NEUROPATHOGENESIS OF JAPANESE ENCEPHALITIS IN A RHESUS MONKEY CHALLENGE MODEL

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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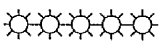
ABSTRACT

Japanese encephalitis (JE) is a major cause of childhood mortality and morbidity in Asia and for which there is no treatment. In addition to direct viral cytopathology, a poorly regulated inflammatory response is postulated to contribute to the pathogenesis, possibly through bystander death of uninfected neurons; but there have been few studies examining this. We used a validated macaque model of JE to characterize the inflammatory response and cytopathic effects of JE virus (JEV) in the brain. There was strong perivascular infiltration of leukocytes, particularly T cells, with endothelial cell activation, vascular damage and leakage of serum across the blood brain barrier (BBB). Adjacent parenchyma exhibited macrophage rich infiltrates, with astrocyte and microglia activation. JEV antigen was seen mostly in neurons, but there was no correlation between intensity of viral infection and degree of inflammatory response. Apoptotic cell death was seen in both infected and non-infected neurons. Interferon (IFN)-α, which is a microglial activator, was also expressed by both. Tumour necrosis factor-α (which can induce neuronal apoptosis), inducible nitric oxide synthase and nitrotyrosine (involved in nitric oxide production) was expressed by microglial cells, astrocytes and to some extent macrophages. The same cells expressed matrix metalloproteinase (MMP)-2 whilst MMP-9

was expressed by neurons; both MMPs cause BBB disruption. The results are consistent with JEV inducing neuronal apoptotic death and release of cytokines such as IFN-α; these are postulated to initiate microglial activation, and release of pro-inflammatory and apoptotic mediators with subsequent apoptotic death of both infected and uninfected bystander neurons. Activation of astrocytes, microglial and endothelial cells likely contributes to inflammatory cell recruitment and BBB breakdown. These results provide strong evidence that neuronal apoptotic death and activation of microglial cells and astrocytes play a crucial role in the pathogensis of JE, and suggest new paths for targeted therapies.

DEDICATION

This work is dedicated to members of my family and in particular to my parents and my princes and princess.



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DECLARATION

Except for the assistance as outlined in the acknowledgements above, the work described is my own work and has not been submitted for a degree or other qualification to this or any other university.

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ABBREVIATIONS

ABC	avidin-biotin-peroxidase complex
AIF	apoptosis-inducing factor
Apaf-1	apoptosis protease activating factor-1
BBB	blood brain barrier
C	core
CNS	central nervous system
CSF	cerebrospinal fluid
CTL	cytotoxic T cell
DAB	diaminobenzidine
E	envelope
EAE	experimental autoimmune encephalomyelitis
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
FADD	FAS-associated death domain
FAS-L	FAS-Ligand
GFAP	glial fibrillary acidic protein
H-E	haematoxylin-eosin
HIER	heat-induced epitope/antigen retrieval

HRP	horseradish peroxidase
IFN-α	interferon alpha
IFN-γ	interferon gamma
IgM	immunoglobulin M
IH	immunohistology
IL	interleukin
iNOS	inducible nitric oxide synthase
IP	indirect peroxidase
JE	Japanese encephalitis
JEV	Japanese encephalitis virus
M	membrane
MAC-ELISA	IgM capture enzyme linked immunosorbent assay
MHC II	major histocompatibility complex class II
MIP-1α	macrophage inflammatory protein -1α
MMP	matrix metalloproteinase
NO	nitric oxide
NS	non-structural
NT	nitrotyrosine
PCR	polymerase chain reaction
prM	pre-membrane

PRNT	plaque reduction neutralization titre assay
qRT-PCR	quantitative real time polymerase chain reaction
RNA	ribonucleic acid
ROS	reactive oxygen species
SLEV	St. Louis encephalitis virus
TBEV	tick-borne encephalitis virus
TMB	tetramethylbenzidine
TNF-α	tumour necrosis factor alpha
TNFR	tumour necrosis factor receptor
TUNEL	terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end *in situ* labelling
VEGF	vascular endothelial growth factor
WNV	West Nile virus

CHAPTER 1

INTRODUCTION

1.1 Japanese encephalitis overview

Japanese encephalitis virus (JEV) continues to be the leading cause of viral encephalitis in Asia and the Western Pacific, where it is a significant cause of mortality and disability (Solomon, 2006). Annually there are estimated 35,000-50,000 cases world-wide with 10,000-15,000 deaths (Solomon *et al.,* 2003). Approximately one third of patients die, and half of the survivors have severe neuropshychiatric sequelae. A renewed concern for the disease followed the outbreak of JE in India in 2005 with more than 1000 fatalities (Kumar *et al.*, 2006). Although vaccination is the most viable option to prevent the disease, affordable vaccines are still not widely available (Beasley *et al.,* 2008), and there is no established treatment for JE.

1.2 Historical perspective

Epidemics of encephalitis were described in Japan since from the 1980s (Innis, 1995). Major epidemics were reported approximately every ten years, with more than 6000 cases reported in the 1924 epidemic (Miyake,

1964). JEV was isolated in 1934 from the brain of a fatal case of encephalitis (Monath, 1988) and this virus isolate was characterized as the prototype (Nakayama) strain of JE virus. The virus was later classed as a member of the genus *flavivirus* (Family Flaviviridae) named after the prototype yellow fever virus (in Latin yellow = flavi). Although of no taxonomic significance, the ecological term "arbovirus" is often used to describe the fact that JEV is insect (arthropod) borne.

1.3 Epidemiology

JE is a major public health problem in several parts of Asia particularly China, India, Nepal, Sri Lanka, Vietnam, Cambodia and Thailand. JE is also reported from other Asian countries such as Indonesia, Malaysia, Myanmar, Philippines, Republic of Korea and Japan albeit to a lesser extent. It is predominantly reported from rural areas, especially among the lower socio-economic groups. JE exhibits a seasonal pattern with incidence of disease peaking during or shortly after the rainy season. However, variations within the same region are known. For instance, epidemics occur during or soon after rainy seasons in the northern regions of Vietnam and Thailand, while the disease has been reported throughout the year in the southern parts of these countries (Umenai *et al.*, 1985).

The epidemiology of JE has undergone a remarkable change in recent years and it is emerging in many new geographic regions of Asia. Until a decade ago Indonesia was the southern limit for JE while the Eastern States of India were the western limit. JE has now emerged in some countries beyond these limits; consequently the geographic boundaries have now extended to Pakistan in the West (Igarashi *et al.*, 1994) and Australia in the South (Hanna *et al.*, 1996) (Figure 1.1).

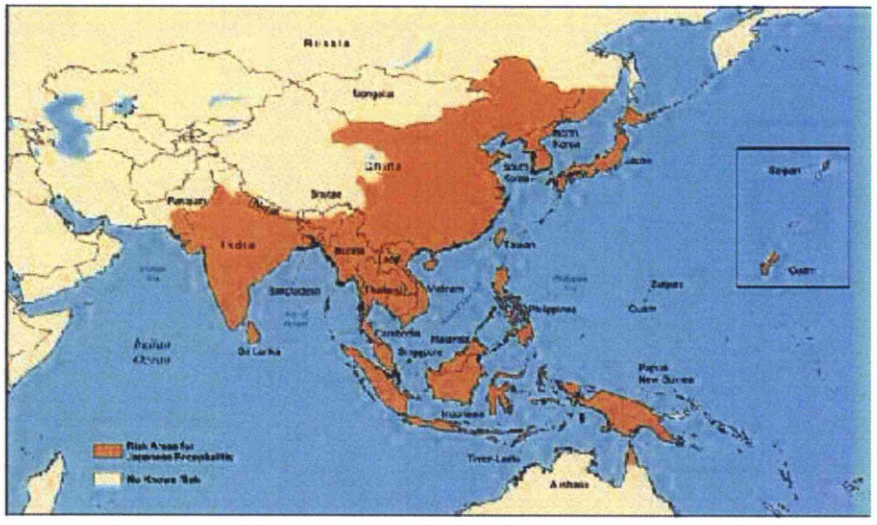


Figure 1.1 Global distribution of Japanese encephalitis (http://www.phac-aspc.gc.ca/publicat/ccdr-rmtc/11vol37/acs-1/index-eng.php)

1.4 Transmission cycle of Japanese encephalitis

JEV is zoonotic and transmitted naturally between wild and domestic birds,

and pigs by Culex mosquitoes - the most competent vector for human infection being Culex tritaeniorhynchus (Figure 1.2). All Culex species are zoophilic, preferring to feed on animals and birds to humans. Environmental temperature and available pools of water as created in rice fields in most of Asia provide an ideal environment for vector replication and virus replication in the mosquito host. Although many animals can be infected with JEV, only those which develop high viraemias are important in the natural transmission cycle. Birds are the most important vertebrate host in maintaining the virus. As well as maintaining and amplifying JEV in the environment, birds may also be responsible for the spread of JEV to new geographical areas (Paul *et al.*, 1993). Pigs act as important amplifying hosts in the transmission of JEV to humans. They are often kept close to human dwellings, have prolonged and high viraemias, and produce many offspring - thus providing a continuous supply of previously uninfected hosts. Humans become infected with JEV coincidentally when living or travelling in close proximity to the virus' enzootic cycle. Although the virus has occasionally been isolated from human peripheral blood (Chan & Loh, 1966) viraemias are usually brief and titres low; thus humans are considered a dead end host from which transmission does not normally occur.

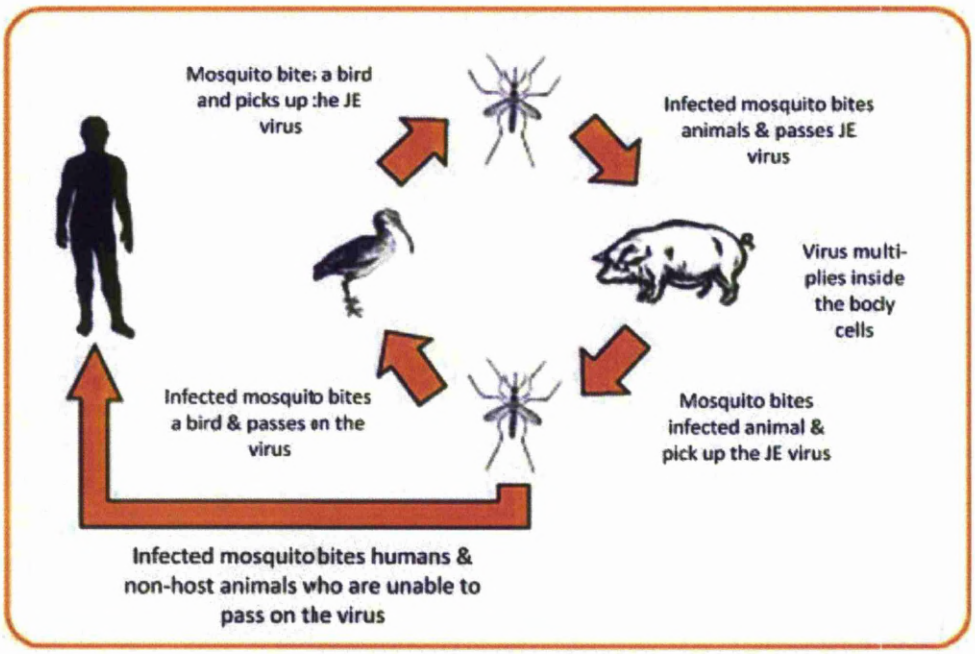


Figure 1.2 Transmission cycle of Japanese encephalitis virus (http://infosehatbugar.blogspot.com/2009/06/penyakit-japanese-encephalitis.html)

1.5 Virology

In common with all flaviviruses, JEV has a small (50nm) lipoprotein envelope surrounding a nucleocapsid comprising of core protein and 11 KB single stranded RNA (3800 kD) (Figure 1.3A). At least five genotypes of JEV occur in Asia, which relate approximately to the geographical area of isolation (Chen *et al.*, 1990, 1992).

Like the other flaviviruses, the JEV virion consists of a single strand of

positive-sense RNA, wrapped in a nucleocapsid and surrounded by a glycoprotein containing envelope. The RNA comprises a short 5' untranslated region (UTR), a longer 3' UTR, and a single open reading frame between them. This codes for a single polyprotein which is co- and post-translational cleaved by viral and host proteases into three structural proteins (core-C, pre-membrane-PrM, and envelope-E), and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Figure 1.3B).

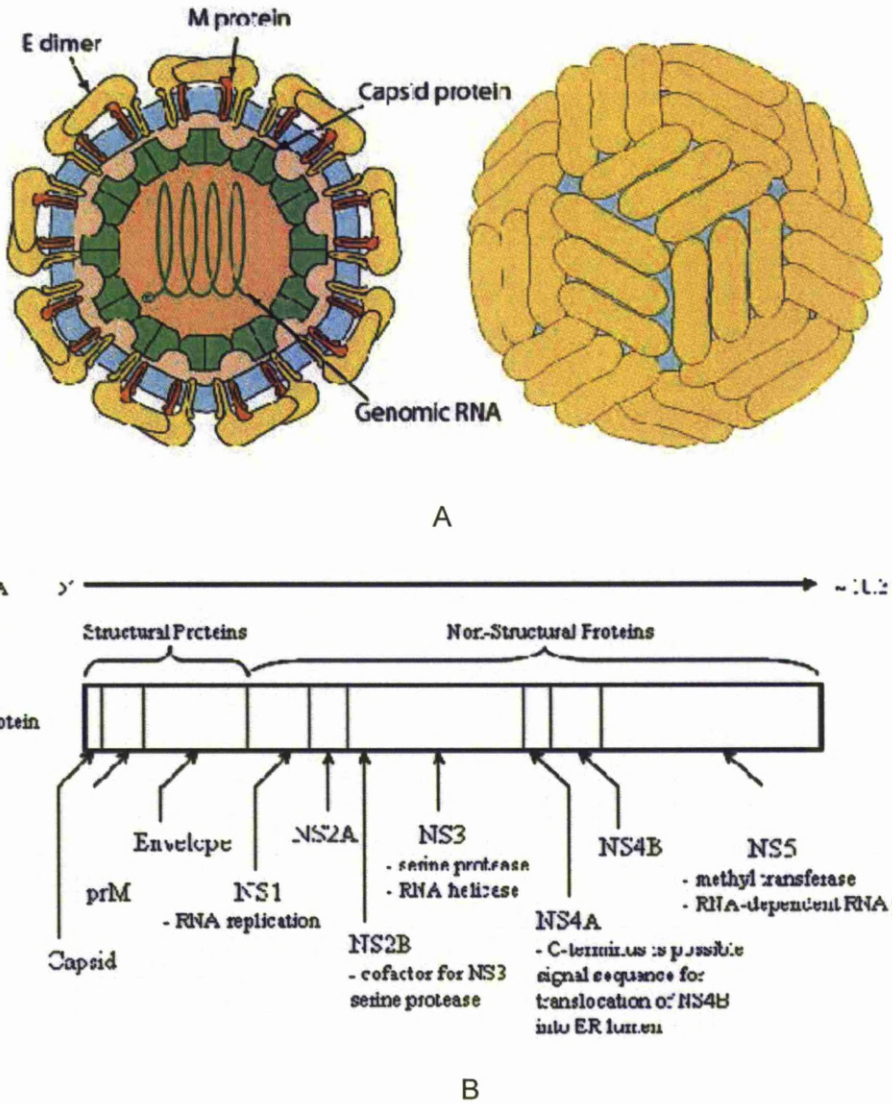


Figure 1.3 A. The mature virion; M, membrane; E, envelope; (http://viralzone.expasy.org/all_by_species/24.html); B. The flavivirus genome (http://carnot.utmb.edu/flavitrack/faq.php)

1.6 Clinical Features

Patients with JE typically present after a few days of non-specific febrile illness, which may include coryza, diarrhoea and rigors (Solomon *et al.*, 2000). This is followed by headache, vomiting and a reduced level of consciousness, often heralded by a convulsion. In some patients, particularly older children and adults, abnormal behaviour may be the only presenting feature, resulting in an initial diagnosis of mental illness. A proportion of JE patients make a rapid spontaneous recovery. Others may present with aseptic meningitis and have no encephalopathic features. Convulsions occur frequently in JE, though no association with outcome has been noted. The classical description of JE includes a dull flat 'mask-like' facies with wide unblinking eyes, tremor, generalised hypertonia, cogwheel rigidity and other abnormalities of movement (Misra & Kalita, 1997). Opisthotonus, and rigidity spasms, particularly on stimulation, occur in about 15% of patients and are associated with a poor prognosis (Kumar *et al.*, 1990).

Approximately 30% of hospitalised patients with JE die, and around half the survivors have severe neurological sequelae. In areas with better hospital facilities there is a reduction in mortality, but a concomitant

increase in the number of patients with sequelae (Ooi *et al.*, 2008). Approximately thirty percent of survivors have frank persistent motor deficits. These include a mixture of upper and lower motor neuron weakness, cerebellar and extrapyramidal signs. Fixed flexion deformities of the arms, and hyperextension of the legs with 'equine feet' are common. 20% of patients have severe cognitive and language impairment, and 20% have further convulsions (Kumar *et al.*, 1993). In addition, more detailed studies showed that there might be subtle sequelae such as learning difficulties, behavioural problems and subtle neurological signs (Kumar *et al.*, 1993).

1.7 Laboratory assays for diagnosis of JE

1.7.1 Serology

For most practical purposes JE is diagnosed serologically. In the 1980s simple enzyme linked immunosorbant assays (ELISA) were developed and IgM antibody capture ELISA (MAC ELISA) for serum and CSF of patients within 4-7 days of onset of disease has become the accepted standard for diagnosis of JE (Innis, 1989). The haemagglutination inhibition test was used for many years, but it had various practical limitations, and since it required paired sera could not give an early diagnosis (Clark & Casals,

1958).

1.7.2 Plaque Reduction Neutralization Titre Assay (PRNT)

This technique directly measures the infectivity of a virus preparation or stock as plaque forming units based on the finding that JE virus produces cytopathic effects (plaques) in certain cell lines. The endpoint is the titre of antibody that will reduce a certain percentage of viral plaques such as a 50%, 70%, or 90% plaque reduction ($PRNT_{50}$, $PRNT_{70}$, $PRNT_{90}$ respectively) compared to control cultures. $PRNT_{50}$ is the most commonly used endpoint. Today a number of PRNT protocols are being used to measure JE neutralizing antibody using a variety of cell lines, including LLC-MK2, Vero, and BHK21 cells. In a patient with suspected acute JE infection, a titre increasing by fourfold suggests an acute infection. A titre of 1:10 or more suggests past exposure or vaccination, and protective immunity.

1.7.3 Virus isolation

Even with the best laboratory facilities attempts to isolate JEV from clinical specimens are often unsuccessful because of low viral titres and the rapid production of neutralizing antibodies. JEV can be diagnosed by injecting post mortem brain tissue into cultured cells as it forms plaques in LLC-MK

(rhesus monkey kidney) cells, vero (African kidney monkey green) cells, and BHK-21 (baby hamster kidney) cells, and in mosquito cell lines such as C6/36 line (Aedes albopictus mosquitoes) and LSTM AP-61 line (Aedes pseudoscutellaris mosquitoes). It was also shown that virus could also be isolated from cerebrospinal fluid (CSF) but it is slow and technically difficult (Leake *et al.*, 1986).

1.7.4 Immunohistology

Immunohistological staining techniques of CSF cells or autopsy tissue with anti-JEV antibodies provide another way of detecting virus (Desai *et al.*, 1995; Mathur *et al.*, 1990; Johnson *et al.*, 1985; Myint *et al.*, 1994). The value of immunohistology is in diagnosing fatal cases of JE when sera or CSF are not available.

1.7.5 Molecular techniques

JEV ribonucleic acid (RNA) has been detected in human CSF samples using reverse transcriptase polymerase chain reaction (Igarashi *et al.*, 1994), however its reliability for regular diagnostic purposes has not been shown. Currently there is no simple rapid diagnostic test that is appropriate for use in the field.

1.8 Pathogenesis of JE

The course and outcome of the disease is thought to be influenced by the dose, the route of inoculation, the species, the age of the host, immune sensitization by earlier infection by the same group of cross-reacting viruses in the endemic areas and the virulence of the virus. Host factors that affect the outcome include the immune status, reticuloendothelial clearance mechanism, nutritional status and probably genetic factors play a role.

