THE ROLE OF MACROPHAGES AND ANTI-VIRAL ANTIBODIES IN WEST NILE VIRUS PATHOGENESIS

A Dissertation presented to the Faculty of the Graduate School

University of Missouri-Columbia

In partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

DAVID GARCIA TAPIA

Steven B. Kleiboeker, Dissertation Supervisor

MAY 2006

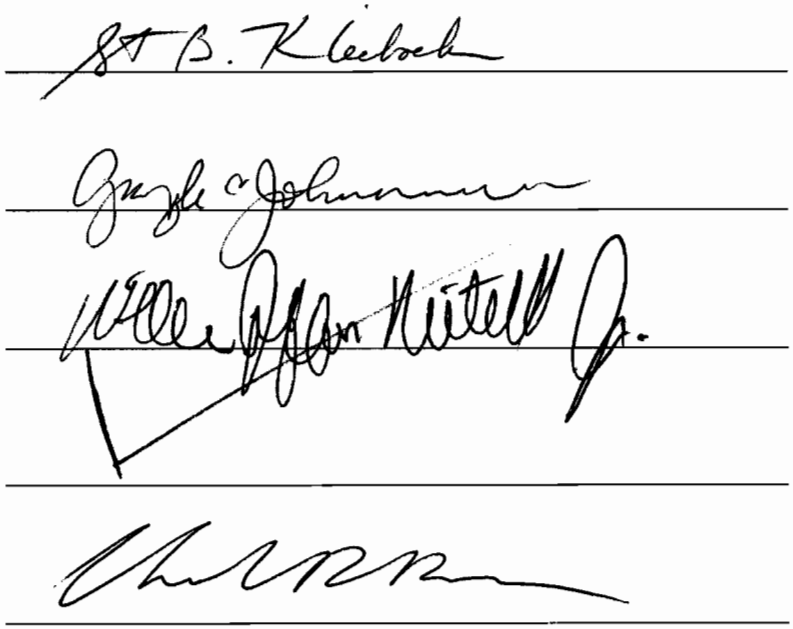
The undersigned, appointed by the Dean of the Graduate School,
have examined the dissertation entitled

THE ROLE OF MACROPHAGES AND ANTI-VIRAL ANTIBODIES IN WEST NILE VIRUS PATHOGENESIS

Presented by David Garcia-Tapia

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worth of acceptance.



THE ROLE OF MACROPHAGES AND ANTI-VIRAL ANTIBODIES IN WEST NILE VIRUS PATHOGENESIS

David Garcia-Tapia

Dr. Steven B. Kleiboeker, Dissertation Supervisor

Abstract

Since the introduction of West Nile virus into the United States in 1999, the virus has spread throughout the continental territory of the United States and the virus has moved into Mexico and Canada. The emergence of this flavivirus in North America has resulted in an intense interest in its study, leading to research efforts focused on the pathogenesis, possible treatments and methods for prevention of the disease. In the present studies, we demonstrate the ability of WNV to productively infect horse monocytes, CD4+ T lymphocytes and monocyte-derived macrophages. Along with these findings we report the ability of immune horse serum to induce antibody dependent enhancement of WNV infection of horse macrophages and mouse macrophages *in vitro*. The question of ADE *in vivo* was also addressed, and it was found that sub-neutralizing dilutions of anti-WNV immune horse serum which induce ADE *in vitro* fail to induce the same effect *in vivo*. The serum induces protection, which is perhaps driven by up-regulation of IL-12 in spleen during the earliest phase of infection. In brain, the chemokines MIG, MCP-5 and IP-10 were secreted earlier than other chemokines and cytokines, suggesting that those chemokines play an important role in the initial stages of the encephalitis caused by WNV. High titers of virus in blood and spleen, as well as the ability of the virus to infect horse monocytes and CD4+ lymphocytes suggest that this virus spreads from subcutaneous tissue to the brain by a hematogenous route.

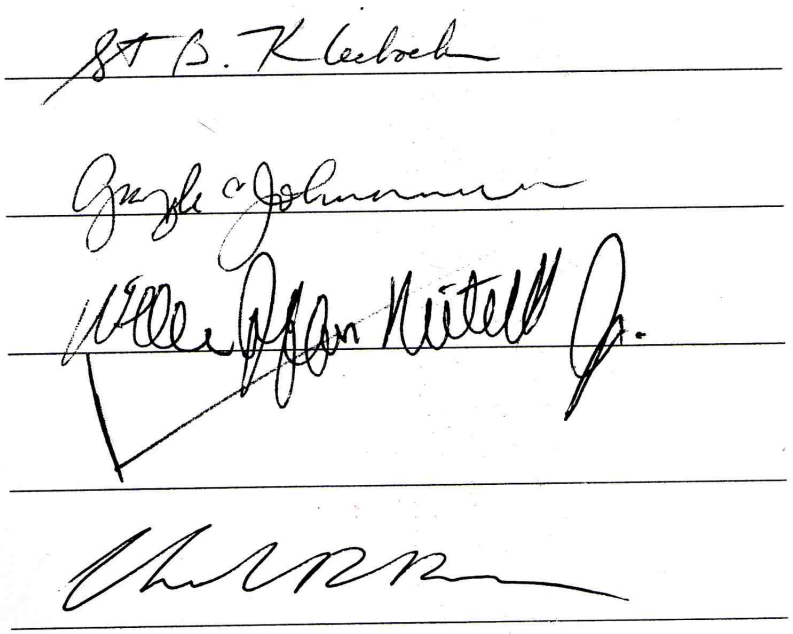
The undersigned, appointed by the Dean of the Graduate School,
have examined the dissertation entitled

THE ROLE OF MACROPHAGES AND ANTI-VIRAL ANTIBODIES IN WEST NILE VIRUS PATHOGENESIS

Presented by David Garcia-Tapia

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worth of acceptance.



DEDICATION

A Giseel:

Gracias por todo tu amor y tu incondicional apoyo. Recuerda que esto no seria posible sin tu ayuda.

Te amo

A Giselle, Daniela Alejandra y David:

Gracias por cada momento de felicidad que ustedes traen a mi vida, el tener su amor me da el animo necesario para seguir trabajando. Recuerden que ustedes son la bendición más grande que Dios me ha dado. Perdón por el tiempo que les he robado para poder terminar este trabajo

Les amo

A Pilar y David (mis padres):

De no haber sido por todo su amor, su ejemplo, sus enseñanzas y por sus sacrificios jamás hubiera crecido en mi la intención de estudiar y terminar lo empezado. Gracias porque aunque soy un adulto ustedes siguen mostrando su amor y su preocupación, y sobre todo, me siguen enseñando.

Les amo

A Homero, Melissa, y Claudia (mis hermanos):

Cada uno de los recuerdos maravillosos de nuestra infancia y el amor que entre nosotros sentimos me recuerdan a cada instante que no estoy solo y que he sido grandemente bendecido al tenerles como mis hermanos.

Les Amo

ACKNOWLEDGEMENTS

I would like to express my gratitude to my dissertation supervisor Dr. Steven B. Kleiboeker, because without his support this dissertation would not be possible. Dr. Kleiboeker, thanks for all the help, all the teaching and especially for your great patience during the time that I worked as your student. I am especially grateful for the opportunity to work and to be trained in your laboratory. I am also grateful to Sang Myeong Lee and Susan Schommer for all their kindness and help since the day I joined to Dr. Kleiboeker's lab. I extend my gratitude to Dr. Andria Cogswell for her great help providing blood from her horses. I would like to extend appreciation to Dr. Daniel Hassett for the invaluable help I received during the development of the last experiment in this dissertation. I appreciate the support that I received from the Veterinary Medical Diagnostic Laboratory, during the time in which I was finishing these experiments. I would like to express my gratitude to my committee members: Drs. Gayle C. Johnson, Charles Brown, Philip Johnson and William J. Mitchell Jr., for their flexibility and for all their guidance. My deepest appreciation is for Giseel, my wife, who has supported me without any complaint during the last ten years, in which she has been practically alone taking care and educating our three children. Moreover, I show my love and gratitude to Giselle, Daniela Alejandra and David Ammon, my three children, who quietly accepted being away from me while I was working on this dissertation. Finally, I want to express my gratitude to my parents, thanks for your love, teachings and great support.

Study financial support was provided through the study loan CONACyT-94100 by Consejo Nacional de Ciencia y Tecnologia-Mexico.

TABLE OF CONTENTS

ACKNOLEDGEMENTS…….. i

LIST OF FIGURES……...… v

LIST OF TABLES……...…….. ix

LIST OF ABBREVIATONS…….....… x

ABSTRACT…… xii

Chapter I.
Review of literature……... 1

I. Introduction…….... 1
II. Epidemiology……. 1
III. Genome and Structure…… 2
IV. Ecology……..… 5
V. WNV and birds……..… 7
VI. WNV and horses……..... 7
VII. WNV and humans……..… 8
VIII Transmission……..… 9
IX West Nile virus: Pathogenesis and immunity……..….. 11
A. Viral pathogenesis……..... 11
B. Innate immunity……..... 16
C. Adaptive immunity……... 22
D. Antibody-dependent enhancement…….…….. 28

Chapter II.
Replication of West Nile virus in equine peripheral blood mononuclear cells 35

I. Introduction……..….. 36
II. Material and Methods……...…. 38
A. Virus cell culture……... 38
B. Plaque reduction neutralization assay……... 40
C. RNA extraction and quantitative RT-PCR…… 40
D. Preparation of peripheral blood mononuclear cell cultures……..… 41
E. Sorting of cell populations……..... 43
F. Assessment of cell purity by flow cytometry……..... 44
G. Fluorescent antibody detection of WNV non-structural protein and Identification of cell type……..…. 45

H. Detection of NF-κB translocation plus nitric oxide and type I interferon production following LPS treatment of adherent cells and sorted monocytes……..…… 45
III. Results……...……. 47
A. Anti-WNV serologic values in horses used as blood donors……... 47
B. WNV replication in equine peripheral blood mononuclear cells….. 47
C. Dual detection of WNV antigen and CD antigen in infected cells... 49
D. WNV in replication in LPS-treated monocytes…… 66
IV. Discussion…….. 71

Chapter III.
Induction of Antibody-dependent enhancement (ADE) by sub-neutralizing dilution of immune horse serum in an *in vitro* infection of horse macrophages and murine TIB-186 cell line by West Nile virus………... 77

I. Introduction……..... 78
II. Material and Methods……..….. 81
A. Virus and cell culture……..…….. 81
B. Purification of equine monocytes by cell sorting……..… 83
C. Assessment of antibody-dependent enhancement of infection……. 85
D. Statistical analysis……..…... 85
III. Results……..….. 86
A. The effect of anti-WNV horse serum on WNV I and WNV II viral yields following inoculation of Vero, TIB-186 or equine monocyte cultures……..... 86
IV. Discussion……..… 87

Chapter IV.
Evaluation of WNV pathogenesis and effect of transference of a sub-neutralizing dilution of immune horse serum in an *in vivo* infection by West Nile virus………...…….. 95

I. Introduction……...… 96
II. Material and Methods……..….. 100
A. Virus cell culture……...… 100
B. Mice…….. 101
C. Experimental design……..… 101
D. Tissue homogenization……..... 102
E. RNA extraction, quantitative RT-PCR and RPA……..……..….. 103
F. Plaque reduction neutralization assays……..…... 105
G Immuno fluorescent antibody test …….……..…. 105
H. Histology and immunohistochemistry…….. 106
III. Results……..…..109
A. Evaluation of ADE in a WNV *in vivo* infection……..…. 109
B. Chronology of the pathogenic and immunologic events following infection of C57BL/6 mice with West Nile virus……...…. 125

B.1. Day 1 P.I……..... 125
B.2. Day 4 P.I……..... 126
B.3. Day 6 P.I……..... 129
B.4. Day 9 P.I……..... 131
B.5. Day 13 P.I……..… 136
IV. Discussion……...… 139
A. Evaluation of ADE in a WNV *in vivo* infection……... 139
B. Chronology of the pathogenic and immunologic events during the infection of C57BL/6 mice with West Nile virus……...… 149

Chapter V.
General discussion……..... 155

I. Discussion……...… 155
II. Recommendations for future research……...…. 161

REFERENCES……..…. 162

VITA……...…. 188

LIST OF FIGURES

Figures **Page**

Chapter I.

1.1 Distribution of WNV in North America……..... 3

1.2 West Nile virus biological cycle…..……..…. 6

1.3 West Nile crossing the blood-brain barrier..……..... 24

Chapter II.

2.1 Viral growth curve for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Horse 1……..……..…. 51

2.2 Viral growth curve for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Horse 2……..……... 52

2.3 Viral growth curve for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Horse 3……..……... 53

2.4 Viral growth curve for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Horse 4……..……... 54

2.5 West Nile virus (WNV) RNA concentration in equine peripheral blood mononuclear cells (PBMCs). Horse 1…….. 55

2.6 West Nile virus (WNV) RNA concentration in equine peripheral blood mononuclear cells (PBMCs). Horse 2……..……..... 56

2.7 Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 1..... 57

2.8 Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 2……. 58

2.9 Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 3…..... 59

2.10 Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 4……. 60

2.11 Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 5……. 61

2.12 Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 6……. 62

2.13 Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 7……. 63

2.14 Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 8..... 64

2.15 Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 9..... 65

2.16 Dual fluorescent antibody detection of West Nile virus (WNV) non-structural protein 1 (NS-1) and CD-antigen of infected cells……..... 68

2.17 Effect of bacterial lipopolysaccharide (LPS) on West Nile virus replication in monocytes……..…….……..……..…. 69

2.18 Nitric oxide (NO) and type I interferon (IFN) production plus NF-κB translocation in LPS-treated or untreated cells inoculated with WNV……..… 70

Chapter III.

3.1 ADE in Vero's cells infected with WNV……..…….. 88

3.2 ADE in TIB-186 cells infected with WNV……... 89

3.3 ADE in horse monocyte-derived macrophages infected with WNV… 90

3.4 ADE in horse monocyte-derived macrophages infected with WNV… 91

Chapter IV.

