagocytes/infiltrating macrophages in the healthy brain (Rezaie and Male 2003). My study is the first to report the observation of CD163+ cells in post-mortem human brain tissue from human encephalitis patients. Further studies with several specific markers capable of differentiating between infiltrating macrophages and activated microglia will provide more insight into the distribution of these two cell types within the brain tissue during encephalitis. Furthermore, the application of molecular methods such as gene-expression microarray could be used to determine genes that are significantly differentially expressed in
these cells. In addition to more detailed characterisation of inflammatory cell populations, future studies should include the examination of inflammatory mediators, in order to improve our understanding of the immune response in HSVE. Furthermore, it would also be interesting to consider M1 and M2 neuroinflammatory pathways in future studies of the pathogenesis of HSVE.

3. The genome-wide transcriptome analysis described in Chapter 5, is to my knowledge, the first study that show a marked reduction of mitochondria encoded transcripts in post-mortem brain tissue of patients with HSVE. These findings reflect earlier in vitro studies reporting degradation of cellular mRNA following HSV infection (Latchman 1988, Saffran et al. 2006). There was a more pronounced loss of mitochondrial transcripts compared to nuclear transcripts in brain tissue of HSVE cases indicating a preferential loss of mitochondrial transcription during HSV infection.

4. The results in Chapter 6 extend the microarray observations. The mitochondrial gene expression that was severely affected in HSV encephalitis was further associated with the reduction of mitochondria encoded protein, CO1. The examination of sequential slides stained with anti-CO1 and HSV-1 demonstrated a reciprocal pattern between the intensity of CO1 and HSV-1 signals across all the HSVE brain regions. This findings indicates that HSV infection is directly linked to the loss of mitochondrial CO1 protein, and this process is most possibly HSV-
mediated. The results suggest a mitochondria-mediated mechanism of cell death during HSVE. Further studies with double immuno-labelling of post-mortem brain tissue for CO1 and HSV-1 may further confirm my findings. More detailed studies, with the use of in vitro models, could also be undertaken to investigate the relationship between HSV infection and mitochondrial death pathway in more detail.

5. A limitation of my study is that the examination was only performed on formalin fixed paraffin embedded (FFPE) brain tissue. It is well known that fresh frozen samples are the best source of human material for molecular gene expression studies, as gene signatures in FFPE samples might be lost due to fixation processes. Furthermore fixation processes also affect the RNA integrity and yield. Despite these concerns, my array findings appeared valid, and were confirmed by both QPCR and measurement of the corresponding mitochondrial protein in the brain tissue.

Other limitations include a low number of HSVE samples/cases available and lack of clinical data for the patients used in the study; this latter was a consequence of the ethical permissions for use of the material. In addition, only limited amounts of information can be obtained from post-mortem FFPE samples. Other types of investigations, such as in vitro and/or in vivo studies would need to be performed in order to fully understand the events occurring during encephalitis.

Many of the findings from my study have since been taken forward and confirmed with the examination of cultures of primary human astrocytes,
performed by Dr Malgorzata Wnek, postdoctoral researcher in the Brain Infections Group. Such in vitro studies, have confirmed that i) mitochondrial, but not nuclear, gene and protein expression decline progressively with an increase of HSV titers; ii) mitochondrial function and structure are severely affected by HSV infection; iii) functional and ultra-structural changes to mitochondria were a selective process that occurred in the infected cells before nuclear function has been impaired (Wnek et al. to be published).

Thus, drugs that modify mitochondrial function may offer a new therapeutic avenue and could be used as an adjunctive treatment in HSVE. This current study significantly contributes to the knowledge on the pathogenesis of HSVE and extends our understanding on the involvement of mitochondria in cellular death during HSV infection.
REFERENCES


express the stem cell antigen cd34 in response to acute neural injury. Glia 50 (2), 121-131.


Masuda, N., Ohnishi, T., Kawamoto, S., Monden, M., Okubo, K., 1999. Analysis of chemical modification of rna from formalin-fixed samples and optimization
of molecular biology applications for such samples. Nucleic Acids Research 27 (22), 4436-4443.


Petty, R., 1994. Recent advances in the neurology of hiv infection. Postgraduate Medical Journal 70 (824), 393-403.


Steiner, I., 2011. Herpes simplex virus encephalitis: New infection or reactivation?” Ultrastructural pathology 24 (3), 268-274.


Wnęk, M., Ismail, Z., Ricci, E., Ressel, L., Kipar, A., Solomon, T., Griffiths, M., To be published. Minocycline protects mitochondria in human herpes simplex virus infection