Following the bite of an infected mosquito, JEV is thought to amplify peripherally, causing a transient viraemia before invading the central nervous system (CNS). Based on data from mouse and macaque monkey, the site of peripheral amplification is thought to be dermal tissue and then lymph nodes. WNV is reported to have a larger dermal cell reservoir in keratinocytes in mice (Lim *et al.*, 2011). In mice, high rates of JEV replication within dendritic cells are linked to higher mortality (Wang & Deubel, 2011). After entering the body through a mosquito bite, the virus reaches the CNS via leukocytes (probably T lymphocytes); it is still not clear whether macrophages can also harbour JEV (Ghosh & Basu, 2009). The means by which JEV crosses the blood brain barrier (BBB) is not

known but the presence of diffuse infection throughout the brain in humans indicate a haematogenous route of entry (Desai *et al.*, 1995; Johnson *et al.*, 1985). This was supported by an intranasal monkey challenge model, in which virus replication was widespread in the CNS and not always identified in the olfactory bulb (Myint *et al.*, 1999; Raengsakulrach *et al.*, 1999). Whether the virus is passively transported, or actively replicates in the endothelium or within infected inflammatory cells that enter the brain parenchyma is unclear. Although experimental evidence suggests replication within endothelial cells may be an important means of crossing the blood brain barrier in some flaviviruses, for JEV passive transfer across the endothelial cells appears a more likely mechanism (Desai *et al.*, 1995; Johnson *et al.*, 1985). The neural invasion in humans is followed by perivascular cuffing, infiltration of inflammatory cells (T cells and macrophages) into the parenchyma, and phagocytosis of the infected cells. Phagocytic microglial cells remove degenerated neurons forming typical microglial nodules (Johnson *et al.*, 1985; Miyake, 1964). Gliosis involving microglial cells and astrocytes is a common feature and microglial nodules are often in close proximity to the affected regions. Neuronal cells constitute the main cellular target population of JEV as in West Nile virus (WNV) and tick-borne encephalitis virus (TBEV).

Electron microscopic studies of the brains of infected mice show that virus replicates in the rough endoplasmic reticulum and Golgi apparatus. There are hypertrophy of the endoplasmic reticulum and degeneration into cystic structures causing extensive cellular dysfunction (Hase *et al.*, 1990).

Although a variety of animal model for JEV exists, the rodent models are the most characterized and a considerable knowledge regarding the pathogenesis has been generated from these studies. Basic histological findings in the CNS lesions in JEV-infected mice are similar to other *flavivirus*-infected mice as well as that of humans. However some JEV infected mice have a prominent infiltration of CD8+ T cells compared to the human autopsy studies in which CD8+ cells are a minority among the T cell population (Johnson *et al.*, 1995; Kimura *et al.*, 2010). The rodent model has shown that activation of microglial cells may play a significant role in the pathogenesis by producing inflammatory mediators like inducible nitric oxide synthase (iNOS) and cytokines inducing neuronal death (Ghoshal *et al.*, 2007). The rhesus macaque has been proposed as a potential model for the evaluation of vaccines and anti-viral treatments. The genome sequence of macaques and humans are 93% similar. The neuropathology of macaques intranasally inoculated with JEV resembles

that of humans with encephalitis that results from naturally acquired infection (Myint *et al.*, 1999), whereas intradermal or subcutaneous inoculation of JEV in macaques generally results in asymptomatic infection (Morris *et al.*, 1955). The apoptosis pathways and the full spectrum of pro-inflammatory factors including matrix metalloproteinases (MMPs) have not been fully characterized apart from cytokines in any previous animal models or in autopsy tissues.

Despite the disease's importance, little is known about the pathogenesis (Solomon & Vaughn, 2002). Infection, dysfunction and subsequent destruction of neurons are presumed to be the main cause of death in JE. Neuronal apoptosis is one of the hallmarks of neurodegenerative infections and 20 years ago was shown to be induced by JEV in an *in vitro* study (Hase *et al.*, 1990). However, its relevance for the disease and its mechanism are still unclear. In addition, the extent of cell injury that occurs as a consequence of the inflammatory response, as opposed to direct viral cytopathology, is unknown.

1.8 Aims

Because of cultural constraints in many of the areas where JE occurs, autopsy tissue from fatal human cases is not often available. Mouse models have shown that microglial activation and subsequent release of pro-inflammatory mediators plays a role in neuronal death in JE (Ghoshal *et al.,* 2007; Das *et al.,* 2008), but detailed studies on the role and mechanisms of apoptosis and the contribution of glial cells have not been reported. The macaque model of JE represents an especially appropriate model for the study of JE since the macaque immune system closely resembles that of humans (Myint *et al.,* 1999).

The present study used material available from prior vaccine studies with the macaque model of JE to focus on the inflammatory response and cascade of events that leads to neuronal damage. I was especially interested in apoptotic pathways and inflammatory mediators because these may point the way to new targeted treatments to control the inflammatory damage, even in the absence of antiviral therapy.

Specific aims and hypotheses

1. To identify the mechanisms of neuronal damage in JE by exploring the role of apoptosis.
2. To identify the predominant inflammatory factors, in particular the cytokines, MMPs and iNOS, involved in JE pathogenesis.
3. To identify the pathways of inflammatory network that leads to neuronal cell death.

Hypothesis 1: Neurons are the main targets of JEV.

Hypothesis 2: In addition to viral cytopathology, bystander effect contributes significantly to neuronal cell death.

Hypothesis 3: Neuronal apoptosis is the hallmark of JE and multiple apoptosis pathways are involved in JE.

Hypothesis 4: Pro-inflammatory mediators contribute to neuronal damage in JE pathogenesis.

Hypothesis 5: Non-neuronal cells and blood brain barrier dysfunction play important roles in the pathogenesis of JE.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

This study was performed on archived paraffin embedded brain tissue of twelve rhesus macaques (*Macaca mulatta*) challenged intranasally with a well characterized JEV strain (KE93) as part of an effort to evaluate second-generation JEV vaccines (Myint *et al.,* 1999). Healthy, young adult macaques of both sexes, weighing 2 – 8 Kg were used for the original challenge study. All animals were bred at AFRIMS veterinary laboratory and the principles of good laboratory animal care were followed throughout the study.

The challenge study had been undertaken in several phases and with different doses (Table 2.1; Raengsakulrach *et al.,* 1999). Animals were screened for neutralizing antibodies to dengue and JE and were found to be seronegative. After challenge, animals inoculated were observed daily for clinical signs of neurological illness. The monkeys were euthanized at the onset of stupor or coma (11-14 days post inoculation). JEV infection was confirmed by virus isolation from the brain; in all cases this was

supported by detection of IgM to JEV in serum or CSF using the double sandwich capture MAC ELISA assay, or an increase in the haemagglutination inhibiting antibody to JEV (Raengsakulrach *et al.*, 1999). Five uninfected control monkeys from an unrelated study served as negative controls for histopathological and immunohistological examinations. All archived specimens used for this study are from unvaccinated monkeys.

Animal models of JEV have the advantage of being more readily available for optimization of tissue procurement and preparation, variables which are inherently difficult to optimize in human post-mortem studies. In conducting the original challenge studies, the investigators adhered to the Guide for the Care and Use of Laboratory Animals, as promulgated by the Institutional Animal Care and Use Committee. My re-use of the archived tissues for these new pathogenesis studies is in keeping with the 3Rs principle of Replacement, Reduction, and Refinement (Zurlo *et al.*, 1996).

Challenge virus

The wild-type JEV (KE-93) was isolated from the brain of a 6 year old Thai male who died of encephalitis in Kamphaeng Phet, Thailand in 1983. Two

monkeys (animals 1 and 2) were inoculated with a virus originally isolated in AP-61 cells and passaged once in C6/36 cells. The remaining 10 monkeys received an isolate prepared from the brain of monkey number 2 (DA 349) that was subsequently passaged twice in suckling mice to increase both virus titre and virulence. Virus titration was performed in *Toxorrhynchites splendens* mosquitoes.

Clinical manifestations in macaques

Clinical signs appeared approximately 7-10 days post inoculation. Early signs included localized mild muscle tremors and fasciculations, usually on the head and arms. The tremors steadily progressed and became more pronounced. While initially able to eat, the animals became progressively more anoretic and lethargic 2-4 days following initial signs. As time continued, the animals would lie in their cages and eventually become comatose and un-responsive.

2.2 Histopathology

Immediately after death, brains from all 12 infected animals and the control monkeys were exenterated and sections of frontal lobe, thalamus, brainstem and cerebellum were prepared and fixed in 10% neutral buffered

formalin for at least 72 hours and processed according to standard histological methods. Following routine paraffin wax embedding, 3-5 µm sections were prepared and stained with haematoxylin-eosin (H-E) or used for immunohistology.

2.3 Immunohistology

For immunohistological studies, sections of thalamus and brainstem were selected as they had previously been shown to be heavily infected by JEV (Myint *et al.*, 1999) and exhibited the most consistent histological changes. The cortex was chosen as a comparison tissue because it exhibited minimal parenchymal inflammatory infiltrates.

Selected sections of the brainstem and thalamus of each animal were stained for the presence of JEV antigen, apoptosis and apoptotic pathways, glial and inflammatory cells, von Willebrand Factor (to confirm BBB breakdown, through the demonstration of plasma protein leakage), and pro-inflammatory markers. Details on the panel of antibodies and the detection methods used are provided in Table 2.2. Briefly, sections were dewaxed in xylene and hydrated through graded alcohols. To inhibit endogenous peroxidase activity, they were treated with freshly prepared

3% H_2O_2 for 15 min and washed three times with distilled water. Sections were then processed according to heat-induced epitope/antigen retrieval (HIER) protocols. This was followed by incubations with normal serum to block non-specific binding sites in tissues, and the primary antibodies (15-18 hrs at 4°C) at the optimized dilutions (Table 2.2). Optimal conditions for antigen retrieval and antibody dilutions (Table 2.2) were independently determined by comparative titration experiments.

Controls

Appropriate controls were included in parallel for each marker: uninfected control monkey brains as negative controls for JEV and to establish constitutive expression of other markers, positive controls (sections with known positivity for the specific marker), and isotype controls (in which the primary antibody was substituted with equal concentration of normal mouse/rabbit IgG) as negative controls.

2.4 Heat-induced epitope/antigen retrieval (HIER)

Formalin is a commonly used fixative for tissue preparation in pathology laboratories. A major adverse effect of this fixative is the concealing of tissue antigens by protein cross-linking (Shigeki *et al.*, 2005). Masking of

epitopes is compounded in archival, paraffin-embedded specimens, which causes considerable loss of immunoreactivity. Pre-treatment with high temperature heating is the most important factor for retrieval of antigens concealed by formalin fixation in archived tissues. HIER is based on the principle that the combination of heat and pH can restore antigenicity to protein epitopes (Shi *et al.,* 2001). The retrieval methods included treatment with microwaves, pressure cooker, and combined action of enzyme digestion and microwaves using solutions like citrate buffer, Tris HCl and EDTA.

In this study HIER was performed almost exclusively with a laboratory pressure cooker (Decloaking Chamber, Biocare Medical, Concord, USA) after the preliminary data which demonstrated that it produced the most consistent results compared to microwaving, enzyme digestion or a common pressure cooker. An advantage of the laboratory pressure cooker is that, temperatures of 120^0C or higher (superheating) can be achieved. Other advantages include short duration of heating, and better reproducibility of results with large batches of slides. For comparison purpose a panel of retrieval solutions were tested for the respective antigens using serial sections from the same blocks; including citrate buffer

pH 6 and pH 9, 0.05% citraconic anhydride solution pH 7 (Tokyo Kasei Co. Ltd., Tokyo, Japan) and Trilogy (Cell Marque, Hot Springs, USA). In all instances I have demonstrated that the combination of citrate buffer pH 6.0 and the laboratory pressure cooker restores the immunostaining of a wide variety of antigens without affecting the tissue morphology.

2.5 Interobserver agreement

For each marker, the extent and intensity of the immunoreactivity was evaluated semi-quantitatively by two examiners independently (Khin Myint and Duangrat Mongkolsirichaikul). It was scored on a 4-point scale as follows: 0 = no difference from the negative control with regard to intensity, distribution and number of positive cells, or no positive reaction, 1+ = occasional individual positive cells, 2+ = a few nests of positive cells, 3+ = frequent positive cells in entire examined brain section. The results in agreement with two observers were considered as final. Identification of cell types based on morphological criteria and the mode of interpretation was confirmed by two pathologists (Anja Kipar and Yvonne Van Gessel).

2.6 Confocal Microscopy

Confocal laser scanning microscope LSM 700 (Carl Zeiss MicroImaging, Germany) with solid state lasers excitation wavelength 488 nm (for FITC) and 555 nm (for Texas Red) and ZEN 2009 software was used to detect immunofluorescent staining, while for all other light microsopic assessments, conventional microscopes were used. Confocal microscopy was kindly performed by Virachai Polsiri and Sansanee Noisakran of the Department of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand.

2.7 Double immunolabelling

Dual staining was performed on selected sections of some monkeys (animals 2, 9, 11) to characterize the populations of cells expressing apoptosis markers (TUNEL and caspase-3, -8, and -9) and pro-inflammatory mediators (cytokines, iNOS and MMPs) and to relate them to the expression of JEV antigen. For this purpose, primary antibodies raised in different species were sequentially localized using non-overlapping secondary reagents and different chromogens (Table 2.3).

2.8 Sequential staining

Sequential staining was performed on consecutive sections, mainly to detect tumour necrosis factor alpha (TNF-α) expression in inflammatory cells and glial cells and to further characterize JEV-infected cells when dual staining could not be attempted because the primary antibody was raised in the same species or when the double immunolabeling was difficult to interpret (Table 2.3).

Table 2.1 Animals, JE challenge virus, infectious doses and time of necropsy. Animals were euthanized at the onset of stupor or coma

Animal No.	Challenge virus	Challenge dose (pfu)	Day necropsied
1	KE93, AP61-1, C6/36-1	2.3×10^7	12
2	KE93, AP61-1, C6/36-1	6.6×10^6	12
3	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^9	11
4	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^9	11
5	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^9	11
6	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^{10}	12
7	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^{10}	10
8	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	2.0×10^{10}	11
9	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	7.5×10^7	12
10	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	7.5×10^7	10
11	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	7.5×10^7	12
12	KE93, AP61-1, C6/36-1, DA-349-1, SM-2	7.5×10^5	13

pfu – plaque-forming unit

Table 2.2 Antibodies and detection methods

Antibody (clone)	Source	Detection method*	Dilution	Specificity
Mouse anti-JEV	AFRIMS	IP&IFA	1:2000/1:160	Japanese encephalitis virus (Henchal *et al.*, 1987)
Mouse anti-Neuronal Nuclei [NeuN]	Chemicon	IP	1:200	Neurons
Rabbit anti-Glial Fibrillary Acidic Protein [GFAP]	DakoCytomation	IP&IFA	1:1000/1:100	Astrocytes
Anti-Human Von Willebrand Factor [Factor VIII]	DakoCytomation	IP	1:1600	Endothelial cells, megakaryocytes, platelets, serum
Rabbit anti-Human CD3	DakoCytomation	IP	1:100	T cells
Mouse anti –Human CD20 cy (L26)	DakoCytomation	IP	1:200	B cells
Mouse anti –Human CD68 (KP1)	DakoCytomation	IP	1:100	Macrophages, monocytes, microglial cells
Mouse anti-Human Myeloid/Histiocyte Antigen (MAC 387)	DakoCytomation	IP	1:1600	Monocytes, neutrophils, recently blood derived macrophages, microglial cells
Mouse anti-Human HLA-DR Antigen [MHC II] (TAL.1B5)	DakoCytomation	IP	1:25	Macrophages, B cells, activated T cells, activated microglial cells, activated endothelial cells
Rabbit anti-Human/Mouse Cleaved Caspase-3 [Asp175]	R & D	IP	1:100	Cleaved Caspase-3
Rabbit Caspase-8 p18 [H-134])	Santa Cruz	IP	1:50	Caspase-8

Rabbit Caspase-9 p10 [H-83])	Santa Cruz	IP	1:50	Caspase-9
Rabbit anti-Human BAX [N-20]	Santa Cruz	IP	1:2000	Baxα, Baxβ, Bax
Mouse anti-Human Bcl-2 (100/D5)	Novocastra	IP	1:80	Human bcl-2 oncoprotein
Rabbit anti-Nitric Oxide Synthase [iNOS]	Thermo Scientific	IP	1:150	Activated macrophages and glial cells
Rabbit anti-Nitrotyrosine [NT]	Chemicon	IP	1:200	Nitrotyrosine
Mouse anti-Human Matrix Metalloproteinase [MMP]-2 (A-GelVC2)	Neo Marker	IP	1:10	Both pro and active forms of MMP-2
Rabbit anti-Human Matrix Metalloproteinase [MMP]-9	Neo Marker	IP	1:750	Both pro and active forms of MMP-9
Rabbit anti-Human Tumour Necrosis Factor [TNF]-α	AbCam	IP	1:1000	Cell bound pre-cursor of TNF-α
Rabbit anti-Human Interferon [IFN]-α	PBL Biomedical Laboratories	IP	1:2000	IFN-α

IP = Indirect peroxidase with peroxidase-conjugated anti-mouse or -rabbit antibody (EnVision Systems; Dako, Carpinteria, CA, USA) and diaminobenzindine (DAB, VECTOR, Burlingame, CA, USA), NovaRED (VECTOR) or tetramethylbenzidine (TMB, VECTOR) as substrate IFA = Indirect immunofluorescence with goat anti-rabbit FITC (kpl, Gaithersburg, MD, USA), sheep anti-mouse IgG FITC conjugate (Sigma, St. Louis, MO, USA), goat anti-rabbit Texas Red (Molecular Probes, Eugene, OR, USA) or goat anti-mouse Texas Red (Molecular Probes).
*pre-treated with heat-induced antigen (epitope) retrieval (HIER) in citrate buffer pH 6.0 (VECTOR) for all antigens except for Bcl-2 (citrate buffer pH 9)

Table 2.3 Double and sequential immunostaining of Japanese encephalitis virus infected monkey brains

Antibody		Detection Method (chromogen/fluorochrome)	
First	Second	First	Second
Double Immunostaining			
TUNEL	JEV	IFA/FITC IP/DAB	IFA/TR ABC/AP
	NeuN	IP/DAB	ABC/AP
	CD68	IP/DAB	ABC/AP
	MAC387	IP/DAB	ABC/AP
JEV	GFAP	IFA/FITC ABC/AP	IFA/TR IP/DAB
	IFN-α	ABC/AP	IP/DAB
	TNF-α	ABC/AP	IP/DAB
	MMP-9	ABC/AP	IP/DAB
	iNOS	ABC/AP	IP/DAB
	Cleaved Caspase-3	ABC/AP	IP/DAB
	Caspase-8	ABC/AP	IP/DAB
	Caspase-9	ABC/AP	IP/DAB
	BAX	ABC/AP	IP/DAB
CD68	iNOS	ABC/AP	IP/DAB
	MMP-9	ABC/AP	IP/DAB
	TNF-α	ABC/AP	IP/DAB
Sequential Immunostaining			
JEV	CD68	IP/DAB	IP/DAB
TNF-α	GFAP	IP/DAB	IP/DAB
	CD3	IP/DAB	IP/DAB
	CD68	IP/DAB	IP/DAB

IP/DAB = Indirect peroxidase with peroxidase-conjugated anti-mouse or -rabbit antibody (EnVision Systems; Dako, Carpinteria, CA) and diaminobenzindine (DAB, VECTOR, Burlingame, CA, USA) or NovaRED (VECTOR) as substrate
ABC/AP = Avidin Biotin Complex – Alkaline Phosphatase with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (VECTOR Blue) as substrate
IFA/FITC = Indirect immunofluorescence with goat anti-rabbit FITC (kpl, Gaithersburg, MD, USA) or sheep anti-mouse IgG FITC conjugate (Sigma, St. Louis, MO, USA)
IFA/TR = Indirect immunofluorescence with goat anti-rabbit Texas Red (Molecular Probes, Eugene, OR, USA) or goat anti-mouse Texas Red (Molecular Probes)

Sources:
AFRIMS, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand; Chemicon International, Inc, Temecula, CA, USA; DykoCytomation, Carpinteria, CA, USA; R&D, Minneapolis, MN, USA; Santa Cruz Biotecnology, Santa Cruz, CA, USA; Novocastra, New Castle, UK; Thermo Scientific, Rockford, IL, USA; Neo Marker, Fremont, CA, USA; AbCam, Cambridge, UK; PBL Biomedical Laboratories, Piscataway, NJ, USA.

CHAPTER 3

HISTOPATHOLOGY, PHENOTYPING AND JEV TARGET CELLS

3.1 Introduction

This chapter aims to examine the immune response and JEV targets in the brain of JEV infected monkeys.

3.1.1 Flavivirus encephalitis

In human autopsy studies the histopathological lesions in the brain showed variable degree of mononuclear cell infiltrate in the leptomeninges extending to the deep parenchyma, along the perivascular space. JEV induce neuroinflammation with features typical for viral encephalitides, including leptomeningitis, perivascular and parenchymal infiltration of lymphocytes, and development of glial nodules associated with neuronophagia in regions of viral replication (Johnson *et al.*, 1985). These lesions are consistently seen from the third day of illness and are commonly seen in the medulla oblongata, substantia nigra, thalamus, molecular layer of cerebellum and cerebral cortex. During the first few days of infection the brain shows a severe degree of vascular congestion and

oedema. In patients who die rapidly from JE, there may be no histological sign of inflammation.