4.1 Antibody dependent enhancement in an *in vitro* infection of TIB-186 cells by West Nile virus……. 110

4.2 Viral growth curves for West Nile virus (WNV) in mice…….…..... 115

4.3 Mortality in mice infected with West Nile virus……..……. 116

4.4 Titers of anti-WNV antibodies in WNV-infected mice……..…… 117

4.5 Quantification of IFN-γ expression in spleens from mice infected with WNV……..… 119

4.6 Quantification of iNOS expression in spleens from mice infected with WNV…….....120

4.7 Quantification of IL-12 expression in spleens from mice infected with WNV……..… 121

4.8 Detection of cytokine expression on brain from infected mice……. 122

4.9 Detection of chemokine expression on brain from infected mice……. 123

4.10 Main features of the pathology of WNV infection in mice

on day 1PI……...……... 127

4.11 Main features of the pathology of WNV infection in mice

on day 4PI……..……..….. 128

4.12 Main features of the pathology of WNV infection in mice

on day 6 PI……..……..……. 130

4.13 Mortality in mice infected with West Nile virus in experiments

1 and 2……..……..133

4.14 Main features of the pathology of WNV infection in mice

on day 9 PI……..….... 134

4.15 Histopathological changes in a WNV-infected C57BL/6, which

succumbed to the disease……..……. 137

4.16 Main features of the pathology of WNV infection in mice

on day 13 PI…….……..…… 142

4.17 Cytokine expression on brain from naive and infected mice……..... 144

4.18 Chemokine expression on brain from naïve and infected mice…….....145

LIST OF TABLES

Table **Page**

Chapter II.

2.1 Serologic values and West Nile virus titers following inoculation of sorted immune cells……...…… 50

2.2 Purity of cell population following sorting by specific antibody and magnetic beads……..…. 66

Chapter IV.

4.1 Experimental design…….……. 107

4.2 Sequences of primers and probes used for the detection of the expression of murine cytokines and iNOS by RT-PCR……..….. 108

LIST OF ABBREVIATIONS

ADE, antibody-dependent enhamcement
APC, antigen-presenting cells
BSA, bovine serum albumin
BSL-3, biosafety level 3
CD, cluster of differentiation
CNS, central nervous system
ConA, concanavalin A
COX, cyclooxygenase
CR, complement receptor
DAB, diaminobenzidine
DC, dendritic cell
DC-SIGN, dendritic cell specific ICAM3 grabbing nonintegrin
DHF, dengue hemorrhagic fever
DMEM, dulbecco's minimal essential medium
ER, endoplasmic reticulum
FACS, fluorescence activated cell sorting
FBS, fetal bovine serum
FcR, Fc receptor
FDA, food and drug administration
FIPV, feline infectious peritonitis virus
FITC, fluorescein isothiocyanate
HIV, human immunodeficiency virus
HLA, human leukocyte antigen
HP, histopathology
IFA, immunofluorescence antibody
IFAT, immunofluorescence antibody test
IFN-α, Interferon alpha
IFN-ß, Interferon beta
IFN-γ, Interferon gamma
IgM, immunoglobulin M
IgG, immunoglobulin G
IHC, immunohistochemistry
HIS, immune horse serum
IL, interleukin
INDO, indoleamine 2,3 dioxygenase
iNOS, inducible Nitric Oxide synthetase
IRF, interferon regulatory factor
ISGs, interferon stimulated genes
ITIM, immunoreceptor tyrosine-based inhibition motif
JAK1, janus kinase 1
JEV, Japanese encephalitis virus
LPS, lipopolysaccharide

MCP, monocytes chemoattractant protein
MHC, major histocompatibility complex
MIG, monokine induced by interferon gamma
MIP, macrophage inflammatory protein
MOI, multiplicity of infection
mRNA, messenger RNA
MVEM, Murray Valley encephalitis virus
NFκB, nuclear factor κ B
NHS, normal horse serum
NK, natural killer
NO, nitric oxyde
NS, non structural protein
OAS, Oligoadenylate synthetase
PBMC, peripheral blood mononuclear cells
PFU, plaque formation units
PrM, precursor protein M
PRNT, plaque reduction neutralization assay
PRRS, porcine respiratory and reproductive syndrome
RPA, RNAse protection assay
RT-PCR, reverse transcriptase-PCR
SAT, supermidine/spermineN1-acetyltransferase
SDHC, succinate dehydrogenase SLE
SOCS, suppressor of cytokine signaling
STAT, signal transduction and activation of transcription
TAP, transporter associated with antigen processing
TBEV, tick-borne encephalitis virus
TCR, T cell receptor
TGF-ß, transforming growth factor
Th, T helper
TLR, toll-like receptor
TNF-α, tumor necrosis factor alpha
TNFSF14 tumor necrosis factor superfamily
TRAF TNF receptor-associated factor
WNV West Nile virus
WNV I West Nile virus lineage I
WNV II West Nile virus lineage II
WNV-E, West Nile virus envelope protein

THE ROLE OF MACROPHAGES AND ANTI-VIRAL ANTIBODIES IN WEST NILE VIRUS PATHOGENESIS

David Garcia-Tapia

Dr. Steven B. Kleiboeker, Dissertation Supervisor

Abstract

Since the introduction of West Nile virus into the United States in 1999, the virus has spread throughout the continental territory of the United States and the virus has moved into Mexico and Canada. The emergence of this flavivirus in North America has resulted in an intense interest in its study, leading to research efforts focused on the pathogenesis, possible treatments and methods for prevention of the disease. In the present studies, we demonstrate the ability of WNV to productively infect horse monocytes, CD4+ T lymphocytes and monocyte-derived macrophages. Along with these findings we report the ability of immune horse serum to induce antibody dependent enhancement of WNV infection of horse macrophages and mouse macrophages *in vitro*. The question of ADE *in vivo* was also addressed, and it was found that sub-neutralizing dilutions of anti-WNV immune horse serum which induce ADE *in vitro* fail to induce the same effect *in vivo*. The serum induces protection, which is perhaps driven by up-regulation of IL-12 in spleen during the earliest phase of infection. In brain, the chemokines MIG, MCP-5 and IP-10 were secreted earlier than other chemokines and cytokines, suggesting that those chemokines play an important role in the initial stages of the encephalitis caused by WNV. High titers of virus in blood and spleen, as well as the ability of the virus to infect horse monocytes and CD4+ lymphocytes suggest that this virus spreads from subcutaneous tissue to the brain by a hematogenous route.

CHAPTER I

REVIEW OF LITERATURE

I. Introduction

The recent introduction of the West Nile virus into the United States has led to a growing interest in the development of novel prevention and treatment strategies for disease caused by this virus. Since its introduction in 1999, WNV has spread throughout the continental United States with surprising speed. The number of the cases occurring in the last 5 or 6 years has led to a growing interest in the impact on public health and the equine industry. This interest has led to research efforts, which are mainly focused on development of vaccines or possible treatments for patients suffering clinical disease caused by this virus. To best identify vaccine candidates or effective therapeutic agents, it is necessary to understand the biology and pathogenesis of the WNV, as well as the immunological responses induced following infection.

II. Epidemiology

West Nile virus is a positive sense, single-stranded RNA virus that is grouped in the Flavivirus genus of the Flaviviridae family. This virus is member of the Japanese Encephalitis serocomplex, which also includes Japanese Encephalitis, Saint Louis Encephalitis, Murray Valley Encephalitis and Kunjin viruses. The virus was first isolated from serum of a febrile Ugandan woman in 1937. Since its original isolation, the virus had been identified as the cause of sporadic outbreaks of mild viral illness, mainly in Africa, Middle East, western Asia and Australia (Kunjin virus subtype) (Smithburn et al.,

1970; Gould and Fikrig, 2004). Two genetic lineages have been identified, and in 2005 Bakonayi et al. suggested that there is a third lineage, which has been isolated in Central Europe (Berthet et al., 1997; Bakonyi et al., 2005). Viruses in lineage 1 are primarily of West African, Middle Eastern, Eastern European and Australian origin. Lineage 2 is composed exclusively of African isolates and has been reported to be less pathogenic than those classified in lineage 1 (Lanciotti et al. 1999). In 1960 infection of horses was described for the first time. In 1974, 10,000 clinical cases of West Nile fever in horses were reported in South Africa (Komar, 2003a). Between 1996 and 1999 the virus was identified as major cause of encephalitis in horses in Romania, Italy, France and Israel (Castillo-Olivares et al., 2004). In 1999 WNV was first isolated in the State of New York in the United States. Currently the virus has been identified throughout the United States (Anonymous, 1999; Marfin and Gubler, 2001). In Mexico seropositive birds was reported in 2003 in the northern state of Tamaulipas and seropositive horses were identified in the southern state of Yucatan (Fernandez-Salas et al., 2003; Lorono-Pino et al., 2003). Antibodies against the virus have been identified in birds from Jamaica (Dupuis et al., 2003), Dominican Republic (Komar et al., 2003), and Guadeloupe (Quirin et al., 2004), suggesting the potential of the virus to spread throughout the entire American continent and the Caribbean islands. The current distribution in North America is represented in the Fig. 1.1.

III. Genome and structure

Flavivirus virions are spherical, 50 nm in diameter and consist of a tightly adherent lipid envelope covered with indistinct peplomers surrounding a spherical

FIG 1.1. Distribution of WNV in North America. The shaded areas represent the zones where WNV activity has been detected. 1) Chihuahua, 2) Coahuila, 3) Tamaulipas, 4) Veracruz, 5) Tabasco, 6) Yuctan**. (Adapted from Gould and Fikrin, 2004).**

nucleocaspid with icosahedral symmetry (Murphy, 1999; Mukhopadhyay et al. 2003). The genome consists of a single linear positive-sense single-stranded RNA 10.6 to 10.9 Kb in length. The genomic RNA itself is infectious. The 5' end of the genome is capped and the 3' end is not polyadenylated, but instead has a 3'-hairpin loop. In WNV this loop

is composed of the final 84 nucleotides, which form a double-stranded structure (Markoff, 2003).

The viral genome contains a single open reading frame that encodes for 10 proteins. The virion is made up of three structural proteins, which are encoded in the 5' end of the genome while the non-structural proteins are encoded in the 3' end of the genome. The structural proteins are: C, the nucleocapsid protein; PrM, a glycosylated precursor that is cleaved to produce the M transmembrane protein; and E, the envelope protein. The flaviviruses have seven or eight non-structural proteins: NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5. The NS5 protein is the RNA-dependent RNA polymerase, and the NS3 functions as a helicase and protease and is part of the RNA polymerase complex. NS3 is responsible for a majority of viral protein cleavages, although host proteases are responsible for some viral protein cleavage as well (Mukhopadhay, et al. 2003). The E protein has been identified as the outer most virion protein, and has been associated as key player in the virus-cell adhesion and invasion processes (Deubel et. al., 2001).

Flavivirus replication occurs in the perinuclear region of the endoplasmic reticulum (ER) of infected cells. As replication progresses, infected cells become vacuolated and hypertrophic. Translation of the poly-protein occurs in association with the rough ER and results in production of the viral proteins PrM, E and NS1. The nascent virion is then translocated into the lumen of the ER. The virions are transported through the secretory pathway to the cell membrane (Barth et al., 1999). Cleavage of PrM occurs during the final stage of exocytosis, resulting in production of mature virions. Replication

of flaviviruses may be lytic for the host cells and lead to syncytium formation, or alternatively may result in persistent non-cytopathic infection (Deubel et al., 2001).

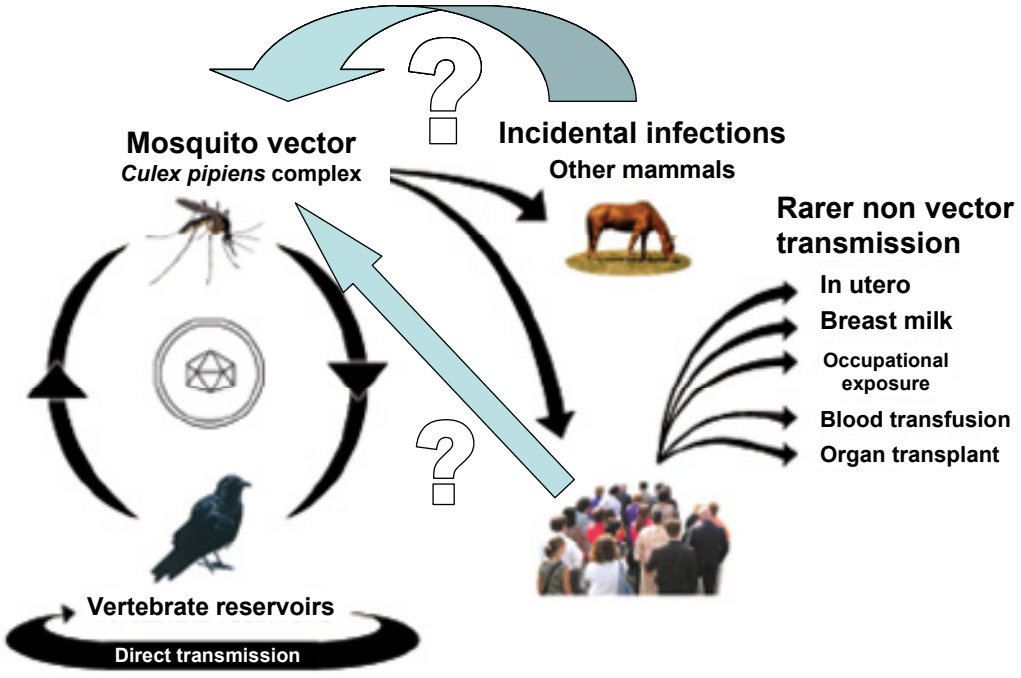
IV. Ecology

West Nile Virus is considered one of the old world *Culex*-vectored Flaviviruses. The virus is successfully transmitted by mosquitoes and has been isolated from both Argasid and Ixodid ticks, although the ability of ticks to successfully transmit the virus at ecologically significant levels in nature is unclear (Monath and Heinz, 1996; Anderson et al., 2003; Mumcuoglu et al., 2005). The major mosquito vector in Africa and Middle East is *Culex univittatus*; with *Aedes albocephalus* or *Mimomyia spp* as important vectors in some areas. In Europe, *Culex pipens*, *Culex modestus* and *Coquillettidia richiardii* are important vectors. In Asia *Culex tritaeniorhyncus*, *Culex visnui* and *Culex quinquefasciatus* are the predominate WNV vectors (Mackenzie et al., 2004). In the United States, more than 40 species of mosquitoes have been identified as possible vectors. However, only *Culex pipens*, *Culex tarsalis*, *Culex restuans* and *Culex quinquefasciatus* are considered important for the transmission of the virus between birds, although a bridge vector between the bird and human or mammal cycles has not been identified (Gould and Fikrig, 2004).