3.1.2 Rarefaction necrosis

The second most common characteristic microscopic finding of JE is the focal, discrete or confluent, small, round to oval areas of acellular necrosis described using a range of terms – rarefaction necrosis, 'punched-out' lesions (Desai *et al.*, 1995; Johnson *et al.,* 1985). Many of these are seen in abundance in the cerebral gray matter, thalamus, midbrain, pons, corpus striatum and medulla oblongata. These lesions are well circumscribed, pale, rarefied zones in the neuropil, usually around a capillary and are predominant after 2 weeks of illness.

3.1.3 Inflammatory cells

Several lines of investigation suggest that initial induction of the virus-specific T and B cell immune response is generated outside the CNS in peripheral lymphoid tissues. Activated cells leave primary lymphoid organs and travel through the blood to infiltrate CNS. Mononuclear inflammatory cells typically appear in three compartments: the CSF, the leptomeninges and the parenchyma of the brain and spinal cord. The types of cells

making up the mononuclear inflammatory response have been characterized for JEV infection in humans (Johnson *et al.*, 1985). The initial phases of inflammation are detectable at all sites 3-4 days after infection. The numbers of cells reach a maximum first in the CSF at 5-7 days. In the brain parenchyma, activated cells come from the blood and cross the cerebral vascular endothelium and accumulate in the perivascular space mostly T cells, B cells and macrophages. Sensitised T helper cells are presumably the first inflammatory cells to enter the brain, and their release of cytokines attracts macrophages (Johnson, 1971). Microglia and macrophages are thought to play an important role in the immune response in viral encephalitis (Erisi *et al.*, 1995).

Because of the inability to directly interact with infected neurons, T cells in the encephalitic brain are likely to influence the immune response and to produce their local effects by production of cytokines (Griffin, 1995). For many infections, CD8+ cytotoxic T cells (CTL) appear to be the primary mechanism for eliminating infected cells from the tissue site of replication. CTL recognize virus-infected target cells through presentation of virus peptides bound to major histocompatibility complex (MHC) molecules on the surface of the infected cell.

B cells comprised 10% of the cells in the perivascular space, but not outside the perivascular regions (Johnson *et al.*, 1985). Although B cell entry into the inflammatory response is generally slower, it is well documented that B cells produce antibodies against viral CNS infection and clear virus from the brain (Griffin, 2003; Tschen *et al.*,2006). .

In animal models of JE, the cellular immune response appears to contribute to the prevention of disease during acute infection by restricting virus replication before the CNS is invaded. Athymic nude mice have increased susceptibility to experimental infection with JEV; transfer of spleen cells from mice immunized with live attenuated virus conveys immunity to infection (Jia & Huang, 1983). Spider monkeys, which are normally unaffected by intracerebrally inoculated JEV develop rapidly progressive encephalitis when T cell function has been impaired by cyclophosphamide (Nathanson & Cole, 1970). By analogy with other human viral infections, including influenza, HIV, Epstein-Barr virus and dengue, CTL might be important in the control and possibly clearance of JEV (Bukowski *et al.*, 1989; McMichael, 1994). However, recent data from

a mouse model suggested that CTL might be more pathogenic in JE infection (Larena *et al.*, 2011).

3.1.4 JEV Target Cells

In human autopsy studies of JE fatalities many antigen bearing neurons are found to be relatively well preserved, the microglial cells forming satellites. In the acute phase a high density of JEV is seen in the neurons of hippocampus, thalamus, substantia nigra, inferior olivary nucleus, hypoglossal and vagal nuclei, while microglia are devoid of antigen (Johnson *et al.*, 1985; Desai *et al.*, 1995). In the later stages, with active neuronophagia, the viral antigen is largely confined to macrophages in microglial shrubs and perivascular zones. Very rarely JEV can be found in astrocytes, ependymal cell, or capillary endothelial cell (Johnson *et al.*, 1985; Desai *et al.*, 1995). JEV antigen was not found in patients who died early in disease and for those who survived longer there was progressive clearance and was seen only in phagocytic cells (Johnson *et al.*, 1985). The infection and destruction of neurons in brainstem can explain readily the profound coma and respiratory failure.

3.2 Materials and Methods

Following routine paraffin wax embedding, 3-5 µm sections were prepared and stained with haematoxylin-eosin (H-E), inflammatory cell markers and JE antibody as specified in Table 2.2. With the exception of H-E, HIER was a requirement for all markers. Optimal conditions for antigen retrieval and antibody dilutions (Table 2.2) were independently determined by comparative titration experiments. The human CD8+ marker did not work well with macaque tissue sections. Double immunostaining of JEV with glial fibrillary acidic protein (GFAP), apoptosis cell markers and pro-inflammatory markers; CD68 with pro-inflammatory markers, was performed as described in Chapter 2 (Table 2.3).

3.3 Results

3.3.1 Histopathology

All JEV-infected animals exhibited mild to moderate, multifocal to diffuse, non suppurative meningoencephalomyelitis with evidence of neuronal degeneration and death. The inflammatory changes in the macaques were localized largely to gray matter. The inflammatory response was dominated by perivascular cuffs comprised of lymphocytes and macrophages (Figure 3.1A) and meningeal infiltrates predominantly lymphocytes. These were

accompanied by morphological evidence of endothelial cell activation with occasional tombstone-like morphology (Kipar *et al.*, 2005) (Figure 3.1B) and/or vascular damage. The latter was indicated by perivascular haemorrhage and substantial leakage of serum into the parenchyma, as demonstrated by staining for von Willebrand factor (Figure 3.1C). Neuronal cell death was indicated by morphological neuronal changes suggestive of apoptosis, in association with satellitosis or microglial nodules (Figure 3.1D, E).

Reactive astrogliosis, represented by multifocal increase in astrocyte numbers (Figure 3.1F) and evidence of astrocyte activation (presence of gemistocytes) in areas with inflammatory infiltrates was also identified.

3.3.2 Inflammatory cells

The inflammatory infiltrates were widespread throughout most sections. T cells (CD3+) were the predominant leukocytes in both perivascular and meningeal infiltrates. They were also present in small numbers in the adjacent parenchyma (Figure 3.2A). B cells (CD20+) were sparse and primarily seen in the perivascular infiltrates (Figure 3.2B), while moderate numbers of macrophage/microglial cells (CD68+) were identified in

perivascular and meningeal infiltrates and the adjacent brain parenchyma (Figure 3.2C). With the CD68 reaction, positive plump-bodied cells appeared to be macrophages whereas ramified process-bearing small cells were in the form of microglial cells (Esiri *et al.*, 1995). Staining for myeloid/histiocyte antigen, reported to stain macrophages (Esiri & Morris, 1991) and microglial cells (Foster *et al*, 2006), identified a substantial number of cells with a morphological appearance of macrophages (Figure 3.2D), suggesting their recruitment into the tissue (Otani *et al.,* 1999). Staining for CD68 and MHC class II antigen (expressed mainly by activated microglial cells) confirmed the presence of microglial nodules but also demonstrated activation of microglial cells (presence of both reactive and amoeboid microglial cells; Figure 3.2C,E) (González-Scarano & Baltuch, 1999). Furthermore, endothelial cells were shown to express MHC II, suggesting their activation (Figure 3.2E inset; Washington *et al.*, 1994). The cells surrounding degenerating/dying neurons in satellitosis appeared to be CD68-positive microglial cells (Figure 3.2F). For comparison, in brain areas without evidence of viral antigen and inflammation (cerebral cortex), only scattered MHCII-positive microglial cells without morphological features of activation were seen. There was no evidence of inflammatory cells or microglial MHC II expression in control brains (Figure

Appendix1.1). Semi-quantitative analysis of inflammatory cells was summarized in Table 3.1.

3.3.3 JEV Target Cells

JEV antigen expression, seen as finely granular cytoplasmic staining, was observed in numerous neuronal cell bodies and processes disseminated in the thalamic and brain stem nuclei of all animals and in neuronal cell processes throughout the affected parenchyma (Figure 3.3A). Most infected neurons appeared morphologically unaltered (Figure 3.3A inset), but some were surrounded by microglial cells (satellitosis) and exhibited degenerative changes (Figure 3.3B). JEV-positive microglial cells were found in some microglial nodules, but occasionally also as individual cells in affected areas, as confirmed by sequential staining for CD68 and JEV antigen (Figure 3.3C). In contrast, there was no evidence of JEV infection of astrocytes (Figure 3.3D). In one animal with a particularly strong inflammatory response (animal 2), a small percentage of slender perivascular cells (perivascular macrophages) did also express viral antigen (Figure 3.3E). There was no evidence of JEV antigen in endothelial cells in any animal. Nor was there any association between intensity of viral infection and degree of inflammatory response. Negative control brain

sections did not show any positive reaction. Semi-quantitative analysis of JEV antigen in the brain of monkeys following JEV infection was summarized in Table 3.1.

3.4 Discussion

My study has confirmed that as in humans, JEV induces a non-suppurative meningoencephalitis with neuronal cell death, microgliosis and astrogliosis (Akhter, 1999; Iwasaki *et al.,* 1986; Johnson *et al.,* 1985; Miyake, 1964); these are also common findings in other viral encephalitides (Leyssen *et al.,* 2003).

However in my study the 'punched-out' areas as a result of focal acellular necrosis, which are often seen in fatal human JE cases (Desai *et al.,* 1995; Johnson *et al.,* 1985) were not observed in the infected monkeys. It is possible that these changes had not yet developed in the macaques at the time of euthanasia, which was soon after onset of coma; in contrast observations in humans are always on post mortem material which is at the end of the disease process (Desai *et al.*, 1995; Johnson *et al.,* 1985).

My results are in keeping with findings in human material (Johnson *et al.*, 1985), where the inflammatory response was dominated by perivascular mononuclear cuffs with less intense infiltrates in the adjacent parenchyma. While T cells dominated in the perivascular infiltrates and macrophage/microglial cells were the largest population in the parenchymal infiltrates, B cells represented a minority and were restricted to the perivascular cuffs.

3.4.1 Role of macrophages in Japanese encephalitis

In other flaviviral infections, such as West Nile Virus (WNV), macrophages could serve as a reservoir, spreading the virus from the peripheral areas to the CNS (Samuel &Diamond, 2006). In viral encephalitis, macrophages are known to migrate from the perivascular space into the surrounding parenchyma where they become activated (Booss & Esiri, 2003). HIV-infected macrophages are shown to secrete MMP (Webster & Crowe, 2006). JEV-infected macrophages that may infiltrate into the CNS due to chemoattraction are reported to release various inflammatory mediators *in vitro* that may contribute to tissue damage (Nazmi *et al.*, 2011) though their contribution to the inflammatory process *in vivo* is yet to be elucidated. In addition to microglia, known to cause neuronal death in JE (D*aset al.*,

2008; Ghoshal *et al.,* 2007), the relative contribution of peripheral macrophages that migrate into the CNS in JE should be studied.

3.4.2 Role of T cells in Japanese encephalitis

T cells are known to play a key role in CNS infections by destroying virus-infected cells, producing cytokines, increasing phagocytic activity of macrophages, and stimulating the local production of antibodies by B cells (Binder & Griffin, 2001; Chambers & Diamond 2003; Bergmann *et al.*, 2006). CTLs have been reported to play a key role in mouse models of JE reflecting the severity of neuroinflammation (Fujii *et al.,* 2008; Larena *et al.*, 2011), but it remains to be clarified whether they are beneficial (Murali-Krishma *et al.,* 1994 and 1996) or deleterious (Hase *et al.,* 1990; Larena *et al.*, 2011). No association could be found between T-cell proliferation and either the antibody response or the clinical outcome (Burke *et al.*, 1985). In the present study, it was not possible to assess the role of CTLs, due to the lack of antibodies suitable for macaques and better understanding of their role in protection is needed.

3.4.3 Role of B cells in Japanese encephalitis

It is well documented that B cells and antibodies protect against viral CNS infection and contribute to clearing virus from the brain (Griffin, 2003; Tschen *et al.*,2006). Studies of post-mortem brain tissue from fatal human cases of JE (Johnson *et al.*, 1985) reported that infiltrating B cells remained within the perivascular spaces. However in my study it was clearly demonstrated that B cells were able to leave the perivascular compartment and invade the CNS parenchyma. Base on this finding, it is reasonable to suggest that both perivascular and parenchymal localization of B cells is important for virus clearance from CNS. The former might be more relevant within the perivascular spaces where B and T cells most often encounter each other to establish immunological synapses (Barcia *et al.*, 2008).

3.4.4 Target of JEV in CNS

3.4.4.1 JEV in neurons

My study confirmed neurons as the main targets of JEV, as previously shown in fatal human cases (Desai *et al.,* 1995; German *et al.,* 2006; Johnson *et al.,* 1985). There was diffuse distribution of JEV in neurons with greatest involvement of the thalamus and brainstem. The spread of the

antigen along the axons and dendrites was conspicuous. The microglial reaction in the vicinity of the JEV infected neurons was not reported in some human studies (Desai *et al.*, 1995). The rostral preponderance of JEV antigen in human studies suggest invasion of CNS from the blood (Johnson *et al.*, 1985). The presence of JEV antigen in the long dendritic processes and the axons, suggest a transcellular spread of virus to distant but functionally related neurons. The intraneuonal antigen localisation and the absence of neuropil staining, suggests that an extracellular spread of JEV to distant areas may not occur.

In contrary to some human studies (Johnson *et al.*, 1985) the viral antigen did not associate well with the inflammatory changes. Since the animals were euthanized relatively early in the course of infection, phagocytosis of infected neurons was rarely seen unlike in some human studies.

3.3.4.2 JEV in microglial cells

In addition to neurons I also examined and demonstrated viral antigen in microglial cells, mainly within microglial nodules surrounding infected neurons which suggests virus uptake by phagocytosis as microglia have a strong antigen presenting function (Fazakerley & Walker, 2003). JEV has

rarely been reported previously in microglial cells of animal or post-mortem studies. However, it cannot be excluded that microglial cells become productively infected, since they do support viral replication *in vitro* (Chen *et al.*, 2010; Thongtan *et al.,* 2010).

3.3.4.3 JEV in perivascular macrophages

Interestingly, I was able to detect JEV antigen in perivascular macrophages in one animal (Figure 4.2). These cells at the interface between blood and brain parenchyma are resident macrophages with high phagocytic activity, MHC-II expression (Kida *et al.,* 1993), CD 68+ expression, and CD163+ expression (Borda *et al.*, 2008) (Figure 3.5) which suggests that they had phagocytosed virus that entered the brain via the blood. Perivascular macrophages are considered to be different from pericytes, microglia, and peripheral macrophages and thought to play a major role as scavengers in the perivascular spaces (Kida *et al.*, 1993). Further studies are necessary to confirm the functional activity of the perivascular macrophages in JE encephalitis.

3.3.4.4 JEV in other CNS cells

In my study viral antigen was not detected in other glial cells, despite the *in vitro* evidence that at least astrocytes can become infected (Chen *et al.*, 2010). There was also no evidence of endothelial cell infection unlike human infection (Desai *et al.*, 1995; Johnson *et al.*, 1985).

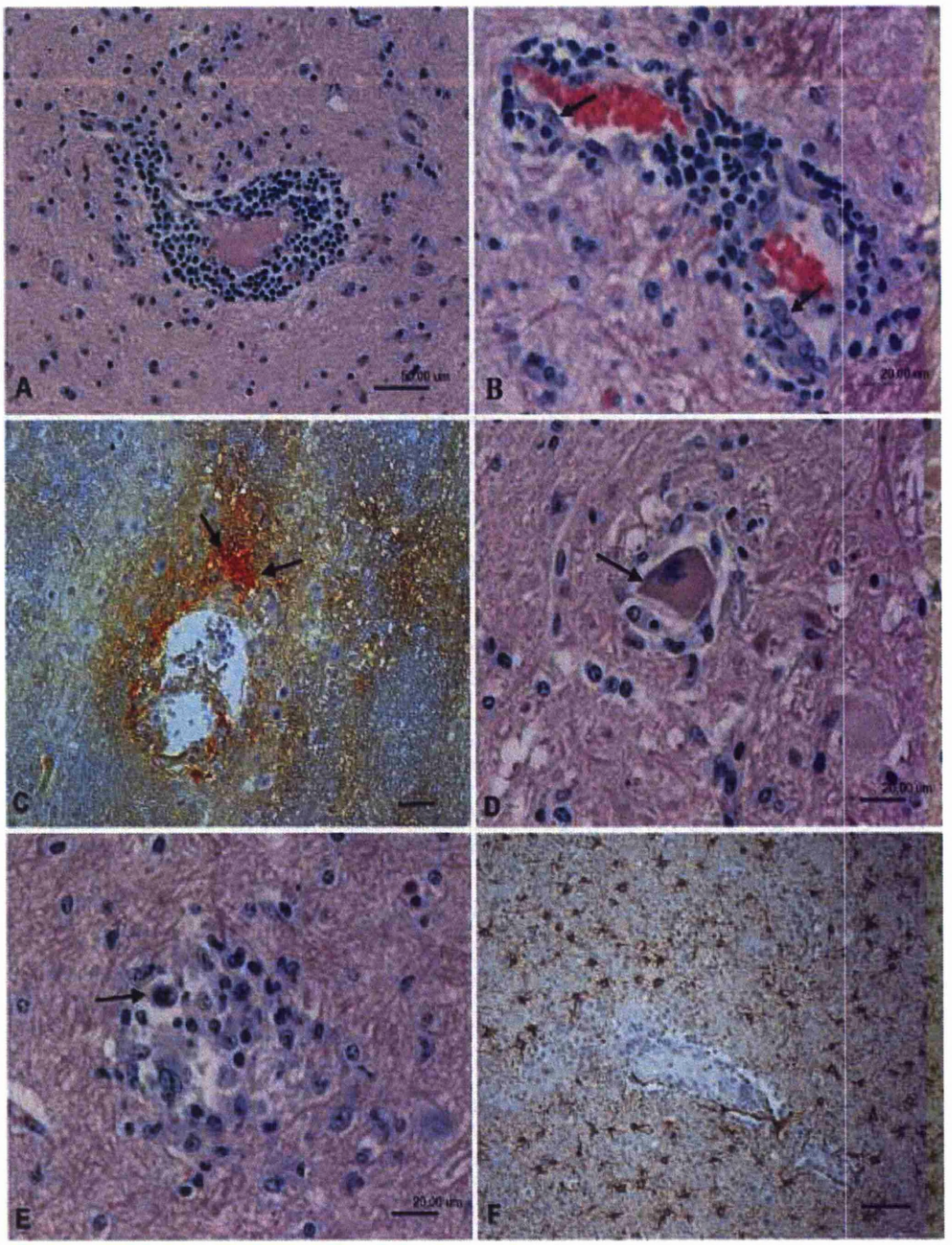
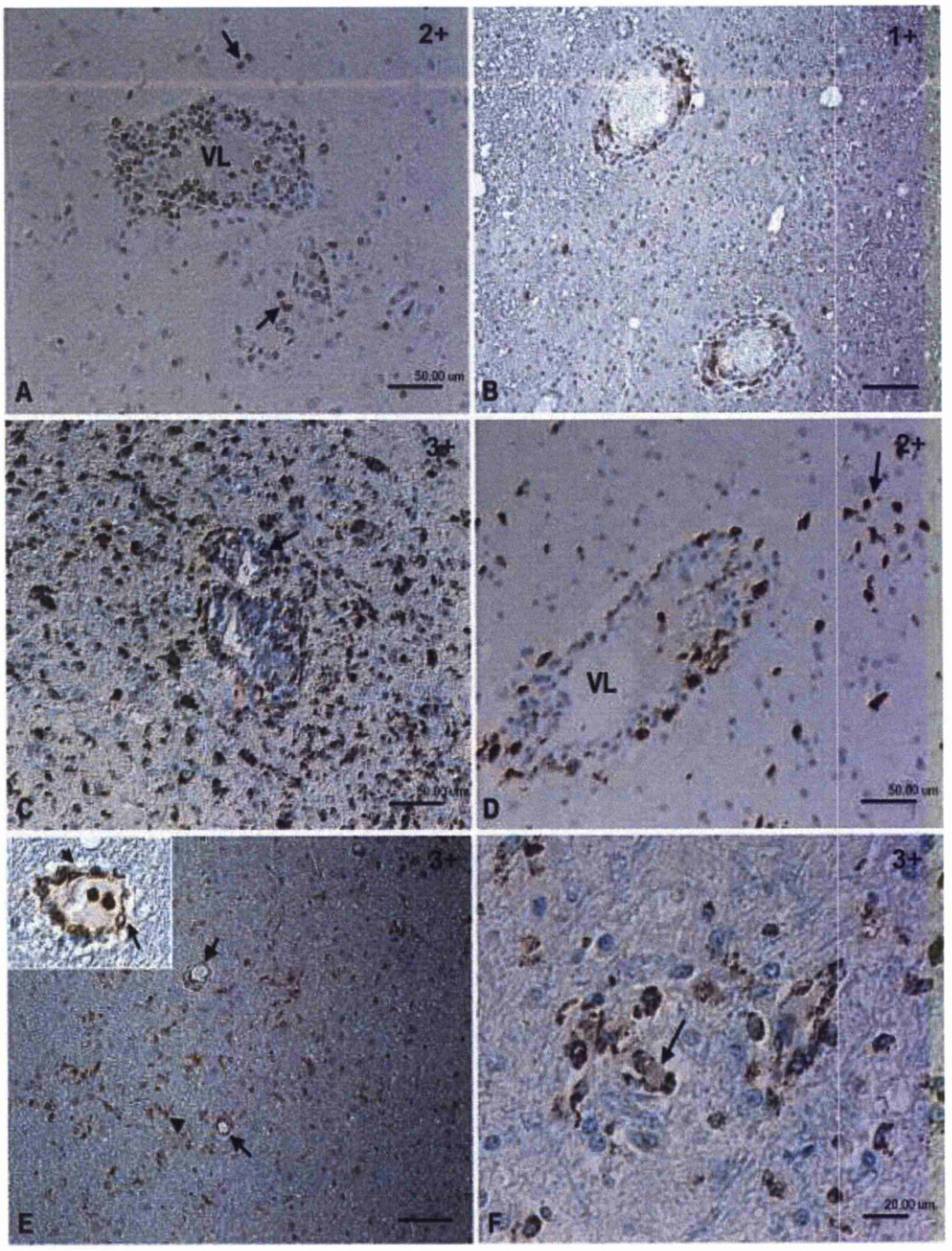

A
B
C
D
E
F

Figure 3.1 Representative histopathological changes in the thalamus of a rhesus macaque (No. 2) after intranasal inoculation with Japanese encephalitis virus (JEV). (A) Non-suppurative encephalitis, represented by moderate, lymphocyte-dominated perivascular infiltration. (B) Small vein with moderate perivascular infiltration and activated endothelial cells with tombstone-like morphology (arrow). (C) The presence of serum, indicated by staining for von Willebrand factor, in the parenchyma surrounding vessels with perivascular infiltrates (arrows) indicates marked vessel leakage. (D) Degenerating neuron (arrow) surrounded by microglial cells (satellitosis). (E) Microglial nodule with occasional apoptotic cells (black arrow). (F) Staining for GFAP highlights the presence of large numbers of activated astrocytes (astrogliosis). A, B, D, E: Haematoxylin-eosin stain. C, F: Indirect peroxidase method, NovaRed (C), DAB (F), haematoxylin counterstain. Scale bars: A, C, F = 50 µm; B, D, E = 20 µm.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B
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C
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E
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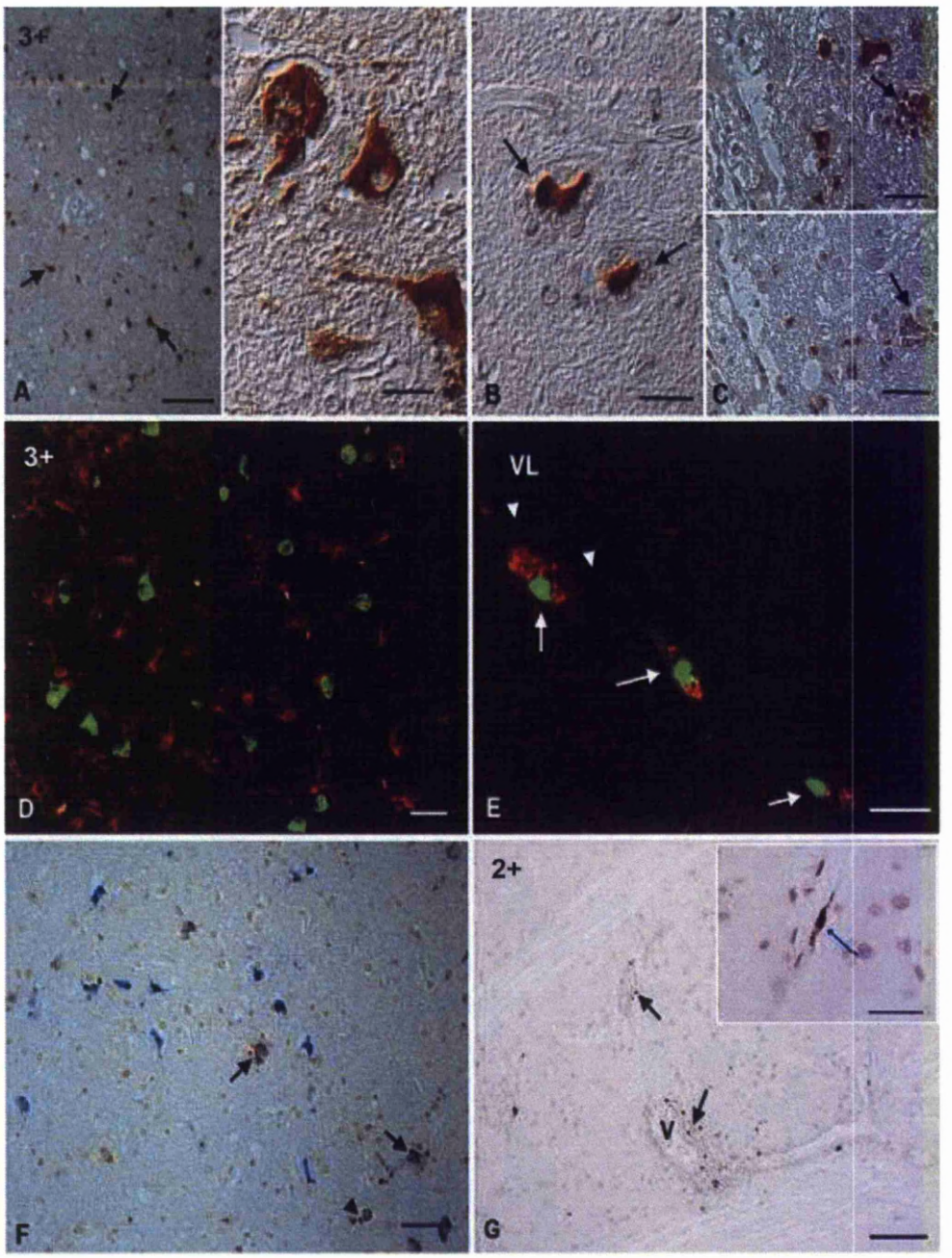
Figure 3.2 Characterization of the inflammatory response in the thalamus of rhesus macaques after intranasal inoculation with JEV (No. 2 (A, B, E) and No. 9 (C, D, F). (A) CD3+ T cells dominate the perivascular infiltrates and are present in smaller numbers in the adjacent parenchyma (arrows). VL: vessel lumen. (B) CD20+ B cells represent a minority in the perivascular infiltrates. (C) Staining for CD68 identifies moderate numbers of macrophage/microglial cells within and surrounding the perivascular infiltrates (arrows) and in the adjacent parenchyma. (D) Some positive cells in the perivascular infiltrates and the adjacent parenchyma (arrow) with the appearance of macrophages also express the myeloid/histiocyte antigen which suggested that they have recently been recruited from the blood. VL: vessel lumen. (E) Activated microglial cells also express major histocompatibility complex (MHC) class II antigen (arrowheads). MHC II is also expressed by vascular endothelial cells (inset, arrows), confirming their activation. (F) Microglial nodule with central degenerate neuron (arrow), surrounded by CD68-positive microglial cells. Indirect peroxidise method, DAB, Papanicolaou's haematoxylin counterstain. Scale bars: A-E = 50 µm; F = 20 µm.

(Scores for each marker recorded on upper right corner)


3+
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G

Figure 3.3 Demonstration of JEV target cells in the thalamus of rhesus macaques after intranasal inoculation with JEV [No. 7 (A, B), No. 2 (C-G)]. (A) JEV antigen is seen in the majority of neurons (left: arrows). Right: Infected unaltered neurons express viral antigen in both cell body and cell processes. (B) JEV-infected neurons that are surrounded by microglial cells in satellitosis appear shrunken (arrows). (C) Microglial cells in particular in microglial nodules can be JEV-infected (top; arrow) and are identified based on their CD68 expression (bottom; arrow), as demonstrated in a consecutive section. (D) Dual staining for JEV antigen (FITC) and GFAP (Texas red) indicates that JEV does not infect astrocytes. (E) While endothelial cells (arrowheads) were not found to be JEV infected, perivascular macrophages in one animal were found to express JEV antigen (Texas Red); these cells were also undergoing apoptosis, since they were TUNEL-positive (FITC) (arrows). VL: vessel lumen. (F) Dual staining for JEV antigen (Vector Blue) and TUNEL (DAB) shows both the degenerating neurons and surrounding microglial cells in satellitosis undergo apoptosis (arrows). JEV-infected, apoptotic microglial cells (arrowhead) are also observed. (G) Occasional TUNEL-positive, apoptotic lymphocytes (arrows) are present in the perivascular infiltrates. Rare endothelial cells (arrow) were found to be undergoing apoptosis (inset). V: vessel. Indirect peroxidase method (A-E, G), Vectastain® Elite ABC-Alkaline Phosphatase Kit (F). DAB (A-G), *BCIP*/NBT blue (F), Papanicolaou's haematoxylin counterstain. Scale bars: A (left) = 100 µm; A (right), C = 25 µm; B, E = 20 µm; D, F, G = 50 µm.

(Scores for each marker recorded on upper left corner)

Table 3.1 Semi-quantitative analysis of Japanese encephalitis virus antigen and inflammatory cells in the brain of monkeys following Japanese encephalitis virus infection

Monkey No.	Tissue	JEV antigen	PHENOTYPING			
			CD3	CD20	CD68	MAC387
1	B	2+	2+	2+	3+	3+
	T	3+	2+	3+	2+	3+
2	B	2+	2+	1+	3+	3+
	T	3+	3+	1+	3+	3+
3	B	1+	3+	±	3+	2+
	T	1+	2+	±	3+	2+
4	B	1+	3+	1+	3+	3+
	T	-	2+	1+	3+	1+
5	B	2+	2+	1+	3+	2+
	T	2+	3+	1+	3+	2+
6	B	2+	2+	1+	3+	2+
	T	2+	2+	1+	3+	2+
7	B	-	2+	1+	3+	3+
	T	2+	3+	2+	3+	3+
8	B	2+	2+	1+	3+	3+
	T	1+	2+	1+	3+	3+
9	B	1+	3+	1+	2+	2+
	T	-	2+	1+	3+	2+
10	B	3+	2+	1+	3+	3+
	T	3+	3+	1+	3+	3+
11	B	2+	2+	1+	3+	2+
	T	2+	2+	1+	3+	2+
12	B	1+	2+	1+	3+	1+
	T	3+	2+	1+	3+	1+
Mean		**2+**	**2+**	**1+**	**3+**	**2+**

Extent and intensity of the immunoreactivity was scored on paraffin sections of brainstem and thalamus and recorded as: 0 = no difference from the negative control with regard to intensity, distribution and number of positive cells, or no positive reaction, 1+ = occasional individual positive cells, 2+ = a few nests of positive cells, 3+ = frequent positive cells
JEV Ag = JEV antigen
CD3 = Pan T cell
CD20 = B cell
CD68 = macrophage/microglial cells
MAC 387 = anti human myeloid/histiocyte antigen (reactive macrophage/microglial cells)
B = Brain stem
T = Thalamus

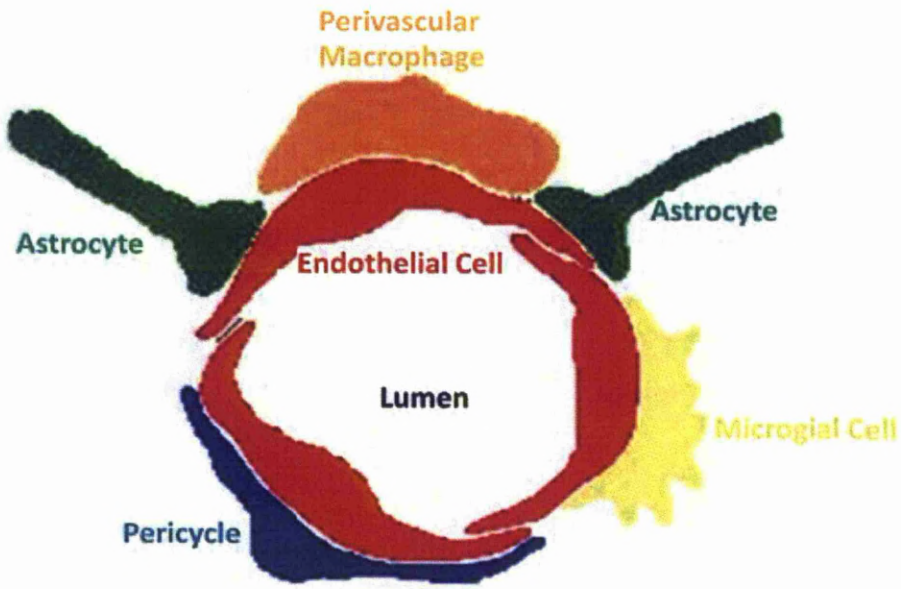

Figure 3.4 Perivascular macrophages

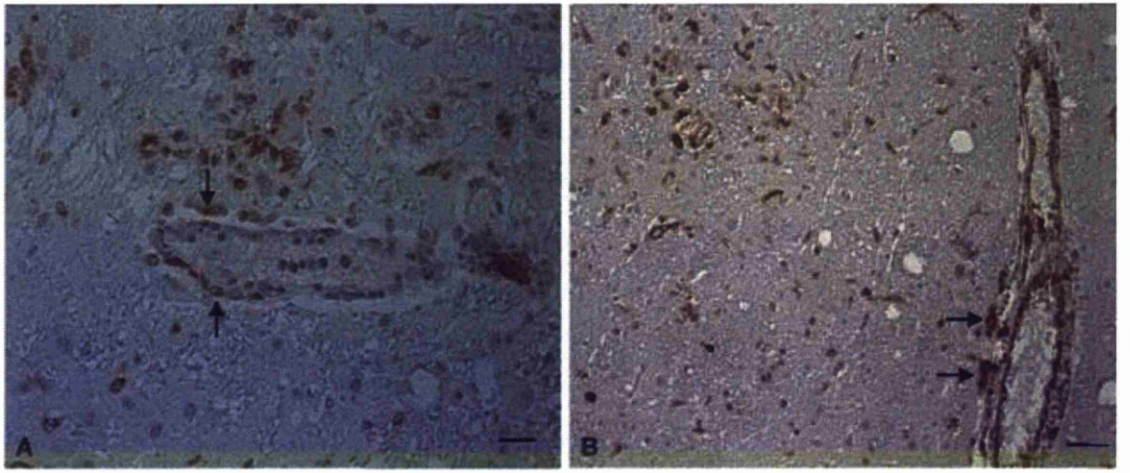

Figure 3.5 Demonstration of perivascular macrophages in the thalamus of a rhesus macaque (No. 2) after intranasal inoculation with JEV. (A) A cerebral vein with CD68+ perivascular macrophages (arrows). (B) Perivascular macrophages also express MHC class II antigen (arrows). Indirect peroxidase method, DAB, Papanicolaou's haematoxylin counterstain. Scale bars: A = 20 µm; B = 50 µm.

CHAPTER 4

APOPTOSIS

4.1 Introduction

This chapter aims to examine the mechanisms of neuronal damage and the apoptosis pathways in JE infection.

After an acute CNS injury early necrotic cell death is known to take place and is followed later by a delayed apoptotic death. Elucidating whether apoptosis contributes to neuronal cell death was one of the main aims of this thesis, therefore its study has provided for the majority of results and discussion.

4.1.1 Apoptotic cell death

Although it is well established that apoptotic mechanisms pay a central role in neuronal death following CNS damage, little is known about the apoptotic pathways in Japanese encephalitis. Apoptosis or programmed cell death is a characteristic process of cell suicide that has distinctive morphological features, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Kerr *et al.*, 1972)

(Figure 4.1). The fragmented nucleus is packaged into vesicles, called apoptotic bodies, which may be phagocytosed.

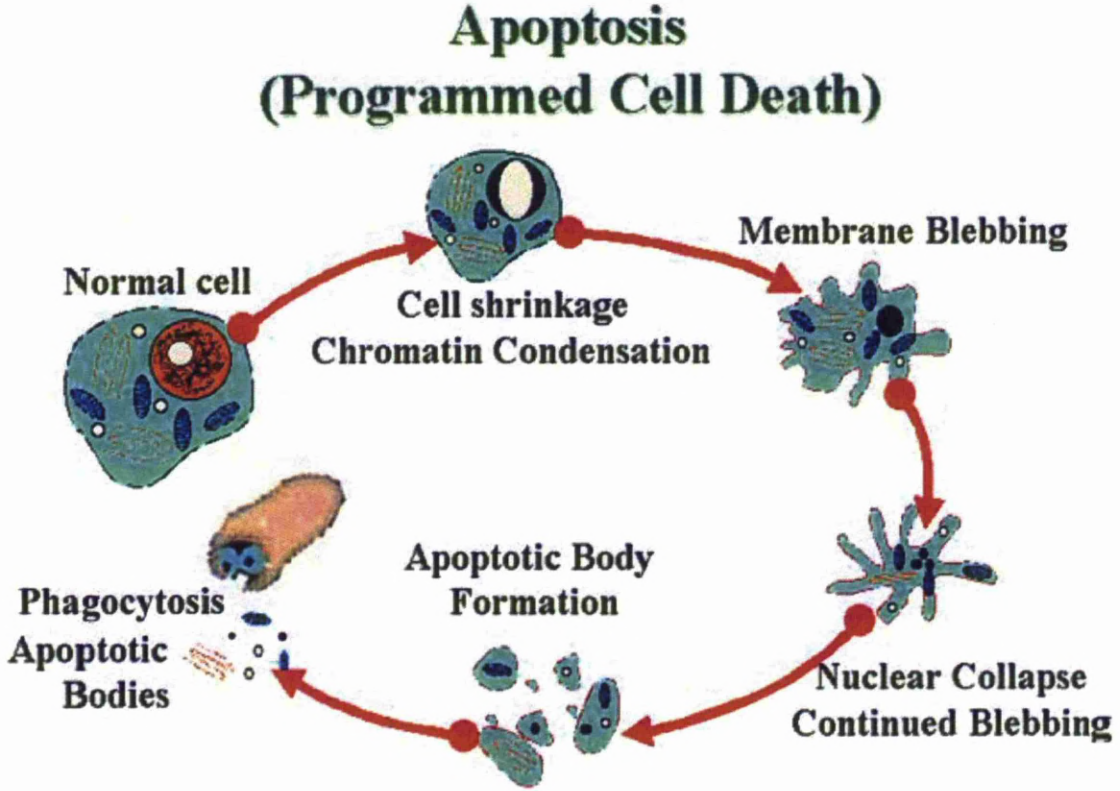

Figure 4.1 Apoptosis

4.1.2 Caspases

TUNEL assay based on DNA fragmentation is subject to problems of sensitivity, but evidence of caspase activation is considered a complementary method for demonstrating the presence of apoptotic features.

Caspases are proteases, which become activated in most forms of cell death. In the cell, caspases are localized in the nucleus, cytoplasm, endoplasmic reticulum, and mitochondrial intermembrane space, and they can be translocated to the plasma membrane. Caspases are categorized into initiator caspases (caspases8, 9, and 10) and effector or executioner caspases (caspases1, 2, 3, 4, 6, 7, 12, and 13) (Salvesen & Dixit, 1997). Caspases exist as latent precursors, which, when activated, initiate the death program by sequential activation. They are present in cells as zymogens and need to undergo proteolytic cleavage in order to achieve their enzymatic activity.

4.1.3 Caspase cascades: two pathways

Two main pathways of apoptosis signalling have been well documented in the mammalian cells; the "extrinsic" or death receptor pathway (Hirata *et*

al., 1998), and the "intrinsic" or mitochondrial pathway (Li *et al.*, 1997) (Figure 4.2).

4.1.3.1 Intrinsic Pathway

Mitochondria play an important role in the execution process of the apoptotic program by acting as a reservoir for a multitude of apoptogenic proteins. After release from the mitochondria, cytochrome c associates with the apoptosis protease activating factor – 1 (Apaf-1) triggering the subsequent recruitment and auto processing of inactive procaspase-9 to form the apoptosome complex, hence initiating downstream caspase activation (Costantini *et al.*, 2002). Activation of caspase-9 precedes the maturation and activation of effector caspase-3 (Figure 4.2).