The primary and amplifying hosts of West Nile virus are various species of birds due to the high level of viremia that develops after infection. Mosquitoes obtain the virus from viremic birds and in turn infect other vertebrates, including horses and humans. These two species develop low viremic titers and therefore are considered not to be

significant hosts for virus amplification. However, Higgs et al. recently reported non-viremic transmission of West Nile virus, by using infected and non-infected *Culex pipens* mosquitoes fed in the same mouse. The mice never developed detectable viremia and the non-infected mosquitoes became infected after the co-feeding with their infected counterparts (Higgs et al., 2005). WNV infection has also been reported in other vertebrate species, including bears, crocodiles, alligators, and bats (Gould and Fikrig, 2004). Figure 1.2 depicts the biological cycle of the virus, including the proposed

FIG 1.2. West Nile virus biological cycle. Shown are demonstrated routes of transmission and proven biological cycles of WNV. The gray arrows with the question marks are proposed routes based experimental transmission of WNV to mosquitoes from non-viremic mice during co-feeding experiments. (**Adapted from Gould and Fikrin, 2004).**



transmission from non- viremic animals, which would suggest a more active role of horses and humans as source of virus to the mosquitoes.

V. **West Nile virus and Birds**

West Nile virus has been isolated from 198 different species of birds through 2002, and mortality may approach 100% in some species. The Passerine birds, including crows, domestic sparrows and blue jays are considered the most important amplifying hosts due to the high and lasting viremia that develops (Komar 2003a; Komar et al., 2003b; Weingartl, et.al., 2005). In Mexico viral activity was demonstrated in four different species of birds (Fernandez-Salas et.al., 2003). West Nile virus infected birds present a broad spectrum of clinical outcomes ranging from inapparent infection to death. The general signs of infection include lethargy, recumbency and hemorrhage (Komar et al., 2003a). The main gross findings in infected birds are vascular congestion in the spleen and brain and hemorrhage in the brain. Microscopically, meningoencephalits, splenitis with necrosis of lymphocytes and mild myositis and myocarditis are observed (Weingartl et al., 2004). In experimental infections of several avian species, numerous cell types were damaged in various tissues. Purkinje cells were particularly targeted, except in corvids. The cause of death in the infected birds is probably multiple organ failure (Steele et al., 2000).

VI. West Nile virus and Horses

In horses, as well as other domestic equids, the clinical course following infection with West Nile virus ranges from unapparent illness to severe encephalitis and death. The

incubation period in horses is 5 to 15 days. Bunning et al. reported that only 10% of experimentally infected horses developed encephalitis (Bunning et al., 2002). The case fatality rate in horses with clinical disease may exceed 30%. During outbreaks, the seroprevalence in horses without clinical signs of encephalomyelitis can reach 8% (Ward et al., 2004). In naturally infected horses with neurological signs, the gross pathologic changes are characterized by petechiae in brain and in the cervical section of the spinal cord. Microscopically, moderate to abundant numbers of macrophages and lymphocytes are observed in the perivascular spaces of the cerebral cortex and in leptomeningeal spaces. The infected lymphocytes were mainly CD3+ (T cells), and WNV antigen was detected in some monocytes sequestered in the perivascular spaces of the brain (Cantile et al., 2003). In the animals which develop clinical signs, these were characterized by attitudinal changes such as somnolence, listlessness, apprehension, depression, or hyperexcitability; and by neurological signs, including muscle fasciculations and limb paresis or paralysis.

VII. West Nile virus and Humans.

In humans, as in other species, infection by WNV has a broad spectrum of clinical manifestations ranging from subclinical infection to death. The incubation period is typically 2 to 6 days, although the beginning of clinical signs can be observed as late as 21 days post-infection even in immunocompromised individuals. Cases without neurological signs are termed West Nile fever, which occurs in approximately 20 to 30% of the infected people. This is characterized by high fever with chills, malaise headache backache, arthralgia, myalgia and retroorbital pain. Other non-specific signs include

nausea, vomiting, diarrhea, cough, and sore throat or pharyngitis. In the cases presented in Africa, a generalized lymphadenopathy is a characteristic sign of the disease; but in the cases presented in North America, this sign is uncommon. Myocarditis, pancreatitis and hepatitis have been reported in severe infections (Solomon et al., 2003; Komar, 2003a). The neurological presentation of the disease is known as West Nile encephalitis. It has been reported that in humans, approximately 1 in 150 infections lead to meningitis or encephalitis, characterized by movement disorders such as tremor, myoclonus, poliomyelitis symptoms with flaccid paralysis, Guillain-Barré syndrome, profound muscle weakness, and incomprehensible speech (Mckenzie et al., 2004; Huang et al., 2002.). Pathologic studies in fatal human cases revealed lesions similar to those observed in Japanese encephalitis virus infections. These include formation of microglial nodules and perivascular cuffing in the brain parenchyma, variable necrosis and loss of neurons. The deep gray nuclei of the brainstem and spinal cord appear to be the most severely affected. Patients with flaccid paralysis have perivascular lymphocytic infiltration of the spinal cord, microglial nodules, and loss of anterior horn cells (Guarner et al., 2004). Inflammation in the spinal cord was observed in 17 of 23 people who died with WNV neuroinvasive disease (Hayes et al., 2005a).

VIII. Transmission

The most common route of West Nile virus transmission is by natural vectors, which are mainly mosquitoes of the genus *Culex*. Transmission is seasonal in temperate zones of North America, Europe and the Mediterranean Basin, with peak activity between the months of July and October (Hayes et al., 2005a). An interesting

phenomenon is presented in tropical areas such as the Caribbean and Mexico, in which the temperatures allow the mosquitoes to survive throughout the year. Due to this occurrence, it would be expected that higher rates of transmission would be present and therefore a higher number of human, equine and avian cases of West Nile virus disease would present. However, since 2003 the data reveal that the incidence of human or equine cases is much lower than in the United States. WNV isolated from a bird in Mexico in 2003 appeared to be attenuated, but whether the attenuation of the virus is related to the low number of cases has not been determined (Beasley, 2004).

Since the outbreak in United States in 1999, alternative routes of WNV transmission have been described. In 2003 Biggerstaff and Petersen calculated that the risk of West Nile virus transmission by blood transfusion was between 1.46 and 12.33 cases per 10,000 donations (Biggerstaff and Petersen, 2003). In the same year Mather et. al. reported that the virus survived in RBC held at 1 to 6º C for up to 42 days (Mather et. al., 2003). In addition, it was observed that the virus was equally distributed in all blood fractions, including plasma and PBMC's (Harrington et al., 2003). Although the virus might be contaminating whole blood or blood fractions, it has also been reported that the virus is susceptible to PEN110 inactivation (Mather et al., 2003), and to the usual viral inactivation procedures, for instance, pasteurization (which is the most effective) and low pH treatment. WNV was found to be more resistant than other viruses to liophilization (Kreil et al., 2003). Based on these results, the FDA stipulated that development and implementation of WNV screening assays should be a priority for the transfusion and organ transplant procedures (Hollinger et al., 2003). In 2002, WNV was identified in one

transplant donor and in four recipients. The donor was infected with the virus after a blood transfusion. Three of the organ recipients developed encephalitis and one suffered West Nile fever (Iwamoto et al., 2003).

The first case of intrauterine transmission was reported in 2002. A pregnant woman acquired the virus in her second trimester of gestation, and subsequently transmitted it to her fetus. The infant was born at term with disseminated choriomeningitis and severe cerebral abnormalities. Transmission of WNV through breast milk has also been demonstrated. The lactating mother acquired the virus through blood transfusion (Gould and Fikrig, 2004). Occupational exposure has been reported in laboratory workers and in workers at a turkey farm in Wisconsin (Gould and Fikrig, 2004). The recent description of new or uncommon ways of West Nile virus transmission, indicates that although the virus was described for first time almost 50 years ago, there are still aspects of the biology and pathogenesis of the virus that remain unknown.

IX. West Nile virus: Pathogenesis and Immunity

A. Viral pathogenesis

West Nile virus is a flavivirus with an exceptionally broad host range which includes mosquitoes, birds, reptiles and mammals, including humans. Although WNV has acquired a significant importance in public health, little is known about the mechanisms of cellular invasion, including the cellular receptors for this virus. In other flaviviruses, domain III of the E protein is a key player in the adhesion and invasion to

Vero and C36/C36 cells. This domain displays a series of ß-sheets and an immunoglobulin-like structure (Chu et al., 2005). Domain III of the WNV-E protein binds to the cellular receptor alpha V beta 3 integrin (αVß3) on the membrane of mosquito and mammalian cells (Lee et al., 2005; Chu et al. 2004). Koschinski et al. demonstrated that after the virus attaches to the plasma membrane, it produces microscopic ion-permeable pores in a pH-dependent manner, which then allows transit of protons from the endosome into the cytoplasm during the viral entry, facilitating cell invasion (Koschinski et al., 2003). Moreno-Altamirano reported four membrane proteins (27, 45, 67 and 87 kDa) from human monocyte-derived macrophages, which have the ability to bind to dengue virus 2, and suggested that those proteins may serve as putative receptors for that flavivirus (Moreno-Altamirano et al., 2002). In 2003, Tassaneetrithep and collaborators reported the use of DC-SIGN (CD209) by dengue virus as a receptor for entry into human dendritic cells (Tassaneetrithep et al., 2003). To date no report has been made to explore the role of DC-SIGN in the infection of dendritic cells by West Nile virus. Although there are reports describing migration of Langerhans cells to local lymph nodes after cutaneous infection with West Nile virus (Johnston et al., 2000), there are no reports about the ability of the virus to infect this or other lineages of dendritic cells.

Once the virus has entered host cells, WNV triggers either up- or down-regulation of several important genes that are important players in the immune response against the virus. It is known that the processing of antigens to be presented in an MHC class I pattern is a key process for developing a cellular immune response. This process is

altered by flaviviruses, including the West Nile and dengue 2 virus. Both viruses alter the activity of the peptide transporter associated with antigen processing (TAP) to the endoplasmic reticulum (ER), and this activity is increased by up to 50% compared to the transport activity in the uninfected cells. Up-regulation of the transport of TAP is dose dependent following *in vitro* infection of Hela cells by WNV or dengue virus 2; however, protein expression is not increased during infection (Momburg et al., 2001). This effect on TAP transportation into the ER is probably related to up-regulation of the expression of HLA-A, -B, and -C genes, which was demonstrated by Arnold, et al. (2004) using an *in vitro* model of infection of human fibroblasts by WNV (Arnold et. al., 2004). Cheng and collaborators (2004a) reported up-regulation of MHC class I molecules in an interferon receptor knock-out mouse embryo fibroblast, which can not respond to type I IFN (Cheng et al., 2004a). These authors suggest that the WNV induces up-regulation of MHC-I by the activation of NFκB in an IFN-independent way (Kesson and King, 2001). These results were confirmed later by Cheng and collaborators (2004b), who demonstrated activation of NF-κB by WNV in TNF-deficient murine embryo fibroblasts, meaning that this virus activates NF-κB in a TNF-independent manner (Cheng et al., 2004b). Based on these results it is unclear why a virus up-regulates expression of MHC class I molecules if these proteins are involved in viral antigen presentation to NK cells. One possible explanation is to induce a weak activation of NK cells which in turn will cause a diminished cellular response, and therefore allow the virus to evade the immune response. In an *in vitro* model of WNV infection using human umbilical cells, expression of E-selectin (ELAM-1, CD62E), ICAM-1 (CD54), VCAM-1 (CD106) was significantly increased. The early enhanced expression of VCAM-1 suggests a sequential adhesion of

leukocyte subpopulations to infected endothelial cells, which may be important in initial viral spread *in vivo* (Shen et al., 1997).

Type I interferons play a key role in the innate immune response against viral infections. These molecules, particularly IFN-α, induce expression of other genes (interferon-stimulated genes [ISG's]), which are effectors of the anti-viral cellular response (Samuel, 2001). Type I IFN's are also affected by flaviviral infections. It has been demonstrated that the IFN-ß promoter is inhibited by NS2A of Kujin virus (Liu et al., 2004). The same virus is able to reduce phosphorylation of STAT-1 and STAT-2, as well as block phosphorylation of JAK1 and Tyk2 (Guo et al., 2005). The same effects are observed in human cells infected *in vitro* by the flavivirus dengue virus and in BHK-21 cells infected by Japanese encephalitis virus. The NS4A and NS2A proteins of dengue may be involved in this inhibitory effect (Munoz-Jordan et al., 2003). All the molecules mentioned (STAT-1, STAT-2, JAK1 and Tyk2) are of major importance in the signal transduction pathway of IFN-α, which stimulates the expression of ISG's. It has been suggested that WNV replication in human cultured cells (HeLa) interferes with both the response to IFN and synthesis of IFN-ß in response to dsRNA by blocking nuclear translocation of IRF3 and inhibition of IRF3 dimerization (Scholle and Mason, 2005).