4.1.3.2 Extrinsic Pathway

Several extrinsic ligands can activate "death receptors" of the tumour necrosis factor receptor (TNFR) family, including FAS/CD95 or TNFR-1. The FAS ligand (FASL/CD95L) is a member of the TNF family of cytokines and its activation of FAS leads to the recruitment of FAS-associated death domain (FADD) and promotes caspase 8 activation (Rytomaa *et al.*, 1999). This then leads to activation of effector caspases (Figure 4.2).

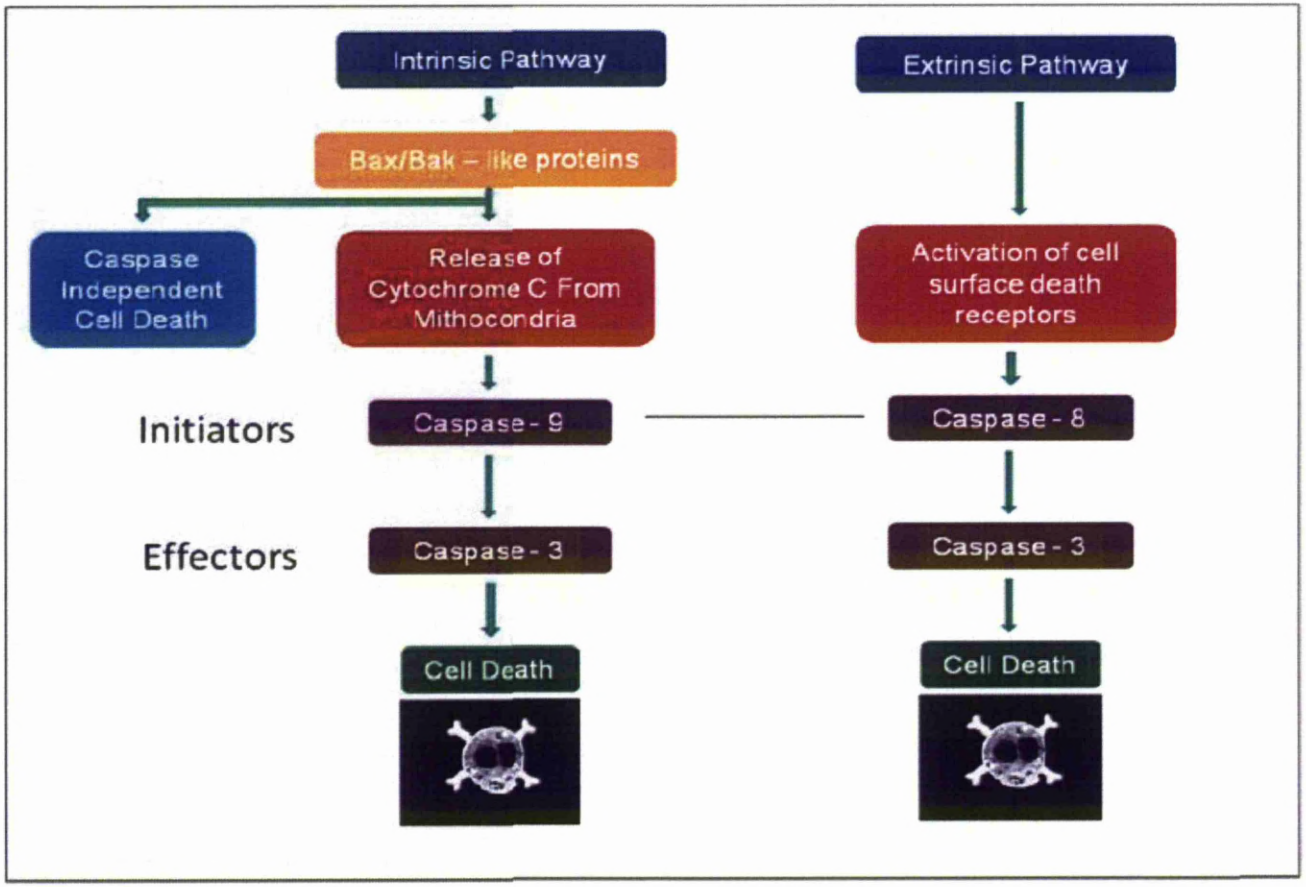

Figure 4.2 Apoptosis pathway

4.1.4 Main executor: Caspase 3

Caspase-3 is a principal downstream effector of the cascade in charge of the execution of apoptotic cell death in both intrinsic and extrinsic signalling pathways (Stennicke & Salvesen, 2000). It appears to be the most abundant of the caspases in the brain and the convergence of all caspase-mediated pathways related to apoptosis. Caspase 3 is activated in several neurodegenerative disorders (Namura *et al.,* 1998, Hartmann *et al.,* 2000, Su *et al.,* 2000). Caspase-3 dependent neuronal damage is also reported in WNV encephalitis (Samuel *et al.*, 2007).

4.1.5 Apoptotic Proteins

The apoptosis-regulating proteins play an important role in the regulation of apoptosis (Cory *et al.*, 2003). Apoptotic factors activated after an insult are usually accompanied by over expression of molecules aimed to inhibit them: the anti- apoptotic proteins. The balance between pro-apoptotic and anti- apoptotic groups will finally determine if the cell will die or survive. The anti- apoptotic group includes Bcl-2, Bcl-xs, Bcl-w and the pro-apoptotic group includes Bax, Bak, Bok, and Bcl-xl. The apoptosis-inducing factor (AIF), a mitochondrial intermembrane flavoprotein with pro-apoptotic effects, involved in a caspase-independent pathway (Joza *et al.*, 2001),

was not studied for this thesis (Figure 4.2).

4.2 Materials and Methods

Following routine paraffin wax embedding, 3-5 µm sections were prepared and stained with apoptosis markers as specified in Table 2.2. HIER was a requirement for all markers. Optimal conditions for antigen retrieval and antibody dilutions (Table 2.2) were independently determined by comparative titration experiments. Apoptotic cells were identified by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end *in situ* labelling (TUNEL) method using the Apoptag *In Situ* Apoptosis Detection kit (Chemicon Inc., Millipore, Billerica, USA) to demonstrate the characteristic DNA changes (Gelbard *et al.,* 1995; Kerr *et al.,* 1972). Double immunostaining of apoptosis markers and JEV was performed as described in Chapter 2 (Table 2.3). Apoptosis related proteins TNFR-1, FAS and FASL did not work well with macaque tissue sections.

4.3 Results

4.3.1 TUNEL

In this study I characterized the type of cells undergoing apoptosis by

TUNEL technique, a marker of DNA damage, which identified apoptotic JEV-infected neurons in microglial nodules and satellitosis as well as apoptotic microglial cells disseminated in the parenchyma, in satellitosis and in microglial nodules (Figure 3.3F). Occasional lymphocytes in the perivascular infiltrates were also apoptotic (Figure 3.3G) and the JEV-infected perivascular macrophages observed in animal 2 did also undergo apoptosis (Figure 3.3E). Very rarely, an apoptotic endothelial cell was seen (Figure 3.3G, inset). Some apoptotic neurons were JEV infected. The extent of apoptotic neurons did not correlate with the presence of JEV.

4.3.2 Caspase activation in JE

Staining for key apoptosis molecules, such as caspases-8, -9 (both initiator caspases) and cleaved caspase-3 (an executor caspase) was undertaken to identify cells undergoing apoptosis that did not yet show the representative morphological features. Among unaltered appearing neurons, cleaved caspase-3 expression was seen in small numbers (mean score = 1+) compared to caspase-8 (mean score = 3+). Both caspases were also expressed by some leukocytes (macrophages and T cells) in the perivascular infiltrates (Figure 4.3A, B). Caspase-3 staining was also seen in nucleus and axonal prolongations. In contrast, caspase-9 activation, was

only detected in astrocytes and microglial cells (Fig. 4.3C). Double staining for JEV and the various caspase markers confirmed that some JEV-infected neurons were undergoing apoptosis (Figure 4.4A,B).

4.3.3 Apoptotic Proteins

In order to gain some understanding of the regulation of apoptotic processes in the relevant cells, the expression of representative pro- and anti-apoptotic proteins was assessed. While numerous microglial cells and occasional neurons stained positive for the pro-apoptotic protein Bax (mean score = 3+) (Figure 4.3D), the anti-apoptotic protein Bcl-2 was mainly expressed by lymphocytes in the perivascular infiltrates (mean score = 1+) (Figure 4.3E). Dual staining showed JEV antigen in some Bax-positive neurons (Figure 4.5A) and occasional Bax-positive microglial cells (Figure 4.5B).

In uninfected control brains TUNEL positive cells were not identified. Caspase and Bcl-2 staining was negligible; weak and infrequent Bax expression was seen in neurons (Figure Appendix1.2). Semi-quantitative analysis of apoptosis markers was summarized in Table 4.1.

4.4 Discussion

4.4.1 Neuronal apoptosis – intrinsic vs. extrinsic pathways

Viral infection and inflammatory response were associated with cytopathic changes, and, although not excessive, neuronal death via apoptosis was clearly observed. The latter was confirmed not only by the TUNEL assay which has been used in the past to demonstrate apoptosis but can provide controversial results in the presence of necrosis and autolytic changes (Grasl-Krauppet *al.,* 1995), but also by staining for cleaved caspase-3 and some apoptotic proteins (Figure 5.3). Apoptotic neurons were often surrounded by microglial cells (satellitosis and formation of microglial nodules) which indicated their impending phagocytosis (Fuller & Eldik, 2008). Some apoptotic neurons were JEV infected. In addition, some morphologically unaltered, infected neurons were shown to express the pro-apoptotic protein Bax, the initiator caspase-8 or the active effector caspase-3 (Yang *et al.,* 1998), which indicates that these cells were also doomed to undergo apoptosis. These results confirm the *in vivo* relevance of previous *in vitro*studies which demonstrated that JEV replication can lead to neuronal apoptotic death (Liao *et al.,* 1997; Raung *et al.,* 2001; Tsao *et al.,* 2008; Yang *et al,* 2009) and support findings in the mouse model that JEV replication contributes to Bax activation (Mishra & Basu,

2008). Taken together, all these findings provide evidence of a direct, although possibly not rapid cytopathic effect of JEV on neurons. The demonstration of caspase-8 in affected neurons also indicates that neuronal apoptosis is initiated by the FAS-mediated or extrinsic pathway (Hirata *et al.,* 1998), a mechanism that is central to the process of immune-mediated viral clearance (Clarke & Tyler, 2009) and seen in a number of CNS viral infections including WNV (Ramanathan *et al.*, 2006).

One of the main candidates of caspase-independent mediators of cell death is AIF (Joza *et al.*, 2001), which is released from the mitochondria and reaches the nucleus where it can directly induce DNA fragmentation. The results from my study emphasize the involvement of the extrinsic pathway over the intrinsic pathway in neuronal death in JE. However it should be mentioned that it might also coexist with other caspases or caspase-independent apoptotic pathways or with necrotic cell death.

Bystander neuronal cell death

Importantly, apoptotic cell death or pre-apoptotic caspase-3 and -8 expressionswere also seen in a proportion of JEV antigen-negative neurons, (Figure 5.4) which suggests some degree of bystander neuronal

death.

4.4.2 Glial cell apoptosis

Both *in vivo* and *in vitro* studies showed that glial cells are also sensitive to apoptotic stimuli. Neurons are described as the most vulnerable cell type to apoptotic damage, followed by oligodendrocytes, astrocytes, endothelial cells, and microglia (Li *et al.*, 1995; Petito *et al.*, 1998). In our study a proportion of microglial cells, often in close proximity to infected neurons but generally not JEV-infected, were shown to undergo apoptosis. Further, morphologically unaltered microglial cells expressed caspase-9 which suggests that microglial apoptosis is initiated by the mitochondria or the intrinsic pathway. While a recent *in vitro* study showed that JEV infection can lead to apoptosis of microglial cells (Thongtan *et al.*, 2010), our results indicate that *in vivo* this direct mechanism is probably less relevant and that pro-inflammatory factors are more relevant, as in other CNS conditions, such as experimental autoimmune encephalomyelitis (EAE) where microglial apoptosis is considered an important homeostatic mechanism to control microglial activation and proliferation (White *et al.*, 1998).

4.4.3 Inflammatory cell apoptosis

Apoptotic cell death was also observed in a proportion of infiltrating inflammatory cells (macrophages and T cells). Considering that these cells were not JEV-infected, this most likely represents a normal mechanism to eliminate activated leukocytes and thereby limit the inflammatory response in the CNS (Baeur *et al.,* 1998). On the other hand, perivascular leukocytes (predominantly T cells) were found to express the anti-apoptotic protein Bcl-2. This supports a murine *in vivo* study that provides evidence of a critical role of Bcl-2 in the survival of virus-specific CTLs (Grayson *et al.*, 2000).

In summary, I have shown in this study that neuronal death is contributed by both the cytopathic and bystander effect and that neurons undergo apoptosis mainly by the extrinsic pathway and microglial cells by intrinsic pathway. The role of apoptotic proteins like FAS, FAS-L, TNFR as well as caspase–independent pathways should be explored to guide the best strategies to limit neuronal damage in JE.

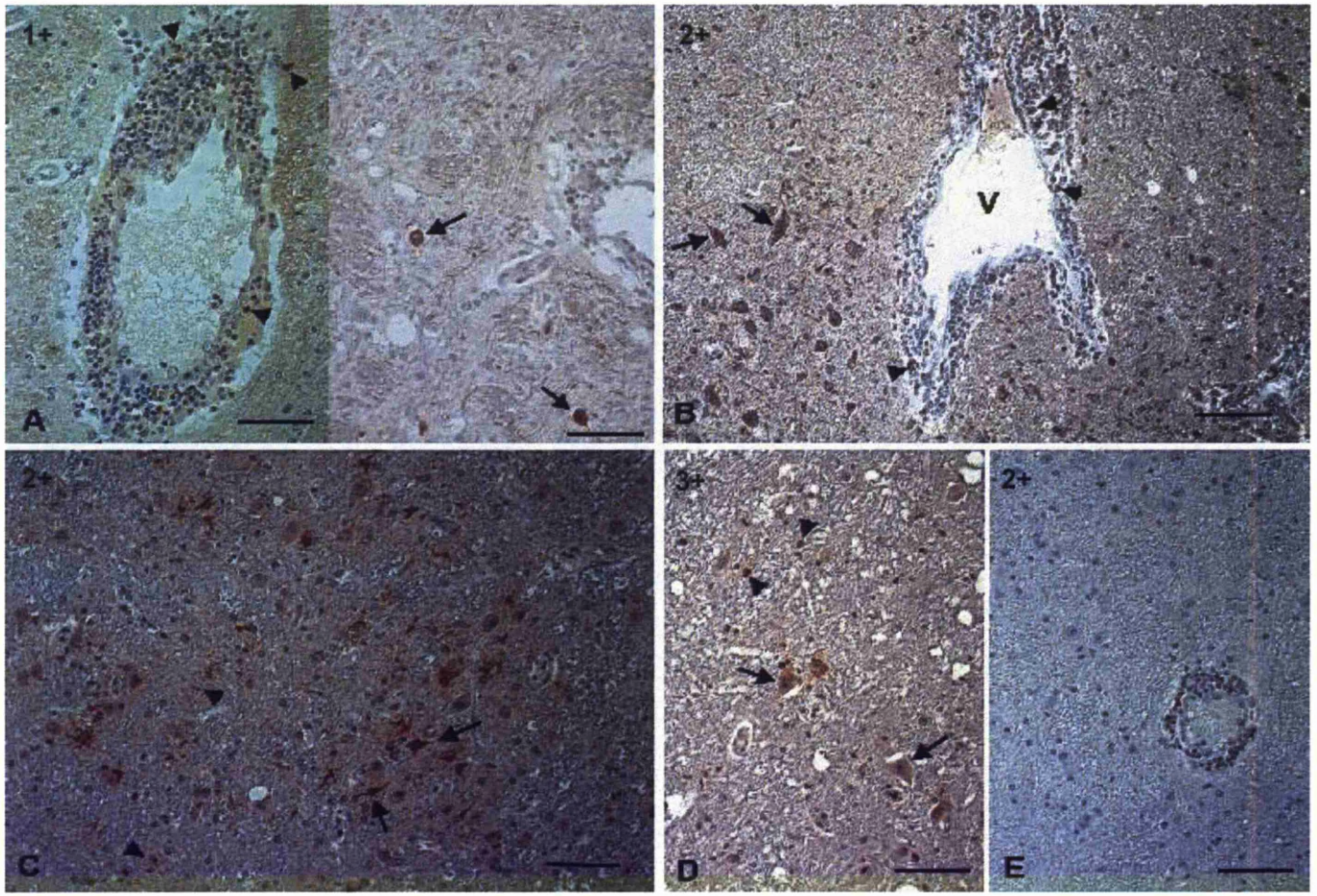
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Figure 4.3 Demonstration of apoptosis related proteins in the thalamus of rhesus macaques after intranasal inoculation with JEV (No. 2 (A, D, E), No. 9 (B), No. 11 (C) (A)Some macrophages and T cells in the perivascular infiltrates (left, arrowheads) and scattered unaltered appearing neurons (right; arrows) express cleaved caspase-3, an executor caspase. (B) The initiator caspase-8 is expressed by unaltered neurons (arrows) and some cells in the perivascular infiltrates (arrowheads). V: vessel. (C) Caspase-9, another initiator caspase, is expressed by microglial cells (arrowheads) and astrocytes (arrows). (D) Bax, a pro-apoptotic protein, is expressed by unaltered neurons (arrows) and microglial cells (arrowheads). (E) Bcl-2, an anti-apoptotic protein, is expressed by T cells in the perivascular infiltrates. Indirect peroxidise method, DAB, Papanicolaou's haematoxylin counterstain. Scale bars = 50 μm.

(Scores for each marker recorded on upper left corner)

Table 4.1 Semi-quantitative analysis of Japanese encephalitis antigen and apoptosis markers in the brain of monkeys following Japanese encephalitis virus infection

Monkey No.	Tissue	JEV antigen	APOPTOSIS					
			TUNEL	Cas-3	Cas-8	Cas-9	Bax	Bcl2
1	B	2+	2+	2+	2+	2+	3+	2+
	T	3+	2+	1+	3+	1+	3+	2+
2	B	2+	2+	1+	3+	2+	3+	2+
	T	3+	2+	1+	1+	1+	3+	2+
3	B	1+	2+	1+	1+	±	1+	-
	T	1+	1+	±	2+	±	±	-
4	B	1+	1+	-	2+	-	2+	-
	T	-	2+	1+	1+	1+	±	-
5	B	2+	1+	1+	2+	1+	3+	1+
	T	2+	2+	-	2+	1+	1+	-
6	B	2+	2+	1+	2+	1+	3+	-
	T	2+	2+	1+	1+	1+	1+	-
7	B	-	2+	1+	1+	±	2+	1+
	T	2+	2+	±	1+	±	-	1+
8	B	2+	1+	1+	2+	2+	3+	1+
	T	1+	2+	1+	2+	2+	2+	1+
9	B	1+	3+	1+	2+	2+	3+	1+
	T	-	1+	1+	-	1+	±	-
10	B	3+	2+	1+	3+	3+	3+	2+
	T	3+	3+	-	1+	2+	3+	-
11	B	2+	2+	1+	2+	2+	3+	2+
	T	2+	2+	1+	2+	±	2+	3+
12	B	1+	2+	2+	2+	-	2+	-
	T	3+	2+	1+	2+	+	2+	2+
Mean		**2+**	**2+**	**1+**	**2+**	**1+**	**2+**	**1+**

Immunohistochemistry of cell markers tested on paraffin sections of brainstem and thalamus and the level of expression recorded as:
0 = no expression observed above the negative control
1+ = occasional isolated positive cells
2+ = a few nests of positive cells
3+ = frequent positive cells in most brain areas
Cas-3 = Caspase - 3
Cas-8 = Caspase - 8
Cas-9 = Caspase - 9
Bax = Pro-apoptosis protein
Bcl2 = Anti-apoptosis protein

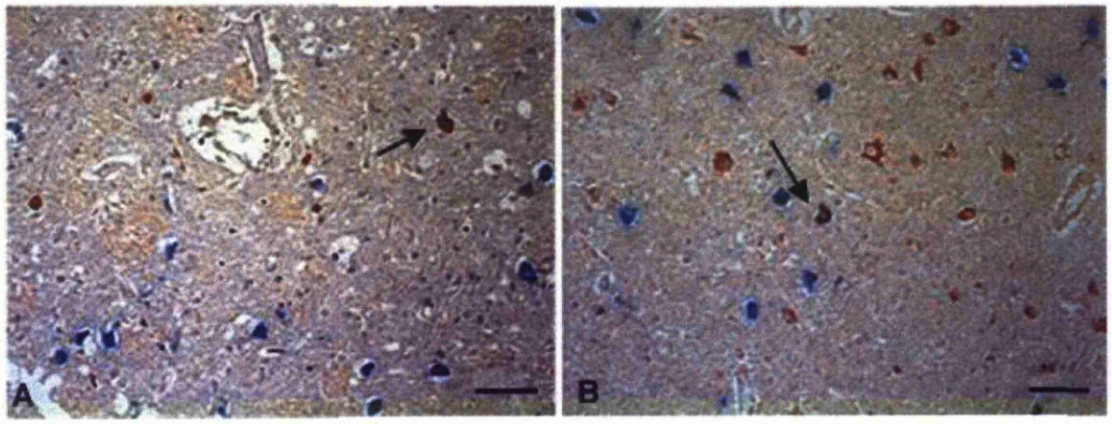

Figure 4.4 Characterization of apoptotic cells in the thalamus of a rhesus macaque (No. 2) after intranasal inoculation with Japanese encephalitis virus. JEV antigen (Vector Blue) and (A) caspase-3 and (B) caspase-8 show that most of the apoptotic neurons are bystander cells. JEV-infected, apoptotic neurons (arrows) are also observed. Vectastain® Elite ABC-Alkaline Phosphatase Kit/NBT blue and DAB. Scale bars = 50 μm.

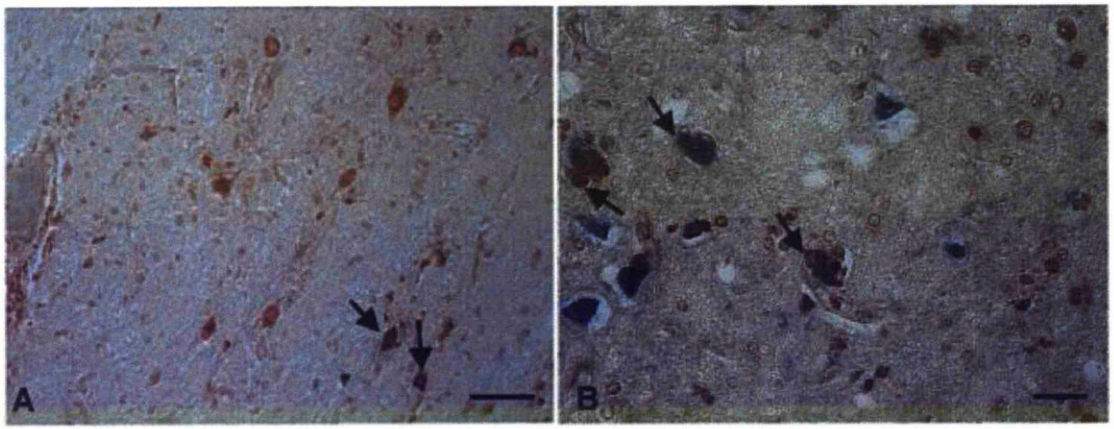

Figure 4.5 Characterization of Bax-positive cells in the thalamus of a rhesus macaque (No. 2) after intranasal inoculation with JEV. MMP-9 (DAB) and JEV antigen (Vector Blue) showing (A) Bax-positive JEV-infected neurons (arrows), (B) Bax-positive JEV-infected microglial cells as demonstrated in satellitosis and neuronophagia (arrows). Vectastain® Elite ABC-Alkaline Phosphatase Kit/NBT blue and DAB. Scale bars: A = 50 µm; B = 20 µm.

CHAPTER 5

PRO-INFLAMMATORY MEDIATORS, GLIAL AND ENDOTHELIAL CELL RESPONSE IN JE

5.1 Introduction

This chapter aims to examine the contribution of pro-inflammatory mediators and the involvement of non- neuronal cells in the pathogenesis of JEV infection.

Having characterized the inflammatory response and the patterns of cell death in JE, I aimed to identify relevant mediators of these processes within the CNS as well as the possible roles of glial and endothelial cells by immunohistology.

5.1.1 Nitric Oxide (NO) production in JE

Because of its biochemically promiscuous nature, NO has been suggested to contribute to a myriad of physiological and pathological processes. NO can act as both a 'mediator and murderer' (Anggard, 1994), acting to regulate a physiological process in one context but disrupt it in another. Like many inflammatory mediators, it is clear that NO is a double-edged

sword, mediating host defence in certain contexts but damaging the host in others. Relatively little is known about the role of NO in mediating inflammation in the pathophysiology of JE encephalitis. It is shown to irreversibly damage lipids and proteins in cells (Iadecola & Zhang, 1996), negatively regulate mammalian neurogenesis (Packer *et al.*, 2003), and enhance glutamate exictotoxicity (Hewett *et al.*, 1996). NO is known to be a potential mediator of neuronal injury and to mediate BBB damage both *in vivo* (Parathath *et al.*, 2006) as well as *in vitro* studies (Wong *et al.*, 2004). It is also implicated as a contributor to the host's innate defense against viral infection in those affecting the CNS. To assess local NO production in the infected CNS sections, I investigated the expression of both indicators of oxidative stress - iNOS and nitrotyrosine (NT) (MacMicking *et al.*, 1997; Jinno-Oue *et al.*, 2003), as not all human antibodies work well for monkey tissues.

5.1.2 Matrix metalloproteinases (MMP) in JE

MMPs are proteins known to degrade components of extracellular matrix (ECM) (Rosenberg *et al.*, 2002) and reported to be secreted by astrocytes, microglia and vascular endothelial cells (Lukes *et al.*, 1999). They are recognized as important immunoregulatory modulators during inflammatory response (Webster and Crowe, 2006). In spite of the accumulating

evidence of MMPs in the pathogenesis of several CNS diseases (Kieseier *et al.*, 1999) very little is known about the MMP expression *in vivo* and its significance in JE. I stained for MMP-2 and -9, which are known to cause BBB disruption by degrading collagen IV, its main component (Rosenberg, 1998). To the best of my knowledge this study represents the first study of MMP expression *in vivo* in JE infection and the significance of these findings is discussed.

5.1.3 Cytokines in JE

Cytokines play a major role in the orchestration of the local immune response by regulating adhesion, chemotaxis, activation and differentiation of cells. Several studies have shown that cytokines are produced by cells intrinsic to the CNS as part of the innate immune response to viral infection before the infiltration of inflammatory cells from the periphery (Griffin, 1997). In CNS infection the cytokines produced locally are likely to influence both the manifestations of disease and the outcome of infection (Owens *et al.*, 1994). IFN-α, a potent antiviral cytokine and microglial activator (Paul *et al.*, 2007.); and TNF-α which has been shown to directly activate microglia (Basu *et al.,* 2002) and induce neuronal apoptosis (Venters *et al.,* 1999) were implicated to play important roles in CNS infections. IFN-α does not seem to be strongly upregulated in acute

encephalitis but has been reported in the plasma and CSF in severe infection with a fatal outcome (Burke and Morrill, 1987). TNF-α is known as a key trigger of the inflammatory response within the CNS and reported to exert direct anti-viral effects *in vitro* (Ramsey et al., 1993). It is also shown to cause MMP-9 increase and subsequent BBB permeability in a murine model (Rosenberg, 1995). Although a number of pro-inflammatory cytokines were shown to be expressed in mouse *in vivo* studies (Gupta *et al.*, 2010; Yang *et al.*, 2011), the role of other cytokines were not investigated in this study due to restriction of antibodies available for monkey paraffin sections.

5.1.4 Involvement of non-neuronal cells

This chapter also summarizes the possible roles of glial and endothelial cells in JE that might be contributing to neuronal damage. The loss of BBB integrity in JE is discussed in detail in Chapter 6.

5.1.4.1 Microglial cells

Microglia, the resident immune cells of the CNS and widely distributed within brain parenchyma constitutes up to 12% of CNS cells, and has been implicated as critical cells in JE encephalitis in a mouse model (Ghoshal *et al.*, 2007). They are known as the predominant source of pro-inflammatory

mediators in CNS infections; the soluble effectors released from activated microglia like IL-1β, IL-3, IL-6, TNF-α, vascular endothelial growth factor (VEGF), lymphotoxin, macrophage inflammatory protein 1α (MIP-1α), MMPs, NO and reactive oxygen species (ROS) (Banati & Kreutzberg, 1993; Colton & Gilbert, 1987; Gehrmann *et al.*, 1995; Kielian, 2004; Smith *et al.*, 2012) are instrumental in inducing neuronal death (Chen *et al.*, 2010; Rock *et al.*, 2004). Activation of microglia induces changes in cellular morphology (from a ramified resting state to an amoeboid activated state) and in the expression of cell surface receptors that results in an appearance very similar to a tissue macrophage (Davis *et al.*, 1994).

5.1.4.2 Astrocytes

Astrocytes are the predominant glial cell population in the CNS and maintain homeostasis in the CNS to support the survival and information processing functions of neurons. They are also known to respond promptly to CNS infection and help regulate neuroinflammation (Kuhlow *et al.*, 2003). GFAP is an intermediate filament protein which is known to be expressed in astrocytes, although its precise contributions to astroglial physiology and function are still not clear. The upregulation of GFAP is the main landmark of reactive astrogliosis in neurodegenerative conditions and acute neuronal damage (Eng & Ghirnikar, 1994). Early astroglial activation

can be seen within hours after injury when the astrocytes generally show an increase of GFAP immunoreactivity associated with cell hypertrophy and sometimes proliferation. Astrocytes are shown to be involved in the repair of ECM by secreting ECM proteins like MMPs (Lukes *et al.*, 1999). Astrocytes, together with microglial cells, endothelium and infiltrating leukocytes are the major players of the inflammatory response after CNS damage and they have shown to produce inflammatory cytokines including TNF-α, and the inflammatory enzyme iNOS. Whether astrocytes are protective or pathogenic is controversial (Singh *et al.*, 2011) and the exact role of astrocytes in the pathogenesis of JE remains unknown. Previously, in studies using H-E staining, an astrocytic reaction was not thought to be a prominent feature in JE although some astrocytes are infected by JEV (Desai *et al.*, 1995).

5.1.4.3 Endothelial cells

The cerebral endothelium is involved both in regulating the influx of immune cells into the brain and in modifying immunological reactions within the CNS. A number of human pathogens may cause encephalitis or meningitis when this important protective barrier is impaired. The endothelial cells lining cerebral microvessels and forming BBB are characterized by limited vesicular transport, limited pinocytotic vesicles,

selective active transport mechanisms and the presence of continuous tight junctions (Joo, 1985; Turner *et al.*, 1987; Goldstein & Lorris Betz, 1983).

5.2 Materials and Methods

Following routine paraffin wax embedding, 3-5 µm sections were prepared and stained with pro-inflammatory markers as specified in Table 2.2. HIER was a requirement for all markers. Optimal conditions for antigen retrieval and antibody dilutions (Table 2.2) were independently determined by comparative titration experiments. The anti-human IL-1β and IL-6 markers as well as CD31 endothelial cell marker did not work well with macaque tissue sections.

5.3 Results

5.3.1 Pro-Inflammatory mediators

Both iNOS and NT were expressed by microglial cells and astrocytes. iNOS expression was also seen in some macrophage/microglial cells in the perivascular infiltrates and the adjacent parenchyma (Figure 5.1A, B) (Table 5.1) where staining for NT was only very weak. MMP-2 was expressed in cells with the morphology of reactive astrocytes (Figure 5.1C)

(Table 5.1) and, to a lesser extent, in microglial cells and in infiltrating macrophages, whereas MMP-9, known to be constitutively expressed in human neurons (Rao *et al.,* 1996), was intensely expressed by neurons and relatively weakly by microglial cells (Figure 5.1D) (Table 5.1). TNF-α expression was seen in macrophage/microglial cells and astrocytes, as dual staining with CD68 and sequential staining with GFAP confirmed (Figure 5.1E). It was also occasionally seen in endothelial cells (Figure 5.1E inset). IFN-α expression, however, was seen both in uninfected and infected neurons, as confirmed by dual staining with JEV antigen (Figure 5.2), and in astrocytes and microglial cells (Figure 5.1F). In control brains, only minimal expression of inflammatory mediators was seen, represented by staining in occasional vascular endothelial cells (iNOS, TNF-α), neurons (MMP-9, iNOS) and vascular smooth muscle cells (TNF-α) (Figure Appendix1.3).

5.3.2. Non-neuronal cells

Chapter 3 confirmed the involvement of microglial cells in JE in the form of microglial nodules (Figure 3.1E), diffuse microgliosis, and activation (Figure 3.2C,E). In Chapter 5, I have identified that they expressed iNOS (Figure 5.1A), NT (Figure 5.1B), TNF-α (Figure 5.1E), and IFN- α (Figure 5.3A) and MMP-9 (Figure 5.3B).

I also described the involvement of astrocytes in the form of reactive astrogliosis (Figure 3.1F). In Chapter 5, I have identified that they expressed iNOS (Figure 5.1A), NT (Figure 5.1B), MMP-2 (Figure 5.1C), TNF-α (Figure 5.1E), and IFN-α (Figure 5.1F and 5.2).

In addition I also provided evidence that endothelial cells might be involved in terms of their activation in Figure 3.1B, 3.2E. They were also identified to secrete TNF-α (Figure 5.1E, inset). Endothelial infection was not observed in any of the animals. Semi-quantitative analysis of pro-inflammatory markers was summarized in Table 5.2.

5.4 Discussion

The upregulation of iNOS and cytokines as well as microglial activation have been reported in earlier studies with a mouse model. This chapter confirms the previous findings in a macaque model and highlights the possible contribution of astrocytes in neuronal death which has never been fully explored in *vivo* in any flavivirus encephalitis.

5.4.1 Role of Cytokines in JE

Most of the cytokine antibodies as well as the mRNA expression work well only in fresh frozen tissues. The expression and possible roles of TNF-α and IFN-α in the inflammatory network were studied in this thesis as it worked reliably in paraffin embedded tissues.

The occurrence of apoptosis in apparently uninfected neurons suggests that indirect mechanisms (bystander cell death) contribute to neuronal damage in JE (Andrews *et al.*, 1999; Schoneboom *et al.*, 2000), and indeed recent *in vitro* and *in vivo* murine studies demonstrated that microglial cells can induce neuronal apoptosis via the release of pro-inflammatory mediators (D*aset al*, 2008; Ghoshal *et al.,* 2007). Also, TNF-α, via its receptor on neurons, has been shown to induce caspase-8

activation in mouse neurons (Badiola *et al.,* 2009). I observed TNF-α upregulation in infected macaques and was able to identify astrocytes, microglial cells, endothelial cells and infiltrating macrophages as the source of the cytokine. It is likely that these cells were also responsible for the TNF-α upregulation observed in JEV-infected mice by bead array and RT-PCR (Biswas *et al.,* 2010; Fujii *et al.,* 2008; Ghoshal *et al.,* 2007; Mishra & Basu, 2008; Saxena *et al.,* 2008). TNF-α related neuronal death is established in a recent *in vitro* study with WNV (Kumar *et al.*, 2010).

IFN was shown to play a part in the innate response to CNS viral infection, the rapid local production of type 1 (IFN-α and IFN-β) could decrease virus replication and prevent neuronal death in a WNV mouse model (Samuel & Diamond, 2005). Type II interferon (IFN-γ) is believed to be a key cytokine in the activation of microglial cells and serves critical functions in both innate and adaptive immunity (Griffin, 2003). The role of IFN-γ in the inflammatory cascade in JE is not determined in this thesis. However, flaviviruses appear to be capable of attenuating some IFN-dependent antiviral effector mechanisms (Chamber & Diamond, 2003). Further definition of the underlying mechanisms will allow us to understand the processes involved in disease progression and to assess the potential of

anti-apoptotic treatment strategies (Clarke and Tyler, 2009).

Cytokine profiling in paraffin sections with quantitative real time – PCR (qRT-PCR)

I attempted to profile the cytokine expression from paraffin sections of JE infected rhesus monkeys although these studies are strongly dependent on sample preparation. It was reported that fixation of tissue samples in formaldehyde leads to extensive cross linking of all tissue components. As a consequence of this cross-linking, the nucleic acids isolated from these specimens are highly fragmented. Studies also showed that extent of fragmentation of extracted RNA significantly increases with archive storage time which in my case is more than a decade. I attempted to study the mRNA expression of gene targets IL-1β, IL-6 and IL-18. While all efforts are made to achieve optimal results by using advanced biomaterials including sophisticated PCR instrumentation, RNA isolation kits, and highly purified Taq polymerase, the assay was unsuccessful. Majority of the tissue used for qRT-PCR were those fixed for undetermined period (more than 1-2 weeks) in formaldehyde that might have caused more fragmentation of RNA. It might also due to the primers, some of which are larger amplicon sizes although I have chosen primers that produce PCR

products as short as possible.

5.4.2 Role of nitric oxide in JE

iNOS and NT expression in my study indicate NO production, which is in accordance with results from a mouse study (Saxena *et al.*, 2001). There, a gradual increase in iNOS activity was observed after intracranial JEV infection, and was considered a consequence of release of cytokines, such as TNF-α or IL-8 which might be beneficial through the inhibition of viral replication and release (Saxena *et al.*, 2001). NO has also been discussed as a potential mediator of pathogenesis in TBEV infection (Kriel & Eibl, 1996), but its role in WNV has not been established (Samuel & Diamond, 2006). The contribution of NO to the inflammatory process is a subject of some controversies and its role in the inflammatory pathway of JE has yet to be proven.

5.4.3 Role of matrix metalloproteinases in JE

I am particularly interested in studying MMP in CNS in JE as unlike NO and cytokines their role in the regulation of neuronal cell death in JE has never been explored. Increased production of MMPs has been demonstrated by WNV in a mouse model (Roe *et al.*, 2012). MMP levels

have been shown to correlate with the severity of some CNS infections (Leppert *at al.,* 2000). MMP-9 is known to be constitutively expressed in human neurons (Rao *et al.,* 1996). However, in my study it was intensely upregulated in neurons of the JEV-infected macaques and weakly expressed by microglial cells, while astrocytes and macrophage/microglia were sources of MMP-2. MMP release is stimulated by pro-inflammatory cytokines including TNF-α (Gottschall and Deb, 1996). In JE, MMPs might play a detrimental role and not only be responsible for BBB disruption through collagen IV degradation (Gearing *et al.*, 1994), but also contribute to neuronal destruction via stimulation of TNF-α release. MMP over expression in my study supports its likely role in mediating BBB disruption, influx of leucocytes into the CNS and secondary neuronal damage. My results suggest that adjunctive therapy with selective MMP inhibitors may prove to be of benefit to prevent neurodegeneration in JE.

5.4.4 Non-neuronal cells

5.4.4.1 Microglial response

Alongside the inflammatory infiltration and the cytopathic effects, I found distinct evidence of activation of a range of cells, namely microglial cells, astrocytes and vascular endothelial cells. Microglial activation was

confirmed by the demonstration of MHC II antigen, iNOS, NT, TNF-α and MMP expression by microglial cells (Rock *et al.*, 2004) and has been reported previously in JEV-infected mice (Ghoshal *et al.,* 2007). To shed light on the potential mechanism of microglial activation, I assessed the expression of IFN-α (type I IFN); this potent antiviral cytokine (Samual, 2001) is an activator of microglia in response to CNS viral infection (Paul *et al.,* 2007), and is elevated in the CSF of patients with JE, where it is associated with a poor outcome (Winter *et al.,* 2004). I demonstrated IFN-α expression in neurons which suggests that they might be responsible for microglial activation early after infection; expression by microglia and astrocytes suggests they might be responsible for sustained microglial activation in JE.

5.4.4.2 Astrocyte response

Astrocytes are the major cellular component of the brain that is capable of intense proliferation and metabolic activity during many inflammatory brain diseases. As described in earlier reports (Chen *et al.*, 2011; German *et al.,* 2006), reactive astrogliosis was observed in this study as in WNV (Lim *et al.*, 2011). Astrogliosis is considered as a non-specific response to degenerative changes including virus-induced damage in the CNS (Booss

& Esiri, 2003). However, a recent study provided evidence that this activation might be an effect of TNF-α release from microglial cells (Chen *et al.*, 2010). Nevertheless, the demonstration of TNF-α, IFN-α, iNOS, NT and MMP-2 expression by astrocytes in my study provides the first *in vivo* evidence that astrocytes may play an important role in the pathogenesis of JE. The same is true for microglial cells and macrophages in the inflammatory infiltrates, since through release of the inflammatory mediators, all these cells might actively contribute to the damage of other cells in the brain and in particular induce bystander apoptotic death of neurons (Das *et al.,* 2008; Ghoshal *et al.,* 2007; Maximova *et al.,* 2008). While a number of studies have documented the importance of microglia in JE encephalitis, little is known regarding the role of astrocytes in neuroinflammation and neuronal death. More research is required to determine the precise role of astrogliosis and its implication on the neuropathogenesis of JE.

5.4.4.3 Activation of vascular endothelial cells

Without a specific marker, the role of endothelial cells cannot be confirmed in my study. However the expression of MHC II antigen and TNF-α in endothelial cells suggests that they are activated and implies they might

have a role in inflammatory cell recruitment and potential contribution to immune reactions, glial cell activation and neuronal apoptosis. Endothelial cells might also be a source of the increase in serum TNF-α seen in JE patients (Winter *et al.*, 2004). Reports on WNV suggest that endothelial cells are activated (Verna *et al.*, 2009) and virus may cause damage to the vascular endothelial cells, leading to disruption of BBB (Diamond & Klein, 2004). However the precise role of endothelial cells in flavivirus encephalitis has not been defined.