Up-regulation of indoleamine 2,3 dioxygenase (INDO) in WNV-infected A172 cells was demonstrated by Koh and Ng (2005). Increased production of INDO by glial cells causes neuronal injury in neuroinflammatory diseases, suggesting that elevated production of this molecule plays a role in the viral pathogenesis in the CNS. Another

important effect of WNV is reduction in the activity of some genes belonging to energy synthesis pathways. These genes include succinate dehydrogenase (SDHC), cytochrome c oxydase (COX5B/COX6B) and various genes of the ATP synthase complex (ATP5G1, ATP5C1, ATP5J, ATP5B, ATP5A1, ATP5O, and ATP5F1). Decreased energy production from down-regulation of these genes is known to cause severe neurodegeneration (Koh and Ng, 2005).

There are several reports concerning the ability of flaviviruses to induce apoptosis have been made. It has been reported that dengue virus adapted to neural tissue will induce apoptosis in the mouse CNS (Despres, 1998). Embryonic stem cells differentiated to a neuronal phenotype have been used to evaluate the neurotropism of WNV and its ability to replicate and induce apoptosis in neurons (Shrestha et al., 2003). Mongkolsapaya et al. (2003) suggested that dengue virus induced apoptosis in CD8+ T cells (Mongkolsapaya et al., 2003). These authors and others have speculated that induction of apoptosis in these cells plays an important role in development of dengue hemorrhagic fever. Another flavivirus, Japanese encephalitis virus, induced apoptosis in BHK-21 cells, N18 murine neuroblastoma cells and in human neuronal progenitor N12 cells (Lin et al., 2004). According to Liao and collaborators (1997), the apoptosis process is not affected by expression of bcl-2 (Liao et al., 1997). Apoptosis is also induced by WNV, as was demonstrated following *in vitro* infection of the K562 and neuro2a cells, a system in which apoptosis was caused by an up-regulation of bax (Parquet et al., 2001). In Vero cells, apoptosis seems to be induced by the release of cytochrome C, which then led to the activation of caspase-9 and caspase-3 (Chu and Ng, 2003). Other authors have

shown that WNV induced apoptosis in murine neurons through the caspase-9 pathway *in vivo* (Yang et al., 2002). Other genes involved in the apoptotic process are up-regulated in an *in vitro* infection of glial A172 cells by WNV. These genes include the tumor necrosis factor superfamily (TNFSF14), nuclear factor of κ light-chain gene (NFκB1A), TNF receptor-associated factor (TRAF1), and supermidine/spermine N1-acetyltransferase (SAT) (Koh and Ng, 2005).

B. Innate immunity

Complement was the first component of the innate immune response that was reported to have an important role in protection against WNV. Complement controls WNV infection *in vitro* primarily in an antibody-dependent manner by neutralizing viral particles in solution and lysing WNV-infected cells. The importance of complement *in vivo* was demonstrated by infection of knock-out mice which lacked the complement receptors CR1 and CR2. These mice developed increased CNS viral loads and were more susceptible to lethal infection at low doses of WNV (Mehlhop et al., 2005). Meyer et al. (2002) reported the potential function of complement as an enhancer of antibody function against flaviviruses after observing increased neutralization properties of the antibodies in an *in vitro* infection of human cells by the flavivirus hepatitis C virus (Meyer et al., 2002).

In vitro and *in vivo* studies have demonstrated that interferon-dependent responses are relevant to protection against flavivirus infections. Libraty and collaborators reported the migration of Langerhans cells to the draining lymph nodes and following WNV

infection, and these cells may be the first source of type-1 interferon after infection by WNV (Libraty et al., 2001). IFN has antiviral activity that functions by preventing translation and replication of viral RNA through different mechanisms, which include RNase L-, Mx1- and Protein Kinase R (PKR)-independent mechanisms (Anderson and Rahal, 2002). Mice retreated with IFN-α or its inducers exhibit a diminished or ameliorated disease when infected with flaviviruses (Brooks and Phillpotts, 1999; Charlier et al., 2002; Lucas et al., 2003). Mice that are deficient in IFN receptor (IFN-α-R -/-) showed an uncontrolled extraneural viral growth, rapid virus entry into the brain and 100% mortality when infected with Murray Valley encephalitis virus (Lobigs et al., 2003). Humans experimentally treated with IFN-α demonstrated a substantial improvement during WNV-induced encephalitis (Kalil et al., 2005). It has also been reported that type I interferons have a critical function in resolving primary infection of dengue virus by limiting the initial replication in extraneural sites and controlling subsequent viral spread into the CNS (Shresta et al., 2004). Recent studies suggested that IFN-α/ß controls WNV infection in mice by restricting tropism and viral burden and by preventing the death of infected neurons. Genetically modified mice that lack of IFN- α/ß receptors (IFN- α/ßR$^{-/-}$) showed higher titers of virus in brain and 100% mortality by day 4 p.i., while the wild type mice exhibited 30% mortality around day 10 to 12 p.i. (Samuel and Diamond, 2005). Priming of cells from congenic resistant mice with IFN-α/ß conferred resistance against WNV infection, and the same treatment failed to induce protection against the virus in cells derived from congenic susceptible mice. In order to be protected against WNV infection, these cells required co-stimulation with double-stranded RNA, which was induced by poly(I:C) (Pantelic et al., 2005). These results

suggest that susceptibility against WNV infection is due in part at least in part to an inappropriate or inefficient IFN- α/ß induced antiviral effect.

In contrast to type I interferons, the effect of the IFN-γ on flaviviral infections is less understood. It is known this cytokine has multiple effects on the host response to viruses, including induction of antiviral and proinflamatory molecules such as nitric oxide. IFN-γ plays an important role in clearing viral RNA in the CNS (Binder and Griffin, 2001). Mice deficient in production of IFN-γ (IFN-γ -/-) or nitric oxide displayed a marginally increased susceptibility to infection with a neurotropic virus (Lobigs et al., 2003). However there are reports suggesting an important role of IFN-γ during infection of mice by WNV. Adoptive transfer of γδ T cells to TCRδ-/- mice reduced the susceptibility of these mice to WNV, suggesting that this effect is primarily due to production of IFN-γ by γδ T cells (Wang et al., 2003b). Further studies are required to understand the role of interferons in WNV infection and to understand the effect of these molecules on the innate immune response as well as subsequent cell-mediated immune responses that are established through their immunoregulatory effects.

One important interferon-inducible gene is oligoadenylate synthetase, which plays a role in the endogenous antiviral pathway. A nonsense mutation of the of 2'-5' oligoadenylate synthetase (OAS1b) is strongly associated with increased susceptibility of mouse strains to experimental WNV infection, and for this reason this gene is called the Flv gene or flavivirus resistance gene (Mashimo et al., 2002; Perelygin et al., 2002). Due to the link between the 2'-5' OAS and viral resistance, it was suggested that this enzyme

is important in the early anti-WNV response. 2'-5' OAS has been detected in the T-lymphocyte fraction prior to appearance of specific antibodies (Bonnevie-Nielsen et al., 1995), and the inactivity of the mutated form of this enzyme does not affect NO activity (Silvia et al., 2001).

Macrophages are one of the most important effector cells of the innate immune response. Activation of macrophages and modulation of their functions are major aspects of flavivirus pathogenesis and the anti-viral response. In addition to their importance in the anti-viral immune response, macrophages are susceptible to infection by flaviviruses, and therefore have a potential role in the pathogenesis of infection, perhaps by antibody dependent enhancement (ADE) through the Fc receptors present on these cells (Peris et al., 1981). The protective role of macrophages is typically based on the ability of these cells to produce immunomodulatory cytokines and their role as professional antigen presenting cells. Some studies have shown that abrogation of phagocytic activity of macrophages induces a higher viremia, increased neuroinvasion and more severe encephalitis following WNV infection (Ben-Nathan et al., 1996). Production of inducible nitric oxide synthetase (iNOS) by macrophages is one of the most important mechanisms of protection against virus (Kreil and Eibil, 1995). Inhibition of Japanese Encephalitis virus (JEV) replication is induced *in vitro* by pretreatment of macrophages with agents that induce production of NO (Linn et al., 1997). Moreover, treatment of mice with an iNOS inhibitor increased mortality after JEV infection (Linn et al., 1997). However, other reports suggest the inflammatory actions of NO may contribute to pathogenesis. For example, administration of iNOS inhibitors improved survival in mice infected with tick

borne encephalitis virus (Kreil and Eibil, 1996), and prolonged the survival rate in mice infected with Murray Valley encephalitis virus (Andrews et al., 1999). Activation of macrophages by flaviviruses promotes not only the production of NO, but also the production of other cytokines and chemokines important for the early immune response. Examples include IL-1ß, TNF-α, IL-8, MIP-1 α, RANTES, Gro-α, -ß and –γ, as well as other important mediators that may contribute to tissue damage where macrophages accumulate (Rothman and Ennis, 1999; Byrne et al., 2001; Bosch et al., 2002; Chen and Wang, 2002; Atrasheuskaya et al., 2003; Moreno-Altamirano et al., 2004). Based on these studies it is likely that macrophages play a role both in the early immune response against flavivirus and in the pathogenesis of these viruses.

Another important participant of the cellular innate immune response are natural killer cells (NK cells), which lyse infected cells by releasing cytotoxic granules that contain granzyme and perforins or by crosslinking of apoptosis-inducing receptors on target cells. NK cells become activated when they encounter a host cell expressing a decreased number of MHC class I molecules. Studies of NK cell activity during WNV infection have revealed decreased cytolytic activity against virus-infected cells as a result of an over-expression of MHC antigen and ICAM-1 on the target cells (Chambers and Diamond, 2003). Although few reports have focused on the role of NK cells in the immune response against flavivirus, evidence suggests that flaviviruses have evolved mechanisms to evade the NK cell responses through augmentation of MHC class I expression.

Dendritic cells (DC) are considered key players in establishing an adaptive immune response due to their role as professional antigen presenting cells. These cells are potential targets for replication of flaviviruses such as dengue virus, and it has been reported that these cells may support viral titers 10-fold or higher than those obtained in monocytes and macrophages (Palucka, 2000; Wu et al., 2000). Dendritic cells express a particular membrane receptor called DC-SIGN (or CD 209), a molecule that has been reported to be the main cellular receptor for dengue virus. Infection of DC by dengue virus was inhibited by using antibodies against DC-SIGN (Tassaneetrithep, et.al., 2003). Skin DCs are considered to be the first target host cell of dengue virus and thus may have an important role in the pathogenesis of disease (Libraty, et.al., 2001; Navarro-Sanchez et al., 2003). However DCs are not only important as potential targets for dengue virus replication, but are also important as effector cells in the immune response. For example, DCs have the ability to present antigen and to drive or induce the immune response through expression of membrane molecules such as B7-1 (CD80), B7-2 (CD86), MHC I, MHC II, CD 11b and CD83 which will induce activation of T lymphocytes. In addition DC are excellent producers of pro-inflammatory cytokines such as TNF-α, and the antiviral molecule IFN-α (Ho et al., 2001). Interestingly, production of IL-12 and IL-6 was precluded or diminished by the infection of DC with dengue virus (Libraty et al., 2001).

Members of the toll-like receptor (TLR) family are key regulators of both the innate and adaptive immune responses. In mammals, the TLR family is composed of at least eleven members and each TLR acts as primary sensor of conserved microbial

components and drives the induction of specific biological responses (Iwasaki, 2004). TLR 3 identifies dsRNA from reovirus, and has been identified as part of the defense against cytomegalovirus. WNV has a ssRNA genome that produces an intermediate dsRNA during replication and thus may be detected by TLR3 (Cook et al., 2004). It has been suggested that this receptor plays an important role in the pathogenesis of WNV, mainly in its invasion of the CNS. TLR3-/- mice are more resistant to infection by WNV when compared to wild-type mice, suggesting that WNV infection leads to a TLR3-dependent inflammatory response which may involve penetration of the virus leading to neuronal injury. It has also been suggested that expression of TNF-α is stimulated in a TLR-dependent manner. Once TNF-α is increased, this molecule facilitates WNV movement across the blood-brain-barrier (Fig. 1.3) (Iwasaki and Medzhitov, 2004; Wang et al, 2004b; Diamond and Klein, 2004).

C. Adpative Immunity

The adaptive immune response is also important in protection against flaviviral infections. B and T lymphocytes are the cellular effectors of this phase of the immune response. The importance of B cells in protection against WNV is based on their role as antibody producers. Passive transfer and active immunization experiments have demonstrated that neutralizing antibodies directed against several epitopes from structural and non-structural proteins (mainly against E protein) can prevent fatal encephalitis (Diamond et al., 2003a; 2003b). Treatment of mice by passive transfer of human antibodies prevents mortality and encephalitis when the serum is transferred prior to or even after infection with WNV (Engle and Diamond, 2003). In other studies passive

transfer worked similarly when monoclonal antibodies specific to JEV are administered to mice prior to the infection (Kimura-Kuroda and Yasui, 1988). Although antibodies induce some protection against flaviviral infections even when administered 5 days after infection (Kimura-Kuroda and Yasui, 1988; Ben-Nathan, 2003). Agrawal and Petersen (2003) suggest that treatment with antibody must be done prior or right after infection to have a maximal effect (Agrawal and Petersen, 2003). The protective ability of monoclonal antibodies against specific epitopes of domain III of the WNV protein E confirms the importance of these epitopes in protection against WNV infection, although antibodies alone can not clear virus from an infected individual (Oliphant et al., 2005). As expected, immunoglobin isotypes M and A are the first classes of antibodies produced after the infection with WNV. IgG appears later and persists longer in the serum of the infected host, although traces of IgM and IgA specific against WNV can be detected as long as 6 months after infection (Prince et al., 2005). Mice that lack B cells are very susceptible to flavivirus infections and encephalitis (Diamond et al., 2003a), perhaps as consequence of the absence of antibodies. However it is still unknown if T cell activity in those mice is completely normal, or whether it is affected by the lack of B cells, due to the possible role that these cells may play as antigen presenting cells or their other immunoregulatory properties.