In summary my study indicated that pro-inflammatory markers produced by glial and endothelial cells are likely to be important in the amplification of inflammatory and immune reactions and may trigger the inflammatory response, neuronal apoptosis and progression of disease.

Table 5.1 A summary of the pro-inflammatory markers examined and their targets

Inflammatory Marker	Targets
iNOS	Microglial cells, astrocytes, perivascular infiltrates (macrophages), parenchymal infiltrates
NT	Microglial cells, astrocytes, perivascular infiltrates (macrophages), parenchymal infiltrates
MMP-2	Reactive astrocytes, microglia, infiltrating macrophages
MMP-9	Neurons, microglia (weak)
TNF-α	Microglial cells, astrocytes, perivascular infiltrates, parenchymal infiltrates, endothelial cells
IFN-α	Astrocytes, microglial cells, neurons

iNOS = Inducible nitric oxide synthase
NT = Nitrotyrosine
INF-α = Interferon alpha
TNF-α = Tumor necrosis factor alpha
MMP-2 = Matrix mettaloproteinase-2
MMP-9 = Matrix mettaloproteinase-9

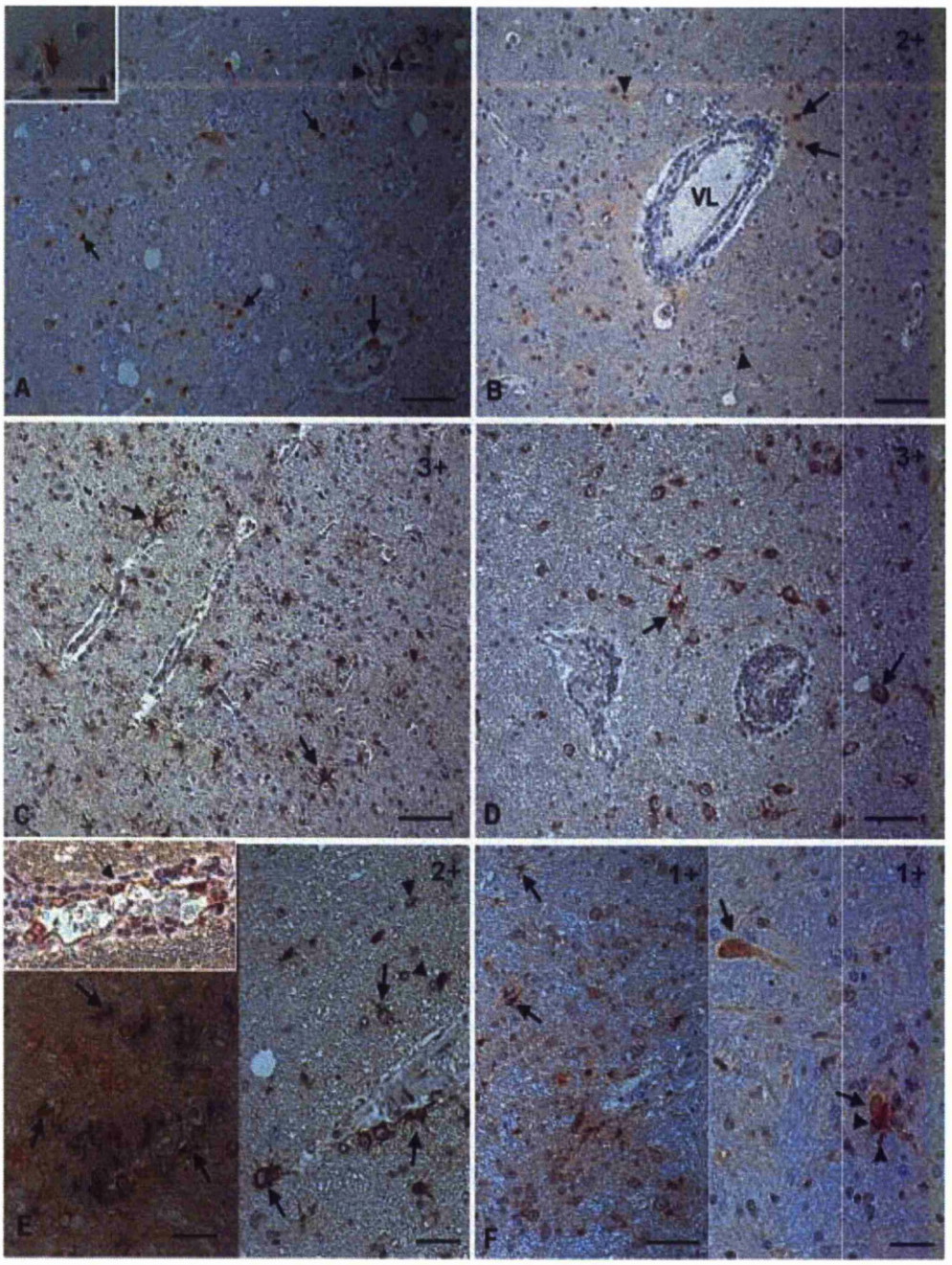
3+
A
2+
VL
B
3+
C
3+
D
2+
E
1+
1+
F

Figure 5.1 Demonstration of pro-inflammatory markers in the thalamus of rhesus macaques after intranasal inoculation with JEV (No. 2 (A, B, D-F), No. 11 (C)). (A) Microglial cells (small arrows), leukocytes in the perivascular infiltrates (arrowheads), perivascular macrophages (large arrow) and astrocytes (inset) express iNOS. (B) Nitrotyrosine expression is observed in microglial cells (arrowheads) and astrocytes (arrows). VL: vessel lumen. (C) MMP-2 expression is diffusely seen in reactive astrocytes. (D) MMP-9 is mainly expressed by neurons. (E) TNF-α (left: brown signal) is expressed by microglial cells (left: arrows; right: arrowheads) that are identified based on their CD68 expression (left: blue signal), endothelial cells (left: arrow, inset), and astrocytes (right: arrows). (F) IFN-α expression is seen in astrocytes (left; arrow) and neurons, both unaltered (left: arrowheads; right: arrow) and degenerating (right: arrowhead), as demonstrated in satellitosis. Microglial cells surrounding the neuron are also positive.Indirect peroxidase method (A-F), Vectastain® Elite ABC-Alkaline Phosphatase Kit (E, left); DAB (A-F), *BCIP*/NBT blue (E, left), Papanicolaou's haematoxylin counterstain. Scale bars A-D, F left = 50 µm. E, F right = 20 µm.

(Scores for each marker recorded on upper right corner)

Table 5.2 Semi-quantitative analysis of cellular markers in the brain of monkeys following Japanese Encephalitis Virus infection

Monkey No.	Tissue	PRO-INFAMMATORY Markers					
		iNOS	NT	TNF-α	INF-α	MMP-2	MMP-9
1	B	1+	2+	3+	2+	2+	3+
	T	1+	3+	3+	1+	1+	±
2	B	2+	2+	2+	3+	2+	3+
	T	3+	2+	2+	1+	3+	2+
3	B	-	±	1+	1+	2+	-
	T	1+	1+	2+	1+	3+	-
4	B	1+	1+	1+	1+	2+	2+
	T	1+	1+	1+	1+	2+	-
5	B	1+	-	2+	3+	2+	3+
	T	1+	2+	2+	2+	1+	-
6	B	1+	2+	2+	3+	1+	3+
	T	1+	2+	3+	2+	3+	3+
7	B	1+	±	3+	1+	2+	1+
	T	1+	-	3+	1+	3+	2+
8	B	2+	2+	3+	2+	3+	3+
	T	2+	1+	3+	2+	3+	3+
9	B	1+	3+	3+	1+	3+	3+
	T	-	1+	1+	1+	3+	2+
10	B	2+	1+	3+	2+	3+	3+
	T	1+	-	3+	2+	1+	1+
11	B	-	2+	2+	1+	2+	3+
	T	1+	±	3+	±	3+	2+
12	B	1+	-	1+	1+	1+	-
	T	2+	1+	2+	1+	2+	1+
Mean		**1+**	**1+**	**2+**	**2+**	**2+**	**2+**

Immunohistochemistry of cell markers tested on paraffin sections of brainstem and thalamus and the level of expression recorded as:
0 = no expression observed above the negative control
1+ = occasional isolated positive cells
2+ = a few nests of positive cells
3+ = frequent positive cells in most brain areas
MAC 387 = anti human myeloid/histiocyte antigen (reactive macrophage)
iNOS = Inducible nitric oxide synthase
NT = Nitrotyrosine
INF-α = Interferon alpha
TNF-α = Tumor necrosis factor alpha
MMP-2 = Matrix mettaloproteinase-2
MMP-9 = Matrix mettaloproteinase-9
B = Brain stem
T = Thalamus

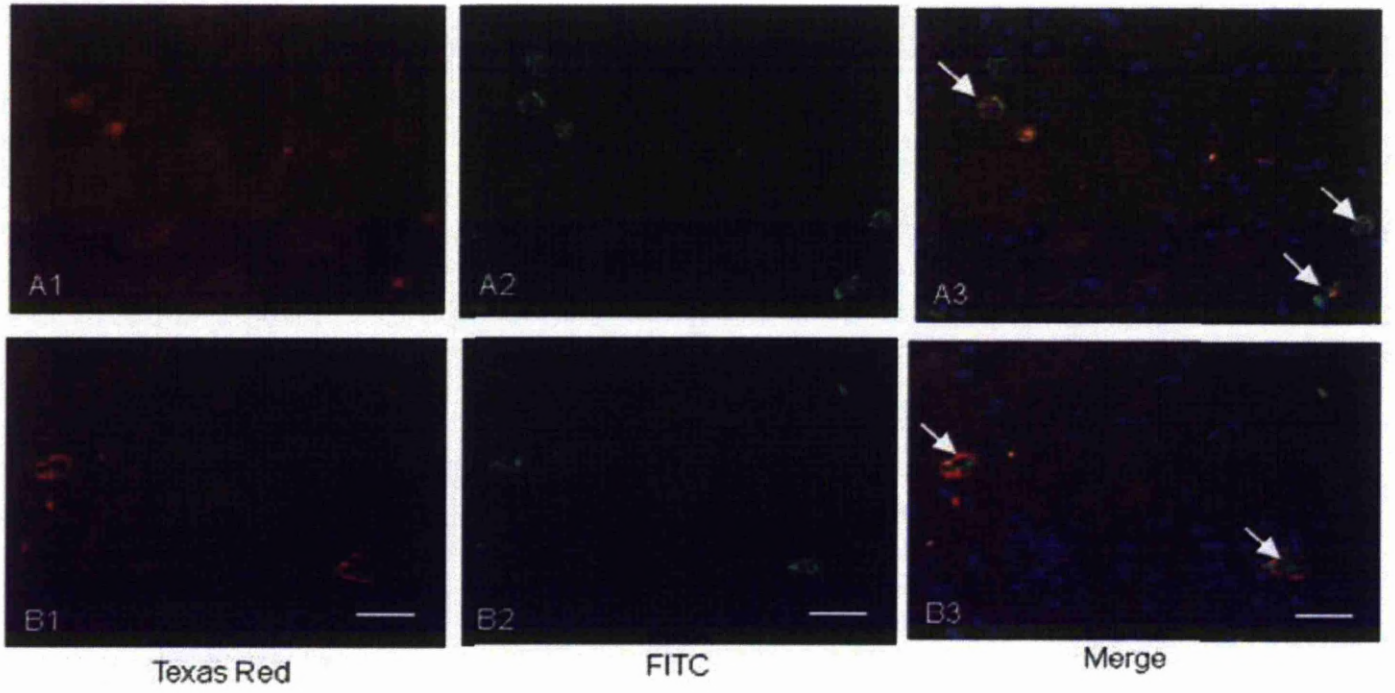

Figure 5.2 Demonstration of JEV infected neurons expressing interferon alpha (IFN-α) in the thalamus of rhesus macaques No. 2 (A 1-3) and No. 9 (B 1-3) after intranasal inoculation with JEV. JEV antigen (FITC) and IFN-α (Texas Red) showing some JEV infected neurons also expressing IFN-α (arrows). Indirect immunofluorescent method, DAPI counterstain. Scale bars = 50μm.

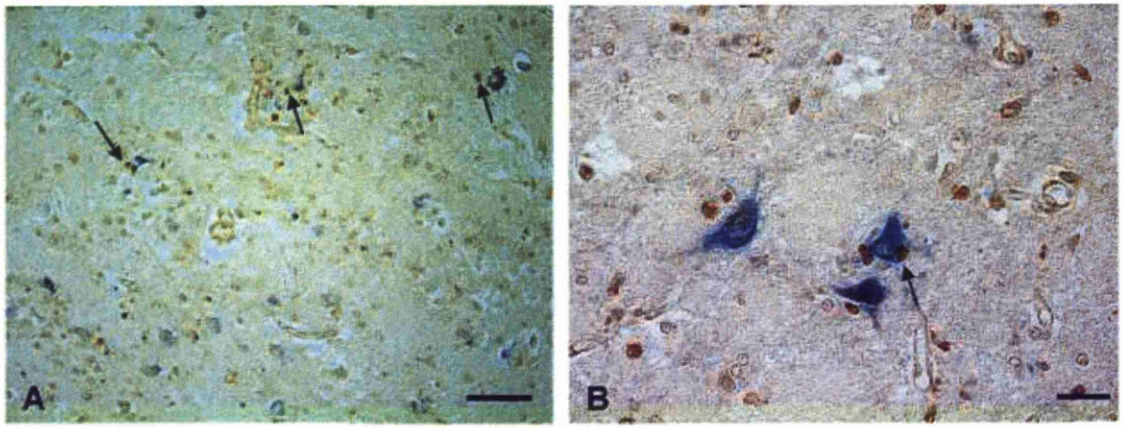

Figure 5.3 Characterization of microglial cells in the thalamus of a rhesus macaque (No. 2) after intranasal inoculation with JEV. (A) IFN-α (arrow, brown signal) is expressed by microglial cells (arrows) in satellitosis and neuronophagia of JEV infected neurons (blue signal). (B) MMP-9 expression seen in microglial cells (arrow, brown signal) in satellitosis of a JEV infected neuron (blue signal). Vectastain® Elite ABC-Alkaline Phosphatase Kit/NBT blue and DAB. Scale bars: A = 50 µm; B = 20 µm.

CHAPTER 6

GENERAL DISCUSSIONS

This chapter aims to examine the possible role of BBB dysfunction and the pathways of neuroinflammatory networks in JE pathogenesis.

Loss of Blood Brain Barrier integrity in JE

In JE, impairment of the BBB is implicated by the widespread perivascular oedema (German *et al.*, 2006). The BBB is both structural and functional, impeding and regulating the influx of most compounds from blood to brain. This barrier is composed of specialized endothelial cells, joined by tight junctions, on a basal lamina, surrounded by pericytes and astrocytic foot processes (Figure 6.1). Breach can occur because of damage of the endothelial lining by trauma, allergic or inflammatory lesions, or because of the release of vasoactive mediators.

Disruption of the integrity of the BBB represents the early event of inflammatory diseases of the CNS and is reported to be caused by pro-inflammatory cytokines, MMPs and ROS (Blamire *et al.*, 2000; Paul *et al.*, 2003; Wang *et al.*, 2004; Yang *et al.*, 1999) which facilitates

additional dissemination of the virus within the CNS. Half of the animals had widespread perivascular oedema indicative of extensive BBB leakage. This was well demonstrated by von Willebrand staining, particularly in animal number 2.

While most agree there is haematological spread in JE, it is not clear whether this spread is passive or involves viral replication in the vascular endothelium. More specifically, although the presence of JEV has been demonstrated in the vascular endothelial cells (Johnson *et al.*, 1995), the mechanism of invasion of the CNS is not clear. Electron microscopic examination showed no evidence of damage to the vasculature (Hase *et al.*, 1990). It has been postulated that specifically sensitised T cells enter the brain and release certain cytokines which then attract the macrophages (Johnson, 1971). A 'Trojan horse' theory for passage of flavivirus across the BBB has also been proposed, whereby JEV (Yang *et al.*, 2004) and WNV (Verma *et al.*, 2009) infiltrate the CNS. My study does not support the 'Trojan horse' theory as viral antigen in the perivascular cell infiltrates and the perivascular macrophages are rare. The distribution of zona occludens (ZO) -1 and occludin, proteins critical for tight junction function, was not explored in this study. Although knowledge of the morphology and physiology of the

BBB has come a long way, the role of inflammatory mediators which induce alteration and loss of integrity of BBB in JE is still unanswered and should be investigated.

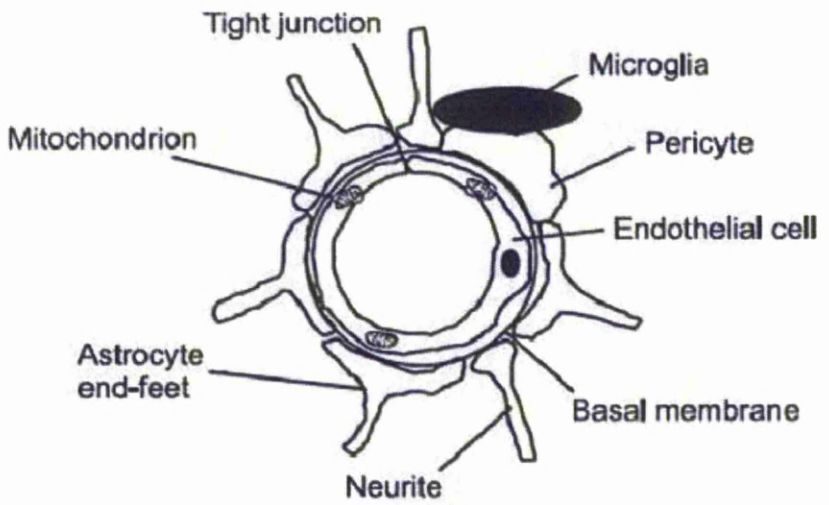

Figure 6.1 Structure of Blood Brain Barrier

Inflammatory network in JE

Based on my findings I postulate that infection of neurons by JEV triggers a network of inflammatory mediators (Benarkis *et al.*, 2009). Through release of IFN-α, neurons activate microglial cells which, via release of cytokines such as TNF-α, activate astrocytes and endothelial cells. Together, these mediators contribute to BBB breakdown,

leukocyte recruitment into the parenchyma and further neuronal apoptosis. Glial cell apoptosis should limit the extent of inflammation. However, the release of further mediators by infiltrating leukocytes, in particular macrophages, results in sustained glial and endothelial cell activation and further leukocyte recruitment, ultimately augmenting the inflammatory response and neuronal cell loss (Figure 6.2). Although the inflammatory response is intended to be protective, and presumably is so in cases which improve and recover, if uncontrolled it can contribute to disease progression in JE.

Flavivirus encephalitis

Flaviviruses such as JEV, TBEV, WNV and St. Louis encephalitis virus (SLEV) are some of the most important pathogens that cause fatal encephalitis in humans. The pathogenesis of flavivirus encephalitis and the cause of neuronal injury *in vivo* remains incompletely understood, especially concerning viral replication, CNS invasion and the nature of immune responses. There is a need for comparative studies between these different viruses. In many cases it appears that the immune response, while crucial to limiting viral spread to the brain, is also responsible for the pathogenesis (Turtle *et al.*, 2012). The mouse model of WNV, a closely related neurotropic and intensively studied flavivirus,

has yielded a significant understanding of WNV pathogenesis (Samuel & Diamond, 2006; Lim *et al.*, 2011). How the neurotropic flaviviruses gain access to the CNS and the specific cell types involved remain controversial, but interactions at the BBB are probably critical. WNV and SLEV are observed to cross the BBB in correlation to the level and duration of viraemia (Lim *et al.*, 2011). Mass release of inflammatory factors including cytokines, might pave the way for CNS infections by compromising BBB-integrity. How much neuronal damage is attributed to direct viral cytopathology and how much is due to the inflammatory process is unknown; the WNV mouse model showed a fine balance between these two factors (Turtle *et al.*, 2012). The role of T cells in flavivirus encephalitis is less clear. CD8+ T cells are reported to contribute to neuronal damage via the induction of bystander damage in a number of flaviviruses including WNV and TBEV (Sips *et al.*, 2012). CD8+ T cells show a dual role for clearing virus and mediating immunopathology in WNV infection; recent research on JEV in a murine model suggested that they might be more pathogenic (Larena *et al.*, 2011). WNV has been demonstrated to produce caspase-3 dependent apoptosis following infection in mice although the different cell death pathways are not defined (Lim *et al.*, 2011). WNV also supports the role of TNF-α in neuronal death as implicated in my study with JEV. The

extent to which involvement of microglial cells and astrocytes contributes to WNV-induced pathology has never been fully investigated although reactive gliosis and infection of these cells are considered key pathogenic features. The role of the inflammatory process in pathogenesis of flaviviruses including JEV remains speculative and more studies need to be done for further evidence of this hypothesis.