The major protective mechanism provided by antibodies in protection against WNV is through neutralization of viral particles, which blocks their entry into host cells. Another mechanism by which antibodies provide protection is through opsonization, which enhances uptake or phagocytosis of virus by phagocytic cells, mainly

macrophages. Although the role of natural antibody in flavivirus infection remains unexplored, mice that genetically lack secreted IgM, (sIgM-/-), but in which cell surface IgM and IgG responses are intact, have increased mortality in certain viral infections,

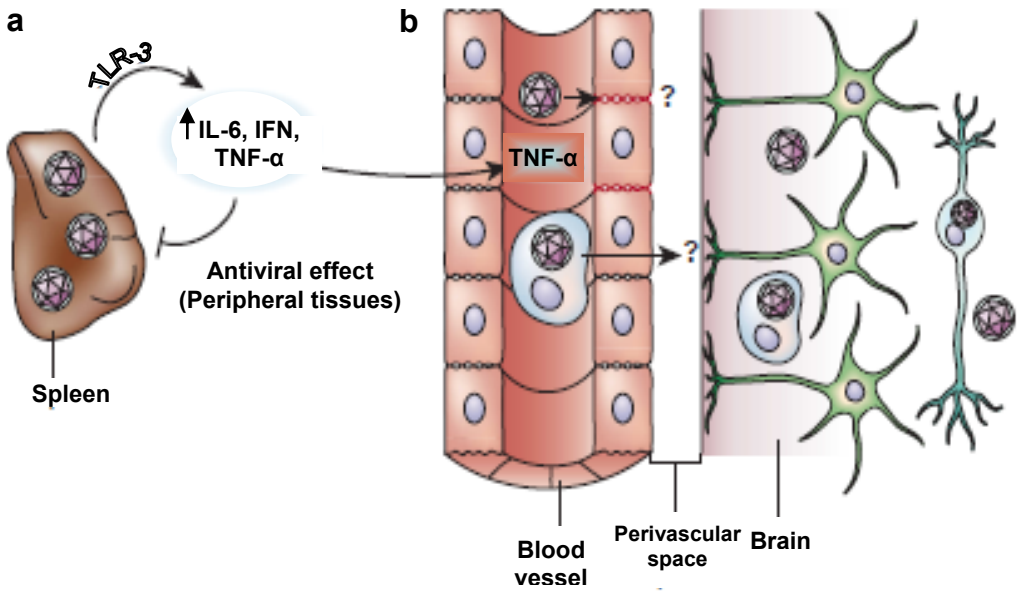


Fig 1.3. West Nile crossing the blood-brain barrier. Infection of macrophages or dendritic cells by West Nile virus in peripheral lymphoid tissue induces TLR-3-dependent secretion of antiviral and immunomodulatory cytokines such as IFN, IL-6 and TNF-α. These cytokines inhibit West Nile virus infection in peripheral tissues. TLR-3-dependent induction of TNF-α, however, also facilitates West Nile virus penetration across the blood-brain barrier. Although the exact mechanism remains unclear, TNF-α may alter endothelial cells or endothelial cell tight junctions so that West Nile virus or West Nile virus–infected leukocytes can move across the perivascular space and glial cell foot processes to infect neurons (Taken from Diamond and Klein, 2004).

involving a defect in the antiviral IgG responses. Those mice are also very susceptible to WNV infections. Within 96 h, markedly higher levels of virus were detected in the serum of these mice. The enhanced viremia correlated with higher viral titers in the CNS, and was also associated with a reduced anti-virus IgG production (Chambers and Diamond, 2003). Similar effects on viral loads in the CNS are observed in mice that genetically lack of B cells and antibody (μMT mice), which are also more vulnerable to lethal infections with low doses of WNV compared to wild type mice (Diamond et al., 2003a; Diamond et al., 2003c).

T-cell responses to flavivirus proteins have been studied for members of the dengue and Japanese encephalitis serogroups. The requirement of both CD4+ and CD8+ T lymphocytes to protect against flavivral infections has been established. In an *in vivo* model of a neurological infection yellow fever 17D virus, it was found that susceptibility to encephalitis was not altered in CD8+ knock out mice and that susceptibility was increased in CD4+ knock out mice. T cells obtained from wild-type mice exhibited a Th-1 pattern of cytokine expression after *in vitro* stimulation by the viral antigen. All these observations suggest that CD4+ T lymphocytes bearing a Th-1 phenotype are a critical factor that is involved in viral clearance (Liu, and Chambers, 2001). In an *in vivo* model of Japanese encephalitis virus, T helper cytokine responses in infected or immunized C57BL/6 and BALB/C mice were dependent on the dose and route of inoculation. The T helper cell cytokine response pattern is clearly indicative of a Th-1 profile when the mice are infected, and the pattern is predominantly a Th-2 when the animals are vaccinated with killed virus (Ramakrishna et al., 2003). A Th-1 T cell response was observed in

natural JEV infections of children. These same authors also found that the T helper immune response is mainly induced by epitopes from the non-structural protein 3 (NS3) (Kumar et al., 2004). However in a different study, CD4+ T lymphocyte responses from two different human patients were analyzed. The individuals were vaccinated 6 or 12 months prior to the study. CD4+ T lymphocytes were cloned, and the clones showed a JEV-specific cytotoxic activity and recognized E protein from JEV while some clones recognized E protein from other flaviviruses such as WNV and three different serotypes of dengue virus (Ahihara et al., 1998). CD4+ T lymphocytes are also important in the immune response against dengue virus. Human peripheral blood mononuclear cells (PBMC) infected *in vitro* by dengue virus showed an early Th-1 cytokine profile with TNF-α, IL-2, and IL-6 production, followed by a delayed Th-2 pattern, characterized by increased production of IL-10, IL-5 and IL-4. Thus dengue virus replication induces a Th-1 type cytokine production, which may be important in the success of the immune response against this virus (Chaturvedi et al., 1999). On the other hand, the role of CD8+T cells is debatable, because although there is a possible protective role of these cells, they may also have a potential role in the pathogenesis of dengue hemorrhagic fever (DHF). It has been reported that variation in the HLA locus is associated with susceptibility to DHF, and the HLA allele expressed as antigen will determine which specific CD8+T cells are protective. Alternatively, activation of cross-reactive CD8+ T cells may play an important role in the development of DHF (Loke et al., 2001). In a mouse model of WNV infection, CD8+ deficient mice suffered more severe clinical signs and increased mortality compared to wild-type mice when infected with a low dose of WNV. Somewhat paradoxically, the same knock out animals (CD8+ -/-) showed an

increased survival rate when infected with a high dose of WNV (10^8 PFU). These results provide evidence that CD8+ T cells are involved in both recovery and immunopathology in WNV infection (Wang et. al., 2003). Shrestha and Diamond (2004) reported that while the antibodies are responsible for terminating viremia in a WNV infection, CD8+ T cells have an important function in clearing infection from tissues and preventing viral persistence. The main observation is that mice lacking of CD8+ T cells or MHC antigens have higher CNS viral burdens and increased mortality after infection with a low dose of virus (Shrestha and Diamond, 2004). The cytolytic effect of T cell responses involves expression of FAS and exocytosis of granules. Mice with defects in FAS or granule exocytosis pathways of cellular cytotoxicity display reduced mortality and increased survival time when infected with Murray Valley encephalitis virus. In contrast these defects increased the percentage of mice which succumbed to encephalitis when infected with a low dose of WNV (Wang et al., 2004). Although the results observed in the different models is still debatable, mainly with regard to the function of CD8+ T cells, it is widely accepted that the presence of CD8+T cells in the CNS is an essential step towards the clearance of WNV from infected neurons. Klein et al. (2005) reported that infected neurons secrete the chemokine CXCL10 which is chemotractant for CXCL3+ CD8+ cells. When CXCL10-/- mice were infected with WNV, these animals exhibited higher mortality and viral burdens in the CNS when compared to wild-type mice (Klein et al., 2005). Another important regulatory molecule is CCR5, which is the cellular receptor for the chemokine CCL5. This chemokine is prominently up-regulated by WNV, and it is associated with CNS infiltration of CD4+ and CD8+ T cells, NK 1.1+ cells and macrophages expressing this receptor. The significance of CCR5 in flaviviral

pathogenesis was established by mortality studies in which infection of CCR5-/- mice was uniformly fatal. These mice had higher viral burdens in CNS and significantly lower numbers of NK1.1 cells, macrophages, CD4+ and CD8+ T cells compared to WNV-infected wild-type mice. CCR5 is an important molecule that acts by regulating trafficking of leukocytes into the CNS (Glass et al. 2005). In summary T cell responses are an important aspect of the immune response against flaviviruses including WNV. If any molecule which plays a role in induction or execution of T cell response is nonfunctional, this may affect the immune response or pathogenesis of flaviviral infection.

D. Antibody dependent enhancement

A potentially important but often controversial aspect of the pathogenesis of flaviviruses is the phenomenon termed antibody-dependent enhancement (ADE). Certain viruses can use preexisting specific antibodies, which potentially neutralize infectivity through the viral receptor-ligand route, and bind to the FcR on phagocytes and thus facilitate infection of Fc bearing host cells. These viruses infect monocytes and macrophages and are able to replicate in those cells, potentially using them as mechanisms of dissemination or as initial replication sites. This route of infection has been demonstrated *in vitro* for models of infection of RNA viruses. Several of those viruses are recognized as some of the deadliest in the world. Some DNA viruses have the ability to productively infect monocytes or macrophages although the ADE phenomenon has not been described in the infections by these viruses. ADE has been widely studied for *in vitro* infections in certain viruses although the correlation between the effect *in*

vitro and the importance of the ADE in the pathogenesis *in vivo* has not been completely described, even in dengue virus infections. This flavivirus is the virus in which the ADE phenomenon has been more widely studied, both *in vitro* or *in vivo*. Because ADE was first suspected in dengue virus infections, other flaviviruses have also been studied in order to evaluate the induction of ADE by those viruses (Suhrbier and Linn, 2003; Takada and Kawaoka, 2003; Tirado and Yoon, 2003). ADE for *in vitro* infections has been reported to occur in several additional viruses within the Flaviviridae, including Japanese encephalitis virus, Murray Valley encephalitis virus (Wallace et al., 2003), hepatitis C virus, West Nile virus (Tirado and Yoon, 2003); and non-flaviviruses such as foot and mouth disease virus (Mason et al.,1994), Ebola virus (Takada et al., 2001; Takada et al., 2003), feline infectious peritonitis virus (Corapi et al., 1995; Hohdatsu et al., 1998;), human immunodeficiency virus (Davis et al., 2001; Guillon et al., 2002; Subbramanian et al., 2002;), Ross River virus (Lidbury and Mahalingam, 2000), human respiratory syncytial virus (Gimenez et al., 1996; Ponnuraj et al., 2003); Coxsackievirus B3 (Girn et al., 2002), B4 (Hober et al., 2001), Auletian mink disease parvovirus, infection salmon anemia virus (Joseph et al., 2003), yellow fever virus, Hanta virus, Sindbis virus, porcine reproductive and respiratory syndrome virus, and equine infectious anemia virus (Tirado and Yoon, 2003).

Interestingly, the mechanisms of ADE are not limited to the use of FcR when the virus is bound to sub-neutralizing or non-neutralizing antibodies. Complement receptors may also be involved in this phenomenon as reported by Cardosa et al. (1983). These authors found that ADE occurred in an *in vitro* infection of P3888D1 cells by WNV, and

was subsequently blocked by the addition of specific antibodies against the complement receptor 3 (CR3), but not by antibodies against FcR. These results suggested independent mechanisms of ADE: FcR-dependent and complement-dependent ADE. In addition to antibody and cellular receptors, viral epitopes may also play a significant role. Both enhancing and neutralizing epitopes have been described in FIPV, HIV, and PRRS in addition to other viruses (Tirado and Yon, 2003).

Recently Mahalingam and Lidbury reported that ADE of macrophage infection by the alphavirus, Ross River virus ablated or diminished the expression of TNF-α, NOS2, and interferon regulatory factor 1 (IRF-1), as well as for IFN inducible protein 10 (IP-10) and IFN-ß. Additionally, transcription factor IFN-α-activated factor (AAF), IFN-stimulated gene 3 (ISFG3), and nuclear factor κB (NFκB) were specifically suppressed in these experiments. Furthermore the expression of IL-10 was elevated by ADE of Ross River virus infection. The ADE infection also abolished activation of the macrophages by LPS (Mahalingam and Lidbury, 2002). Following these observations the question arises as to whether ADE is not only influencing the uptake of the virus into the host cells, but also has some effect on development of the immune and/or cellular response by altering the expression and action of important molecules. Ligation of FcγR will result in an activator or inhibitory signal, depending on the type of the Fc receptor involved. Thus it is widely know that crosslinking of FcγR IIb has an inhibitory effect, due to its immunoreceptor tyrosine-based inhibitory motif (ITIM) which inhibits the activation signals from other FcγRs. The inhibitory effects of this receptor have been demonstrated to be effective to regulate cell activity (Takai, 2002). It is known that LPS activation

induces late expression of IL-10 and that intrinsic IL-10 can regulate the cellular pro-inflammatory activity (Gerber and Mosser, 2001). IL-10 is also up-regulated and IFN-γ is down-regulated by ADE-dengue virus infection in human monocytes *in vitro* (Yang et al. 2001). Therefore IL-10 may also play an important role in the immunomodulation induced by ADE –RRV and ADE-DV infection. IL-10 is recognized as a key cytokine for down-regulation of inflammatory responses by stimulating the expression of the suppressor-of-cytokine signaling 3 (SOCS 3). SOCS3 and SOCS1 have been identified as inhibitors of anti-viral responses (Suhrbier and Linn, 2003). ADE-dengue virus infection of a human mast cells/basophil line has been reported and some immunomodulatory effects were observed. These effects were mainly manifested as up-regulation of the expression of IL-1ß and IL-6 (King et al., 2000). These findings suggest that ADE-dengue virus infection of mast cells *in vivo* may be important in the pathogenesis of the dengue hemorrhagic fever due to the effect of the IL-6 over the endothelial cells. These cells are activated by IL-6 and by TNF-α as well as induction of receptor molecule expression, both of which induce inflammatory cell activation and migration which in turn leads to vascular damage.

The association of ADE with the clinical syndrome of dengue hemorrhagic fever is based on several observations, the most prominent of which is the presence of subneutralizing dilutions of specific antibody in patients that suffer from this disease. Another possible contributing factor is the antigenic variation between the dengue virus serotypes, inducing specific antibodies to one serotype. Those antibodies are neutralizing for autologous virus but have a reduce avidity to other serotypes, thus inducing ADE of

infection by those heterologous serotypes. ADE *in vivo* is more likely present in environments in which more than one serotype of the virus coexist (Ferguson et al., 1999; Kawaguchi et al., 2003; Rothman, 2004; Cummings et al., 2005). ADE of infection has been considered possible after previous vaccination, which may induce the production of enhancing antibodies. Conversely, in a recent report ADE was not induced *in vitro* by serum from Thai children who were vaccinated. The sera did not induce ADE in the infection of any of the four different serotypes of dengue virus (Guy et al., 2004).

Antibody dependent enhancement was demonstrated *in vivo* in a murine model of Murray valley encephalitis virus. In this experiment, the authors used specific anti-JEV mouse hyperimmune serum. Subneutralizing dilutions of this serum were transferred to BALB/C naïve mice, and then the mice were inoculated with MVEV. The immune mouse serum-treated animals showed a significant increase in mortality and viral loads in the brain when compared to non-serum treated mice (Wallace et al., 2003). These observations suggest the possibility taht ADE is present in natural *in vivo* infections by flavivruses in areas where two or more flaviviruses circulate. In contrast ADE could not be induced by anti-tick borne encephalitis virus (TBEV) serum passively transferred to mice which were the infected with the same virus (Kreil and Eibl, 1997).

Antibody dependent enhancement of WNV infection was demonstrated *in vitro* using rabbit hyperimmune serum (Peris and Porterfiled, 1979). ADE occurred through Fc receptors since treatment with anti-FcγR antibodies inhibited the ADE effect (Peris et al., 1981). However, the significance of the ADE for *in vivo* WNV infections is still

unknown. WNV was recently introduced into the United States in 1999, and to date only one genetic lineage is present in this country. However, multiple lineages of WNV are recognized. Lineage II is considered to be mildly pathogenic and its distribution is restricted to southern Africa and Madagascar (Burt et al., 2002). Kunjin virus is genetically very close to the WNV and is considered as a serotype of this virus (Charrel et al., 2003). In addition a recent report suggests the presence of a third lineage of WNV in eastern Europe (Bakonyi et al., 2005). The genetic divergence of the different lineages of WNV may potentially play a role in the presentation of ADE *in vivo* if any of the foreign lineages are introduced into the United States. Since the first report of WNV in the United States, the concern about genetic variation and formation of quasispecies have induced the interest of some research groups. The ability of WNV to change genetically has been evaluated. The results are equivocal, but investigators suggest that currently there is an absence of strong selective pressure to drive the emergence of dominant variants (Beasley et al., 2003). Others suggest the presence of genetic variants that group in a temporally and geographically dependent manner, and propose that a dominant variant has emerged across United States (Davis et al., 2003; Davis et al., 2005). Jerzak et al. (2005) suggested that WNV populations may be structured as quasispecies with a strong purifiying natural selection of the viral populations (Jerzak et al., 2005). Finally Brault et al. (2004), found genetic variations in the NY99 strain compared to Kunjin and Kenyan serotypes, suggesting that those genetic alterations in NY99 WNV are responsible for the crow-virulent phenotype (Brault et al., 2004). The genetic variations in the WNV strain present in United States eventually may yield into new genetic variants, which would then be potentially important in the presentation of ADE *in vivo*.

In the set of experiments described in this dissertation, key aspects of the pathogenesis of infection by WNV are evaluated both *in vitro* an *in vivo* using the following hypotheses:

- Horse monocytes are susceptible to infection by WNV *in vitro.*
- Anti-WNV immune horse serum is able to induce ADE of WNV infection in horse monocyte-derived macrophages and a mouse macrophage-like cell line.
- Immune horse serum is able to induce ADE of WNV *in vivo* using a murine model.

Each hypothesis was addressed and the results are described in the following three chapters.

CHAPTER II

REPLICATION OF WEST NILE VIRUS IN EQUINE PERIPHERAL BLOOD MONONUCLEAR CELLS

Abstract

A cell model of primary monocytes and other mononuclear cells isolated from equine blood was used to study the kinetics of West Nile virus (WNV) replication in a natural host. West Nile virus has emerged on the North American continent as a significant cause of morbidity and mortality in a wide range of avian and mammalian species. While other flaviviruses are known to infect monocytes and lymphocytes, the ability of WNV to productively replicate in specific immune cells of peripheral blood has not been assessed. In this study, enriched populations of monocytes and lymphocytes as well as purified monocytes, CD4+, CD8+ and B lymphocytes were obtained from equine blood. Productive WNV replication was demonstrated by viral growth curves, quantitative RT-PCR for WNV RNA, and indirect immunofluorescence detection of a non-structural WNV protein. Enriched and purified monocytes consistently supported productive viral replication in blood from nine of nine horses tested while a minor subset of CD4+ lymphocytes supported productive replication in cells from three of the nine horses tested. Peak viral titers of 3.2 – 6.6 $\log_{10}$ PFU/ml were reached at 6 days post-inoculation (p.i.) and titers were maintained through 10 – 15 days p.i. Activation of monocytes with bacterial lipopolysaccharide, which resulted in activation of nuclear transcription factor κB (NF- κB) plus elevation of nitric oxide and type I interferon

levels, reduced or eliminated WNV replication. These results suggest that immune cells of the peripheral blood may serve as target cells for initial replication of WNV and may play a role in subsequent viral dissemination. Furthermore, primary equine immune cell cultures represent a potentially useful model of a natural WNV host when testing compounds such as antivirals for use in WNV treatment.

I. Introduction

West Nile virus (WNV) is a single-stranded, positive-sense RNA virus that is classified in the Japanese encephalitis virus serocomplex within the genus *Flavivirus*, family *Flaviviridae* (Monath and Heinz, 1996). The genome of WNV is approximately 11 kb and encodes three structural proteins (C, M, and E) and seven nonstructural proteins. West Nile virus is the etiologic agent of West Nile encephalitis, and similar to other members of the Japanese encephalitis virus serocomplex, WNV is an arbovirus that is transmitted by a variety of mosquito species. The geographic distribution of WNV is widespread and includes much of Africa, central and southern Asia, southern Europe and Oceania. West Nile virus was first detected in North America in 1999 (Anonymous, 1999) and has since spread rapidly throughout the United States and into Canada and Mexico. This virus is now endemic throughout a significant portion of North America, with clinical disease in susceptible hosts likely to occur annually. Since introduction of WNV into the U.S., this pathogen has caused morbidity and mortality in humans plus a wide variety of wild and domestic species, both avian and mammalian (Kramer and Bernard, 2001). The WNV epizootic has been particularly severe in horses with

numerous clinical cases and case fatality rates of 35% or more (Ostlund et al., 2000; Ostlund et al., 2001).

Currently there are no antiviral treatments or human vaccines against WNV. In an attempt to protect horses against WNV, both a formalin-inactivated vaccine and a viral-vectored vaccine have been developed and are currently in use. However, the efficacy of the equine vaccines for protection against clinical neurological disease caused by virulent WNV challenge has not been demonstrated, primarily due to difficulties in developing an experimental model of WNV encephalitis in horses (Bunning et al., 2002). In murine models, protection against WNV challenge has been attributed to both cellular and humoral components of the adaptive immune response as well as components of the innate immune response (Kesson et al., 1987; Kulkarni et al., 1991; Diamond et al., 2003a; Diamond et al., 2003b). For example, both type I and II interferon have been shown to directly inhibit WNV infection both in vitro and in vivo (Anderson and Rahal, 2002; Diamond et al., 2003b). Interestingly, however, it was recently reported that the innate immune response to WNV resulted in TNF-α secretion by peripheral lymphoid tissue, which in turn caused a transient increase in permeability of the blood-brain barrier and provide an opportunity for viral entry into the central nervous system (Wang et al., 2004a).

The pathogenesis of WNV in mammalian hosts is characterized by specific neurotropism. After an initial transient, low-level viremia that coincides with the onset of clinical signs, WNV is rarely detected in tissues outside of the central nervous system

(Lanciotti et al., 2000; Solomon et al., 2003). In a recent study of horses clinically ill with WNV encephalitis and seroconverting herdmates of those horses, only 8% were found to have detectible WNV RNA in peripheral blood (Kleiboeker et al., 2004). However, the role of viremia and hematogenous spread and/or lymphatic spread in the pathogenesis of WNV has not investigated to date. Furthermore, a peripheral site of replication has not been defined, but may involve initial replication in monocyte-derived dendritic cells of the skin, as demonstrated for dengue virus (Wu et al., 2000). In the present study, the ability of WNV to productively replicate in equine peripheral blood mononuclear cells (PBMCs) was investigated using primary cultures purified from heparinized blood. Productive WNV replication occurred in two immune cell types and these results suggest a potential role for these cells in initial amplification and subsequent dissemination of WNV. Given the similarity of clinical disease between horses and other mammalian hosts, the results of these studies provide broad insight into the pathogenesis of WNV in mammalian hosts.

II. Materials and Methods

A. Virus and cell culture

West Nile virus was isolated from the brain of an American Kestrel that died in Missouri (United States) with neurological signs in 2002. The initial virus isolation and subsequent propagation were performed in Vero cell cultures using standard techniques (Burleson et al., 1992). Vero cell cultures were maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and

2 mM L-glutamine, 0.25 µg/ml fungizone, and 0.5 mg/ml gentamycin (cell culture reagents supplied by Mediatech, Inc., Herndon, VA). The cells were maintained at 37°C in a humidified 5% CO_2 incubator. Approximately 4 days post-inoculation (p.i.) with the brain homogenate, a diffuse cytopathic effect was identified in inoculated Vero cells. Identification of the cytopathic agent as WNV was confirmed by indirect fluorescent antibody staining of inoculated cells using hyperimmune anti-WNV ascitic fluid (VR1267 AF, American Type Culture Collection, Manassas, VA) and an FITC-labeled anti-mouse IgG conjugate (Sigma, Inc., St. Louis, MO). Additionally, identification of the isolate as WNV was confirmed by WNV-specific RT-PCR (Johnson et al., 2001) followed by sequencing of 441 bp of the amplified viral envelope glycoprotein (E) gene. Comparison of the sequenced region of the isolate demonstrated 99.8% identity to the prototype U.S. strain NY99-flamingo382-99 (Lanciotti et al., 1999; GenBank accession no. AF196835) from nucleotide 1401 to 1841. The sequenced region was 100% identical over the 441 bp sequenced to a number of WNV isolates from 2002 (Beasley et al., 2003; GenBank accession no. AY185906, AY185907, AY185908, AY185909, AY185911). Quantification of WNV stocks and experimental samples (lysed cells and supernatants from WNV-inoculated cells) was performed using a viral plaque assay. Ten-fold serial dilutions of samples were adsorbed for two hrs onto confluent Vero cell monolayers in six-well plates (10 cm^2/well). Cell monolayers were washed once with cell culture media. Overlay media, which consisted of maintenance media plus 0.5% (w/v) agarose, was then added to each well. Cultures were maintained at 37°C in a humidified 5% CO_2 incubator for three days. Overlays were then removed and cell monolayers were stained with 0.5%

(w/v) crystal violet/70% (v/v) methanol and plaques were counted and viral titers were calculated (Burleson et al., 1992).

B. Plaque-reduction neutralization assays

The plaque-reduction neutralization titer (PRNT) of serum from horses used as donors for peripheral blood mononuclear cell (PBMC) cultures was determined by adding approximately 50 plaque-forming units of WNV to serial two-fold dilutions of heat-inactivated (56°C, 30 min) horse serum. Samples were incubated at 37°C for 1 hour followed by one additional hour at room temperature prior to addition to confluent Vero cell monolayers. After adsorption for two hrs, the inoculum was removed and the monolayer was rinsed once with cell culture media and then replaced with cell culture media containing 0.5% (w/v) agarose. Three – four days following addition of agarose overlay, it was removed and cell monolayers were stained as described above. Viral plaques were counted and the PRNT was recorded as the final serum dilution that reduced the number of plaques by ≥90% compared to control wells to which no horse serum had been added.

C. RNA extraction and Quantitative RT-PCR

Extraction of RNA was performed using silica membrane spin columns (Nucleospin RNA II kit, BD Biosciences, Inc., Palo Alto, CA) according to the manufacturer's instructions. Negative control extractions were performed concurrently with the extraction of samples. For real-time, quantitative RT-PCR amplification, a previously reported dual-labeled probe and oligonucleotide primers (Lanciotti et al.,

2000) were used with modifications previously described (Kleiboeker, 2003). Quantification was performed using WNV heterologous competitor RNA (Kleiboeker, 2003), the concentration of which had been calculated from the spectrophotometric value of the purified stock. Serial ten-fold dilutions of WNV competitor RNA were amplified to generate a standard curve for each set of reactions. The number of viral RNA copies in each sample was calculated from the cycle threshold of the sample and the equation for a straight line that was derived from the standard curve.