The difficulty in obtaining tissue samples from fatal human cases demonstrates the need for an animal model to study the pathogenesis of JE. Because the immune system of the rhesus macaque closely resembles that of humans, the macaque model provides a good system for studying JEV.

My thesis provides insight into the neuropathogenesis of JE. I have characterized the cellular infiltrates, type of cells infected by JEV, apoptosis and pro-inflammatory infiltrates within the CNS by immunohistology techniques and fulfilled my aims/hypotheses to demonstrate that:

1. the immune response in JEV contributes to neuronal death and pathogenesis of infection;

2. cell death of JEV-infected neurons is caspase-3 dependent;

3. the activation of microglial cells and astrocytes plays a role in the pathogenesis through the action of pro-inflammatory mediators.

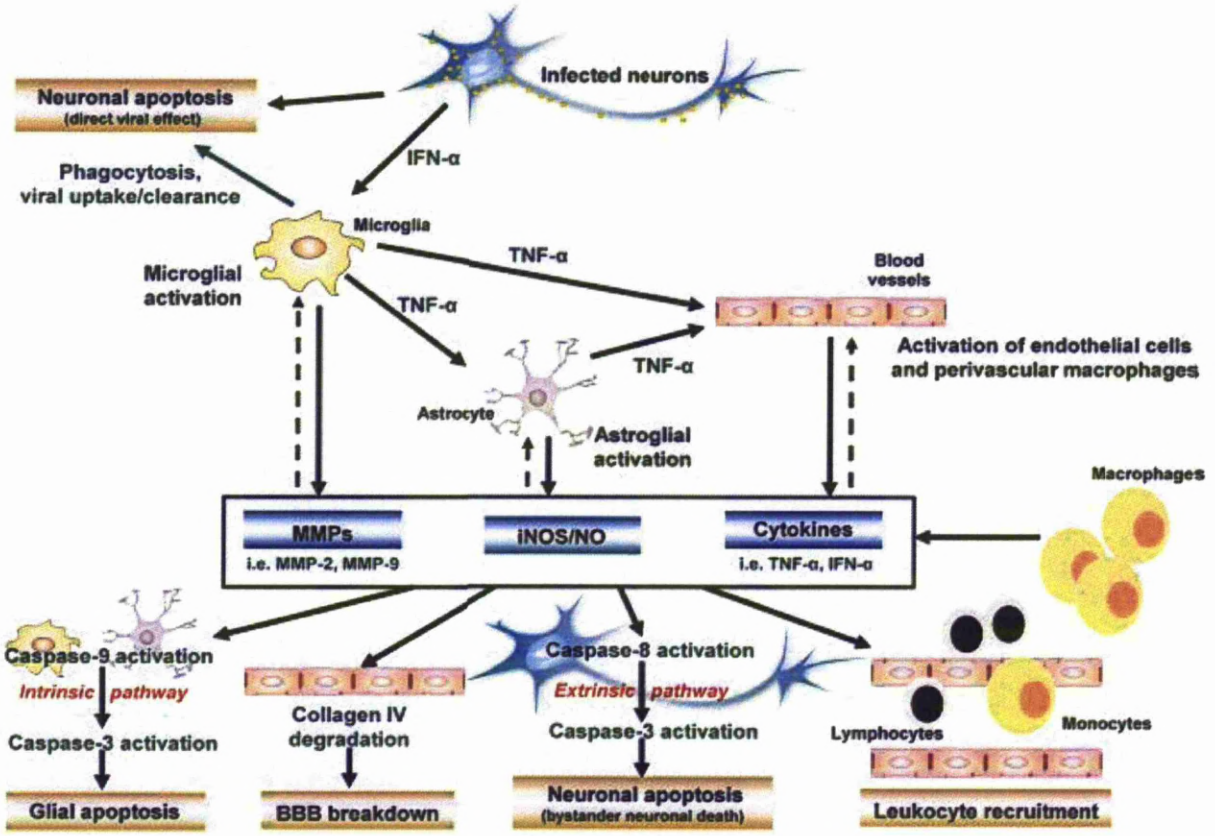

Figure 6.2 Model of neuroinflammatory network in Japanese Encephalitis

CONCLUSIONS

This thesis examined the possible causes of neuronal damage in JE in a macaque model and hopefully improved the understanding of the neuropathogenesis of this infection.

Despite some successes with formalin-inactivated vaccination, and the promise of the newly evaluated vaccines, JE will likely remain an important public health problem. The enzootic nature of JEV means that the 2.8 billion people living in affected areas will continue to be exposed to the virus. Thankfully only a small proportion of those infected develops disease, but we have little understanding of what determines severity and outcome of the infection.

The cascade of events that I have outlined for JE may also apply to other viral encephalitides. Currently there is no proven efficacious therapy for most viral infections of the CNS including JE. Novel strategies for treating viral CNS infections are urgently needed. My thesis suggests that neuronal apoptosis and glial activation are crucial steps in the pathogenesis of JE. My results imply that adjunctive therapy with inhibitors of caspases or

targeted anti-inflammatory treatments might be a promising therapeutic approach for JE in the future. Pathophysiological research described in this thesis has suggested possible therapeutic avenues for JE. Even in the absence of specific antiviral drugs for JE, measures aimed at controlling the apoptosis and inflammation may improve the prognosis of this terrible disease.

FUTURE DIRECTIONS

Only a handful of laboratories are involved in rigorous studies on JEV.

1. My detailed study on Chapter 5 describes the apoptosis pathways involved in JE. The results of my study suggest for the first time that JEV might simultaneously trigger, both directly and indirectly, the extrinsic apoptotic pathway in neurons and the intrinsic apoptotic pathway in microglial cells. The role of apoptotic proteins like FAS, FAS-L, TNFR as well as caspase–independent pathways should be explored to guide the best strategies to limit neuronal damage in JE.
2. A limitation of my study is that only paraffin tissue was available. JE was labelled as a select agent soon after my PhD study started and I had no possibilities to study the detailed cytokine profile (both pro- and anti-inflammatory) including gene expression because most of the cytokine antibodies as well as the mRNA expression work well only in fresh frozen tissues. This led to a change of scope for my PhD studies to focus only on the available cytokines that are reported to work on paraffin embedded tissues like TNF-α and IL-1β. The role of TNF-α in the inflammatory network is studied in this thesis in Chapter 6. The next

step would be to identify other pro- and anti-inflammatory cytokines and their role in the pathogenesis of JE on fresh tissues.

3. The results in Chapter 3 confirm that a number of animals exhibited BBB leakage in this study. Although alterations in vesicular transport and trans-endothelial support may contribute to the increased BBB permeability, defective BBB tight junctions are thought to be the most important process in CNS infections. Distribution of proteins critical for tight junction function like zona occludens (ZO)-1 and occludin should be explored.
4. The results from Chapter 3 and 6 confirm the earlier reports on contribution of microglia to neuronal death in JE. My study highlights that in addition to microglial cells, astrocytes are found to play an important role in the inflammatory cascade. Oligodendrocytes are reported to play a major role in some CNS infections. Their role in JE encephalitis should be studied.
5. The results in Chapters 3 and 4 shows that perivascular macrophages were involved – some are shown to be apoptotic and some as scavenger cells. Further studies are necessary to confirm the functional activity of the perivascular macrophages in JE encephalitis.

6. It has been reported that neuronal death is primarily due to the inflammatory milieu. In addition to microglia the relative contribution of peripheral macrophages known to migrate into the CNS in JE should be studied. The species cross-reactivity information of commercially available antibodies limits the study of macaque tissues. The role of CTL and T-helper cells is not explored in this study due to lack of antibodies for monkey tissue sections. The exact role of CTLs in disease protection and recovery in JE is still unclear and should be evaluated.
7. Many informative studies can be done in animal models. For cultural reasons, obtaining autopsy material is difficult but the use of human pathogenic specimens is needed to investigate whether the animal observations have direct correlation to human disease.
8. Last, but not least, the study of apoptosis and the inflammatory response to JE was conducted on target areas like thalamus and brainstem and neocortex which is less affected, due to limitation of reagents. Thalamus and brainstem were shown previously in my original challenge study to be heavily infected by JEV and exhibited the most consistent histological changes. However less involved areas like the cerebellum and spinal cord should also be evaluated for the complete picture of neuropathogenesis in JE.

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Appendix 1

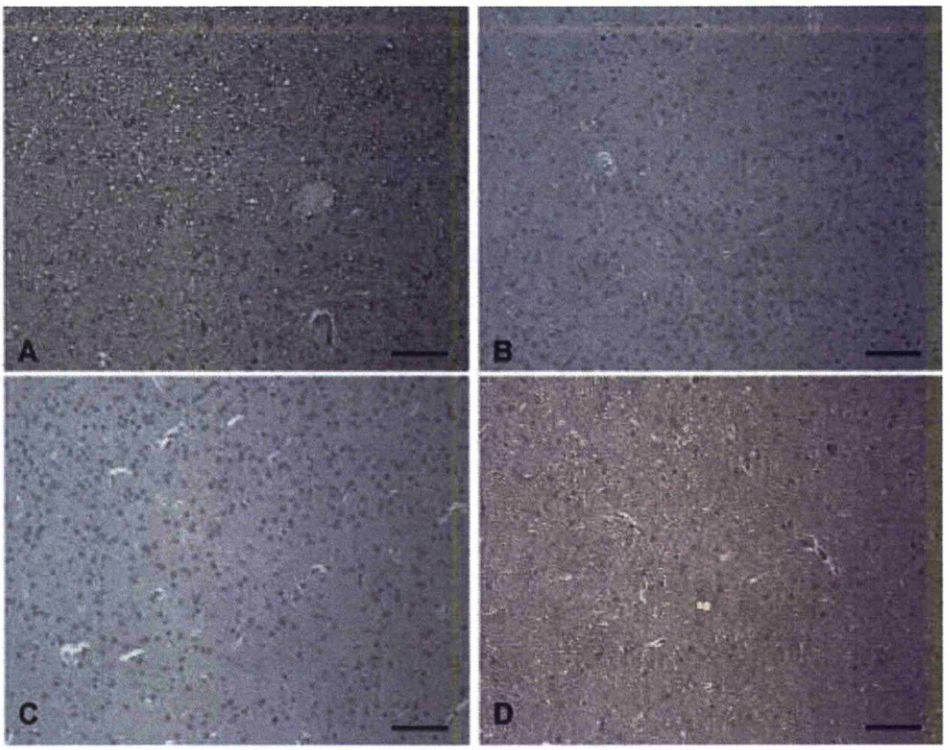


Figure A1.1 Staining with inflammatory cell markers in the thalamus of an uninfected control rhesus macaque show no positive immunostaining for (A) CD3, (B) CD20, (C) CD68, and (D) MHC class II. Scale bars = 50 μm.

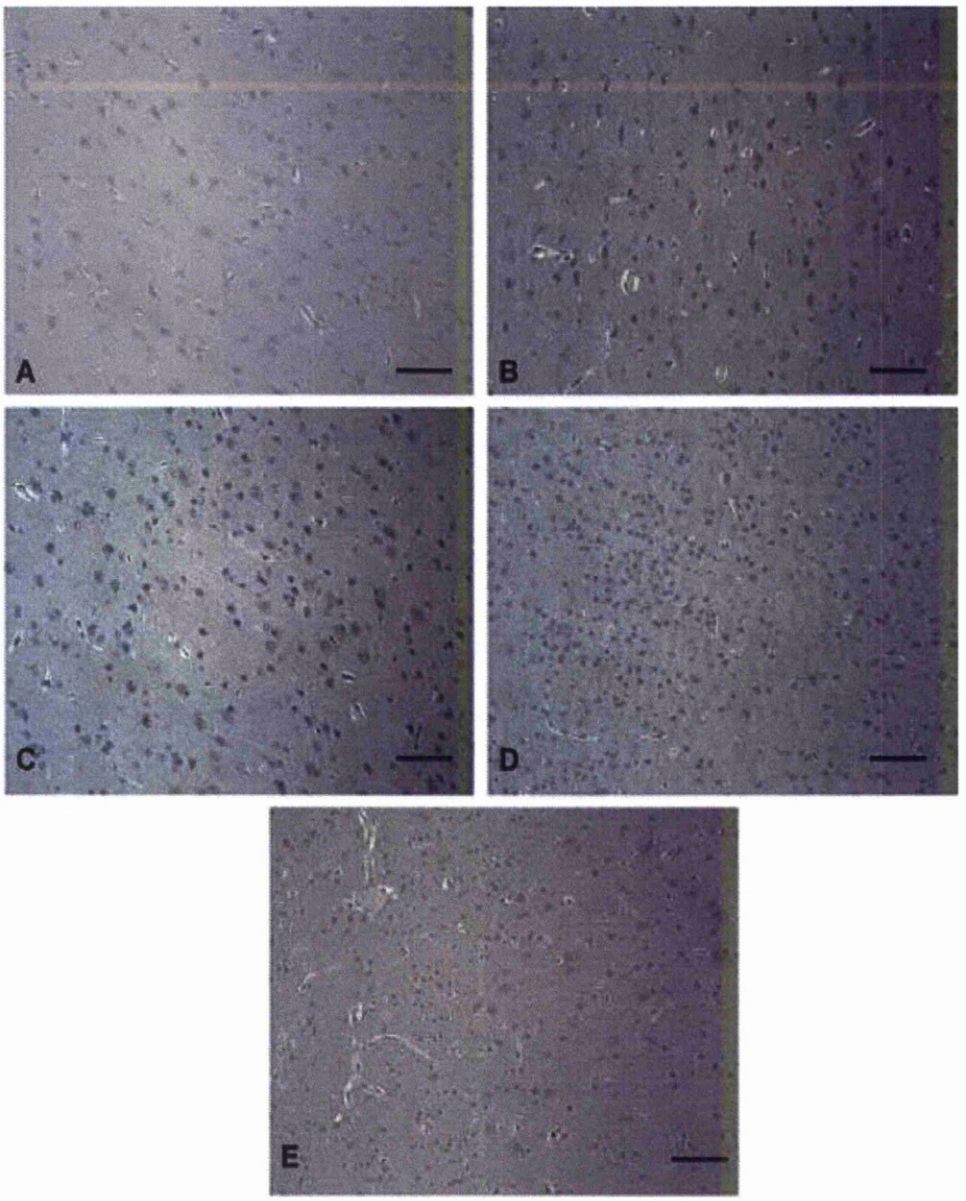
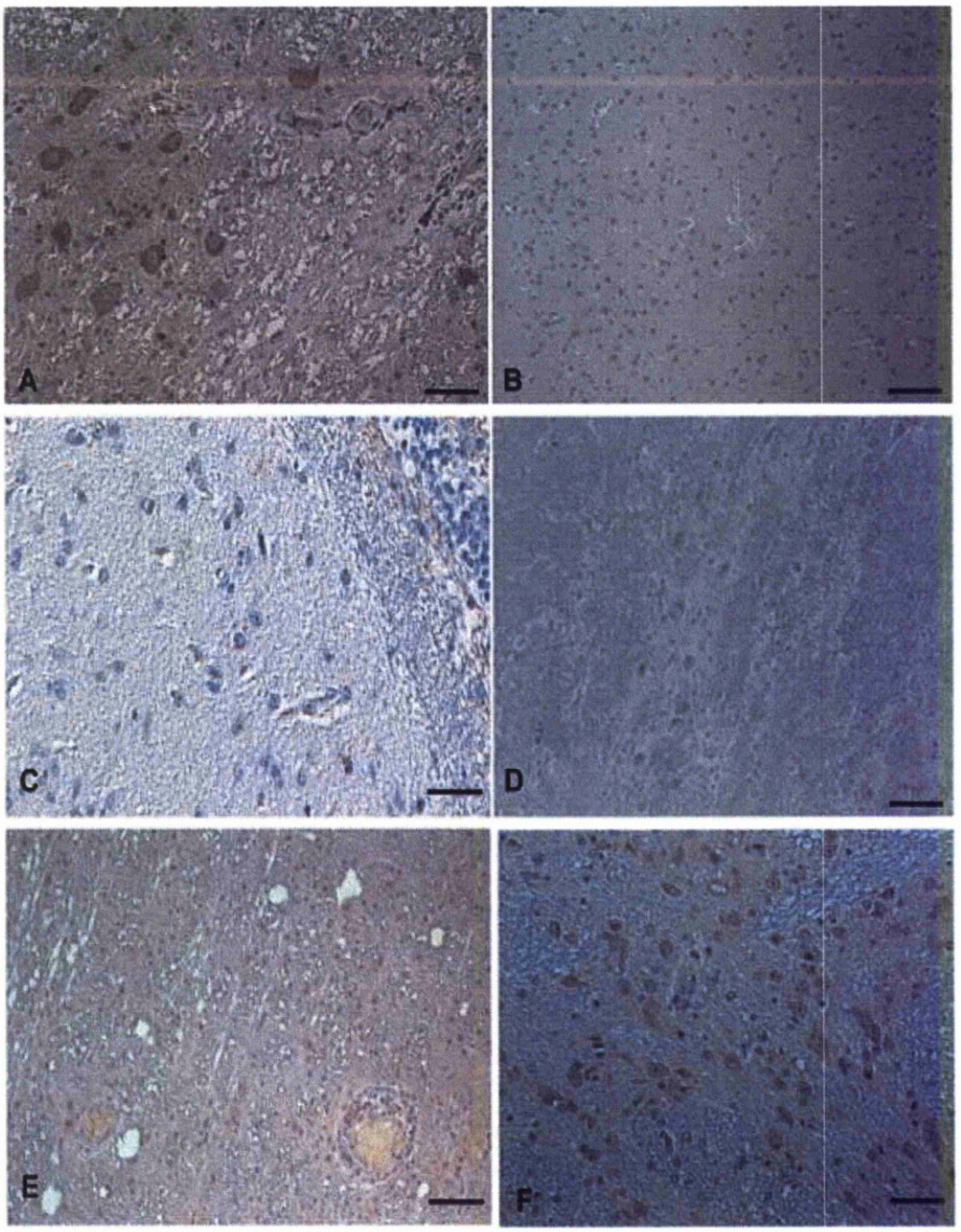

A
B
C
D
E

Figure A1.2 Staining with apoptosis related proteins in the thalamus of an uninfected control rhesus macaque show negligible reactions with (A) caspase-3, (B) caspase-8, (C) caspase-9, (D) Bcl-2 and (E) occasional weak neuronal staining with Bax. Scale bars = 50 μm.


A
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C
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F

Figure A1.3 Staining with pro-inflammatory mediators in the thalamus of an uninfected control rhesus macaque with (A) iNOS, (B) NT, (C) MMP-2, (D) MMP-9, (E) TNF-α, (F) IFN-α show occasional weak neuronal staining with (A) iNOS, (D) MMP-9, (F) IFN-α and vascular staining with (E) TNF-α. Scale bars = 50 μm.

APPENDIX 2

PUBLICATIONS ARISING FROM THIS WORK

Myint KS, Gibbons RV, Perng GC, Solomon T. Unravelling the neuropathogenesis of Japanese encephalitis. Trans R Soc Trop Med Hyg 2007; 101:955-6.

Khin Saw Aye Myint, Anja Kipar, Richard G. Jarman, Robert V. Gibbons, Guay Chuen Perng, Brian Flanagan, Duangrat Mogkolsirichaikul, Yvonne Van Gessel, and Tom Solomon. Characterization of the inflammatory response and cell death in a primate model of Japanese encephalitis. Acta Neurologica Submitted.

APPENDIX 3

PRESENTATIONS ARISING FROM THIS WORK

Apoptosis in Japanese encephalitis virus infected rhesus monkey challenge models, American Society of Tropical Medicine and Hygiene Meeting, 4- 8 November 2007, Philadelphia, US

Japanese encephalitis virus infected neurons enter the apoptotic pathway in the rhesus monkey challenge model, American Society of Tropical Medicine and Hygiene Meeting, 8-10 December 2008, New Orleans, US

Apoptosis and Phenotyping in Japanese encephalitis virus infected rhesus monkey challenge models, Joint International Tropical Medicine Meeting, 13-14 October 2008, Bangkok, Thailand

Inflammation in Japanese encephalitis – too much of a good thing? Association of British Neurologists Meeting, 22-26 June 2009, Liverpool, UK

Astrocytosis in Japanese encephalitis, American Society of Tropical Medicine and Hygiene Meeting, 3-7 November 2010, Atlanta, US

Inflammatory network in a primate model of Japanese encephalitis, Eijkman Symposium, 8-10 November 2011, Jakarta, Indonesia

Inflammation in Japanese encephalitis – a double edged sword?, LBIG

Retreat, 28-30 March 2012, Leek, UK