D. Preparation of peripheral blood mononuclear cell cultures

Blood (120 – 200 ml) was collected by aseptic technique from a total of nine horses. Sodium heparin was present at a final concentration of approximately 50 unit/ml blood. All horses were clinically normal with no known health problems at the times of blood collection and all horses had been previously immunized with a minimum of three doses of killed West Nile virus vaccine (West Nile Innovator, Ft. Dodge Laboratories, Ft. Dodge, IA) in the 12 months preceding blood collection. Heparinized blood was centrifuged at 2000 r.p.m. for 25 min, and the buffy coat layer was removed and diluted 1:1 with phosphate buffered saline, pH 7.4 (PBS). The diluted buffy coat sample was layered onto a Ficoll-Hypaque gradient (Sigma Chemical Co., St Louis, MO) and centrifuged at 850 x *g* for 25 min. The interphase was collected, washed three times with PBS by centrifuging at 50 x *g* for 10 min to eliminate the platelets. After the last wash, the cells were resuspended in DMEM supplemented with L-glutamine, penicillin streptomycin-fungizone and 10% normal horse serum (Life Technologies, Inc. Grand Island, NY) and counted using a hemocytometer. The horse serum used in the culture

media was tested by PRNT and an indirect fluorescence antibody (IFA) assay plus real-time RT-PCR (see above) and found to be negative for WNV neutralizing activity and viral RNA, respectively. The adherent cell population was purified from the PBMCs as described elsewhere (Raabe et al., 1998). Briefly, 100 mm tissue culture dishes were coated with 2% gelatin (Sigma Chemical Co., St Louis, MO) at 37°C in a humidified 5% CO_2 incubator for one hr. The gelatin was removed and the dishes were allowed to dry then washed twice with PBS and held at 37°C until 1.5 x 10^7 PBMCs were added. The cells were incubated at 37°C for 5 hr to allow adherent cells to attach, and after incubation the unattached cells were removed and centrifuged then resuspended to be counted and plated as a non-adherent population. Attached cells were removed from the tissue culture dishes by using 5 ml of Accutase (Innovative Cell Technologies, Inc., San Diego, CA), centrifuged and counted using a hemocytometer prior to being re-plated on 24-well plates. The composition of the adherent cell population was found to be predominantly monocytes (mean 82%) as determined by flow cytometry using antibody recognizing equine monocytes (CD172w, VMRD Inc., Pullman, WA). The composition of the non-adherent cell population was found to be predominantly lymphocytes (mean 96%) as determined by flow cytometry using antibody recognizing equine CD2+ lymphocytes (HB88a, VMRD Inc., Pullman, WA). After 20 hr at 37°C in a humidified 5% CO_2 incubator, PBMCs (seeded at a density of 5 x 10^5 cells per well), the adherent cell population and non-adherent cell population (both seeded at a density of 1.5 x 10^5 cells per well), were infected with WNV at a multiplicity of infection (m.o.i.) of 0.2. After 3 hrs, the supernatant was removed, the cells were washed twice with fresh media, then held at 37°C in a humidified 5% CO_2 incubator for the indicated times. In

experiments where lipopolysaccharide (LPS; *E. coli*O128- B12 derived, catalogue no. L2887; Sigma, St. Louis, MO) was added to cultures, cell viability was assessed in LPS treated and untreated control cells by staining with 0.4% trypan blue (Bio-Whittaker, Walkersville, MD). The total number of cells and the number of dead cells (i.e. those not retaining trypan blue) were quantified microscopically with a hemocytometer.

E. Sorting of cell populations

Positive selection of four different cell populations was performed using aseptically obtained horse blood (200 ml) following purification of PBMCs as described above. Once PBMC's were separated from whole blood, 10^8 cells were incubated with 100 µg of specific monoclonal antibody against either equine CD13 (for monocytes; MCA 1084 Serotec Inc., Raleigh, NC), equine CD4 (MCA 1078 Serotec Inc., Raleigh, NC), or equine CD8 (MCA 1080 Serotec Inc., Raleigh, NC). Equine B lymphocytes were sorted using both anti-human CD79α (M7051 Dako Cytomatation California, Carpinteria, CA) and anti-equine B cell (E18A VMRD Inc., Pullman, WA) antibodies. All antibodies were incubated with cells for 20 min at room temperature in 500 µl of sorting buffer (0.5% BSA, 2mM EDTA in phosphate buffered saline pH 7.4). After incubation with antibody, cells were washed twice with sorting buffer and then incubated with 80 µl of anti-isotype antibody conjugated to magnetic microbeads (rat anti-Mouse IgG1 microbeads catalog no. 130-047-101; rat anti-Mouse IgG 2a+b microbeads catalog no. 130-047-201; rat anti-Mouse IgM microbeads catalog no. 130-047-301; all from Miltenyi Biotech Inc, Auburn, CA) for 20 min at 4°C in with gentle shaking. This incubation was followed by two washes with sorting buffer. Cells were sorted by positive selection,

using magnetic cell sorting equipment (Auto-MACS, Miltenyi Biotech, Auburn, CA). After sorting, cells were washed twice with DMEM supplemented with 10% normal horse serum. Cells then were counted and 1.5 x 10^6 cells were seeded on to ultra low attachment six-well plates (Corning Inc., Acton, MA) before being infected with WNV at an m.o.i. of 0.02. After 3 hours, cells and supernatant were removed and cells were washed twice with fresh medium by centrifugation at 5,000 rpm. Infected and control (uninfected) cells were plated in 24-well plates at a density of 250,000 cells per well and maintained in at 37°C in a humidified 5% CO_2 incubator.

F Assessment of cell purity by Flow Cytometry

Purity of sorted cells was checked by flow cytometry, using biotinylated monoclonal antibodies against equine monocytes (CD172w DH59B VMRD Inc., Pullman, WA), equine CD4 (HB61A VMRD Inc., Pullman, WA), equine CD8 (HT14A VMRD Inc., Pullman, WA), or equine B cells (E18A VMRD Inc., Pullman, WA). Briefly 10^6 sorted cells were incubated with 1 µg of specific antibody for 20 minutes on ice, then cells were washed twice with FACS buffer (0.5% bovine serum albumin, 2% FBS, 0.01% NaN_3 in PBS). Cells were incubated on ice with a streptavidin-FITC conjugate (Becton Dickinson, San Diego, CA) for 20 min (protected from light), and then cells were washed twice with FACS buffer. After the last wash, cells were resuspended in 500 µl of 2% paraformaldehyde and held at 4°C until the samples were read in a FACS Vantage Cell Cytometer-Sorter (Beckton Dickinson, San Diego, CA). Flow cytometry results were analyzed with the WinMDI 2.8 software or with the CellQuest software (Becton Dickinson, San Diego, CA).

G. Fluorescent antibody detection of WNV non-structural protein and identification of cell type

Total equine PBMCs, monocytes or CD4+ cells were purified as described above and plated at a density of 5 x 10^5 cells per well in 8-well glass chamber slides (Lab-Tek II Chamber Slide, Nalge Nunc International, Naperville, IL). Cells were infected with WNV at an m.o.i. of 1. On day 6 p.i. cells were fixed with acetone and indirect immunofluorescence was performed using a mouse-origin monoclonal antibody recognizing the NS1 protein of West Nile/Kunjin virus (MAB8152, Chemicon Intl. Temecula, CA) and anti cellular markers CD-2 (HB 88A, VMRD Inc., Pullman, WA) or CD172w (DH59B, VMRD Inc., Pullman, WA), using fluorescene isothiocyanate labeled anti-mouse IgM (Pharmingen, San Diego, CA) or rhodamine labeled anti-mouse IgG1 (Pharmingen, San Diego, CA) as a secondary antibodies. Slides were incubated at 37°C in a humidified chamber for 30 min with a cocktail of anti-NS1 WNV and anti-CD2 or anti-CD172w, and three washes with PBS were performed between incubations. After the secondary antibodies incubation, cells were visualized with an ultraviolet light epifluorescence microscope and photographs were taken with a digital camera. Uninoculated cells from the same horse and sample preparation were used as negative controls.

H. Detection of NF-κB translocation plus nitric oxide and type I interferon production following LPS treatment of adherent cells and sorted monocytes

Adherent cells or sorted monocytes were treated with 5 ng per well (24-well tissue culture plate; 2 cm^2 surface area per well) of *E.coli* O128 B12-derived LPS (Sigma,

St Louis, MO) for two hours prior to infection with WNV. At the indicated time point following treatment with LPS, translocation of the transcription factor NF-κB was assayed by an indirect fluorescent antibody assay using rabbit anti-NF-κB p105 polyclonal antibody (K59184R, Biodesign Intl., Saco, ME). The reaction was developed using FITC-conjugated goat anti-rabbit IgG polyclonal antibody. The concentration of nitric oxide (NO) was quantified in cell lysates and supernatants by using a colorimetric nitric oxide (NO_2^-/NO_3^-) assay kit, following instructions from the manufacturer (Assay Designs, Inc., Ann Arbor, MI). The concentration of type I interferon (IFN) was determined using a bioassay. Briefly, 100 μL of two-fold dilutions of cell culture supernatant samples from WNV-inoculated cells were added to Madin-Darby bovine kidney cell monolayers for 18 hrs. The cells were then inoculated with vesicular stomatitis virus (Indiana strain) at an m.o.i. of 0.05. The cells were incubated with the virus for 1 hr at 37°C, 5% CO_2 in a humidified incubator and then rinsed with cell culture media. Following incubation for 24 hr at 37°C, 5% CO_2 in a humidified incubator, the plates were stained with 1% crystal violet in methanol. Quantification was performed by determining the 50% end-point of viral inhibition against a standard IFN concentration curve. The final concentration of type I IFN was obtained by calculating the reciprocal of the dilution that induced a 50% viral growth inhibition. For both nitric oxide and type I IFN quantification, each time point was performed in triplicate and the experiments were repeated three times. The experiment detecting NF-κB translocation was also repeated three times.

III. Results

A Anti-WVN serologic values in horses used as blood donors

The plaque-reduction neutralization titers (PRNT) of serum from the nine horses used as blood donors was determined (Table 1). West Nile virus neutralizing antibody was not detected in serum from horse no. 2, suggesting that this horse was seronegative, and potentially immunologically naïve, to WNV. For the other eight horses, a 90% PRNT was observed at serum dilutions ranging from 1:80 to 1:5,120 indicating that these horses were seropositive to WNV and had been previously exposed to WNV antigen either through vaccination or natural exposure. Serum from each horse was also tested by indirect immunofluorescence (IFA) for antibodies recognizing WNV antigen and the titer values correlated well, though were slightly lower than PRNT values (data not shown). Most significantly, horse no. 2 was also negative by IFA at all serum dilutions tested.

B. WNV replication in equine peripheral blood mononuclear cells

Total PBMC's, adherent or non-adherent cells were purified from peripheral blood and inoculated with WNV at an m.o.i. of 0.2. Additionally, monocytes, and CD4+, CD8+ or B lymphocytes were purified from peripheral blood by magnetic cell-sorting and inoculated with WNV at an m.o.i. of 0.02. Inoculations were performed and analyzed in triplicate for each experiment and multiple replicate experiments were performed with independent blood preparations from individual horses with consistent results observed. At various times post-inoculation (p.i.) combined cellular and supernatant samples were collected and held at -80°C until viral titers were determined by plaque assay. For

adherent, non-adherent and total PBMC cell populations, an initial decline in WNV titer of approximately 1 – 3.7 $\log_{10}$ PFU/ml was observed at day 2 p.i. (Figs. 2.1-2.6). Following the decline, WNV titers increased by day 4 p.i. and reached maximum levels by day 6 – 7 p.i. Titer values gradually declined until the final sample collection point at day 15 p.i. For cells derived from peripheral blood of horse no. 1 (Fig. 2.1), maximum titers of approximately 6.6 $\log_{10}$ PFU/ml were detected in adherent cells on day 6 p.i. Titers in PBMCs and non-adherent cells were significantly lower than those detected in adherent cells at several times p.i. For cells from horse no. 2 (Fig. 2.2), maximum titers of approximately 5.1 $\log_{10}$ PFU/ml were detected in both adherent and PBMC cultures. No significant differences were noted in titers from adherent cells and PBMCs from horse no. 2 at all time points p.i. except one (day 10 p.i.). In contrast, WNV titers from non-adherent cells of this horse were significantly lower ($p < 0.05$) than those detected in adherent cells or PBMCs at days 2, 4, 6 and 10 p.i. For cells from horse no. 3 and 4 (Figs. 2.3 and 2.4, respectively), similar patterns were noted compared to horse no. 2 with the exception that WNV titer values from non-adherent cells of horse no. 4 remained near the lower limit of detection from day 2 p.i. through the end of the time course. When quantitative RT-PCR for WNV genomic RNA was performed on aliquots of the same samples (from horses no. 1 and 2) used for plaque assay quantification, similar trends were observed (Fig. 2.5 and 2.6) although viral RNA concentrations tended to decline only slightly even as late as day 15 p.i.

To identify the specific cell type(s) supporting WNV replication, cell-sorting with specific antibodies to immune cell surface markers and magnetic beads was used to

purify populations of cells from the peripheral blood of a total of nine horses (Table 1, Figs. 2.7 – 2.15). Sorted equine monocytes and CD4+, CD8+, or B lymphocytes were evaluated for purity by flow cytometry with mean cell purity values ranging from 83.96 to 97.22% (Table 2). Similar to the results shown in Fig. 1, purified cell populations exhibited a drop in viral titer of approximately 1 – 3 $\log_{10}$ PFU/ml at day 2 p.i. (Fig. 2). Monocytes from all nine horses supported productive replication of WNV following decreased titers on day 2 p.i. The range of maximum titers for monocytes was 3.15 – 5.36 $\log_{10}$ PFU/ml and was consistently observed on day 6 p.i., with titers maintained through day 10 p.i. Productive viral replication occurred in unpurified PBMC cultures in all but two of the horses tested. In general, peak titers of PBMC cultures were lower than those detected in purified monocytes. However, for cells derived from horse no. 1 WNV titers from PBMC cultures were approximately 10-fold higher at day 6 – 10 p.i. (Fig. 2.7). Interestingly, CD4+ lymphocytes from three horses (Fig. 2.9, 2.10 and 2.12) supported viral replication with titers comparable than those observed in monocytes. Productive WNV replication was not detected in CD8+ or B lymphocytes from any of the nine horses tested, although low levels of WNV were detected at most points in the time course experiments.

C. Dual detection of WNV antigen and CD antigen in infected cells

To confirm the cell populations infected by WNV, dual fluorescent antibody staining of monocytes and CD4+ lymphocyte cultures was performed with monoclonal antibody recognizing the WNV NS-1 protein and with antibodies recognizing CD2 or

CD13 antigens. Sorted monocytes or CD4+ cells from horse no. 6 were infected with WNV at an m.o.i. of 1 in glass chamber slides. Uninoculated cells from the same preparation were used as controls. At day 6 p.i., cells were fixed and stained for WNV NS-1 protein and for cell surface antigens then visualized by fluorescence microscopy. In observation of >100 anti-CD13 positively stained cells, 42% of the sorted monocytes were double-stained with anti-NS-1 and anti-CD13 (Fig. 2.16 C and E, respectively). In contrast, observation of >300 anti-CD2 positively stained cells found that only 2% of the sorted CD4+ were double positive for NS-1 WNV and for CD2 (Fig. 2.16 D and F, respectively). Uninoculated sorted monocytes or CD4+ cells were always clearly negative for NS-1 WNV, but uniformly positive for CD13 or CD2, respectively.

Table 2.1. Serologic values and West Nile virus titers following inoculation of sorted immune cells

		Log_{10} PFU/ml at day 6 p.i. (mean ± SEM)				
Horse no.	PRNT titer[a]	PBMC	Monocyte	CD4+	CD8+	B cell
1	1:80	4.03 ± 0.13	3.15 ± 0.11	0.33 ± 0.29	0.63 ± 0.55	0.33 ± 0.29
2	<1:10	3.59 ± 0.05	4.30 ± 0.15	1.86 ± 0.03	0.33 ± 0.24	0.54 ± 0.38
3	1:1,280	3.70 ± 0.12	5.14 ± 0.07	4.26 ± 0.06	2.64 ± 0.12	0.99 ± 0.36
4	1:80	3.83 ± 0.07	3.64 ± 0.08	3.24 ± 0.28	1.55 ± 0.11	1.70 ± 0.04
5	1:1,280	3.85 ± 0.17	4.38 ± 0.12	2.89 ± 0.36	2.87 ± 0.05	2.31 ± 0.17
6	1:1,280	3.85 ± 0.07	3.98 ± 0.01	4.30 ± 0.12	2.50 ± 0.19	2.39 ± 0.15
7	1:5,120	2.36 ± 0.13	5.36 ± 0.04	2.79 ± 0.18	2.42 ± 0.41	2.30 ± 0.15
8	1:80	1.54 ± 0.19	4.00 ± 0.27	2.75 ± 0.08	1.00 ± 0.29	0.77 ± 0.54
9	1:80	2.60 ± 0.09	3.42 ± 0.04	1.59 ± 0.27	2.03 ± 0.11	1.54 ± 0.11
	Mean[b]	3.26 ± 0.41	4.15 ± 0.35	2.67 ± 0.60	1.77 ± 0.44	1.43 ± 0.38

[a]90% Plaque-reduction neutralization titer of serum collected at time of sampling
[b]Mean ± SEM of samples from all horses

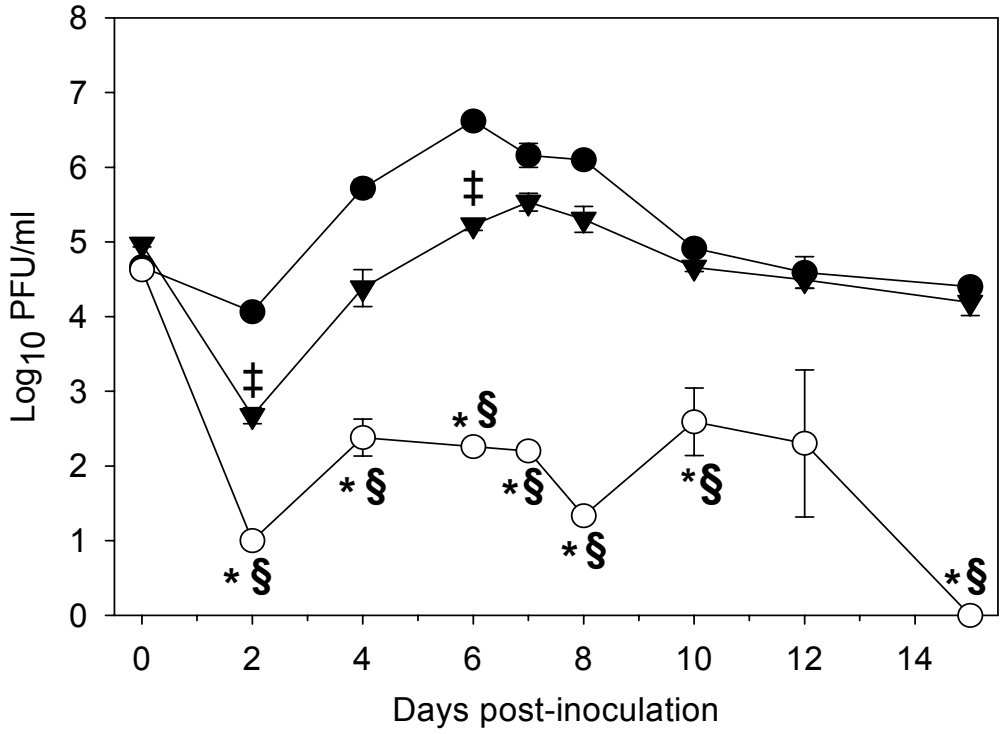


Figure 2.1. Viral growth curve for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Horse 1. Non-adherent (○), adherent (●) or total peripheral blood mononuclear cells (▼) were inoculated with WNV at an m.o.i. of 0.2 and samples were harvested at the indicated days. Values shown are the mean viral titers ± SEM . *****, significant difference ($p < 0.05$) between non-adherent and adherent cells; §, significant difference ($p < 0.05$) between non-adherent and PBMCs; ‡, significant difference ($p < 0.05$) between adherent and PBMCs. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

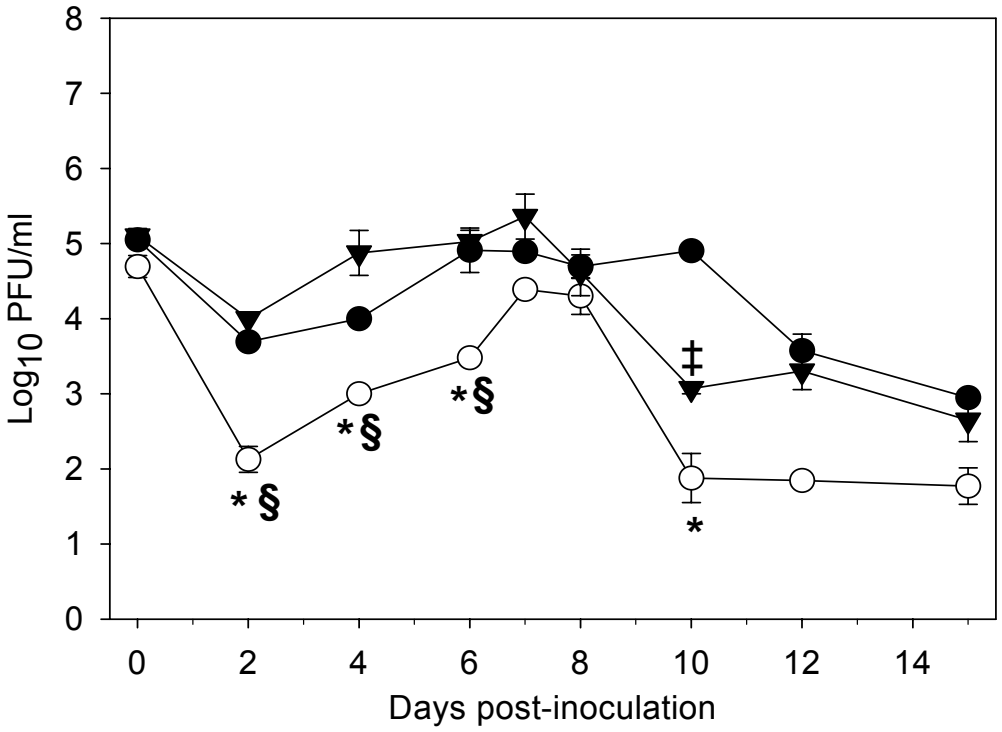


Figure 2.2. Viral growth curve for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Horse 2. Non-adherent (○), adherent (●) or total peripheral blood mononuclear cells (▼) were inoculated with WNV at an m.o.i. of 0.2 and samples were harvested at the indicated days. Values shown are the mean viral titers ± SEM . *****, significant difference ($p < 0.05$) between non-adherent and adherent cells; §, significant difference ($p < 0.05$) between non-adherent and PBMCs; ‡, significant difference ($p < 0.05$) between adherent and PBMCs. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

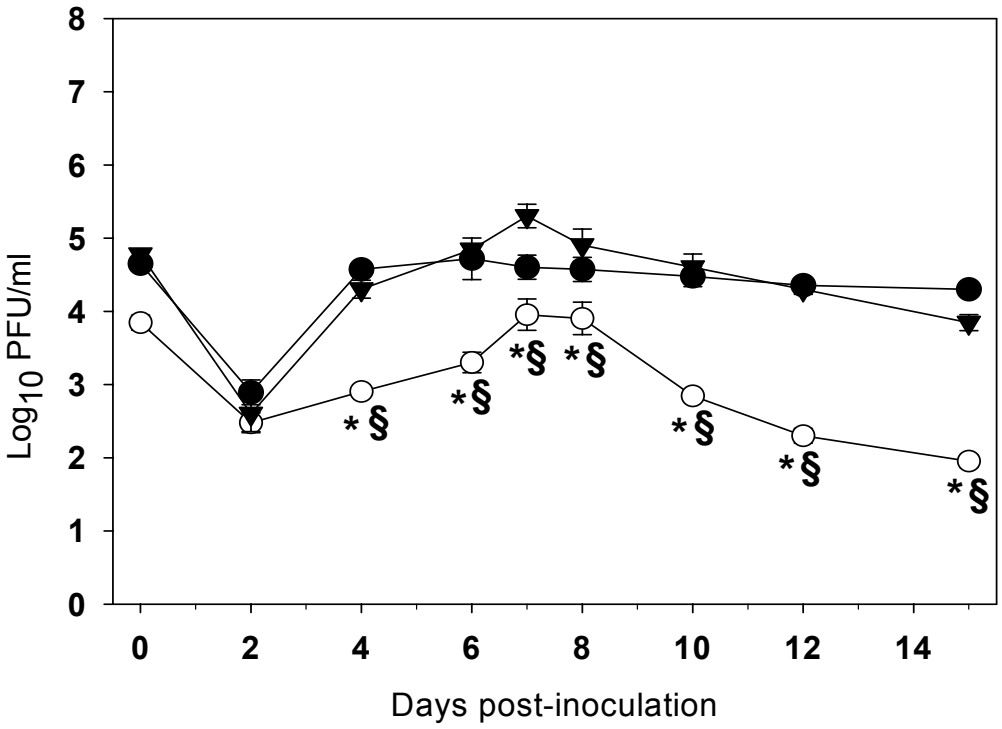


Figure 2.3. Viral growth curve for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Horse 3. Non-adherent (○), adherent (●) or total peripheral blood mononuclear cells (▼) were inoculated with WNV at an m.o.i. of 0.2 and samples were harvested at the indicated days. Values shown are the mean viral titers ± SEM . *****, significant difference ($p < 0.05$) between non-adherent and adherent cells; §, significant difference ($p < 0.05$) between non-adherent and PBMCs. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

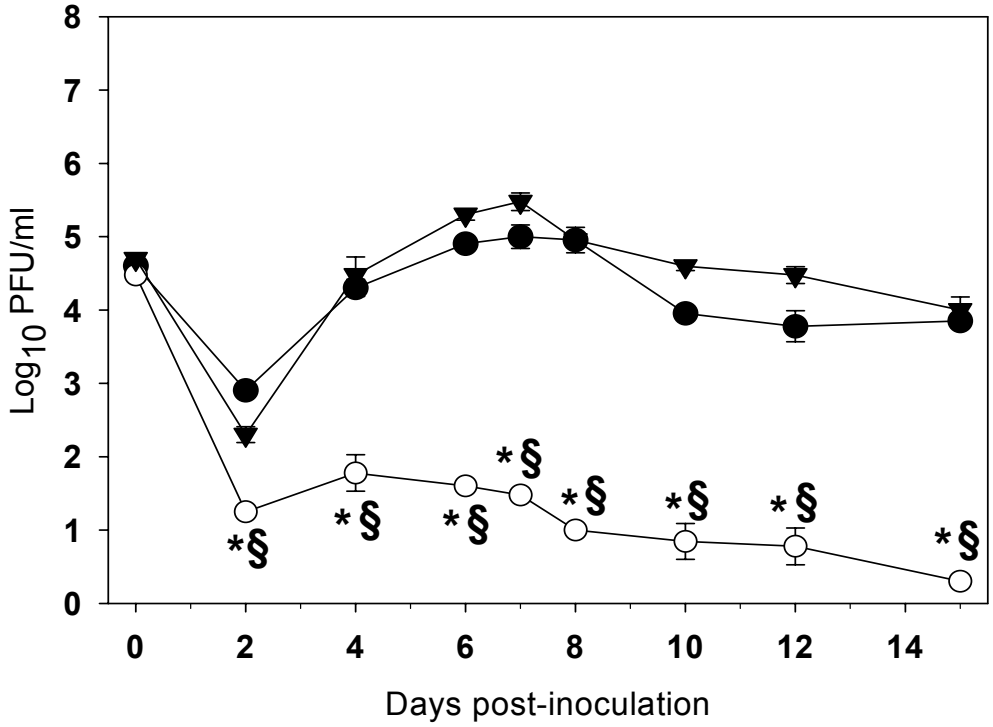


Figure 2.4. Viral growth curve for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Horse 4. Non-adherent (○), adherent (●) or total peripheral blood mononuclear cells (▼) were inoculated with WNV at an m.o.i. of 0.2 and samples were harvested at the indicated days. Values shown are the mean viral titers ± SEM . *****, significant difference ($p < 0.05$) between non-adherent and adherent cells; §, significant difference ($p < 0.05$) between non-adherent and PBMCs. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

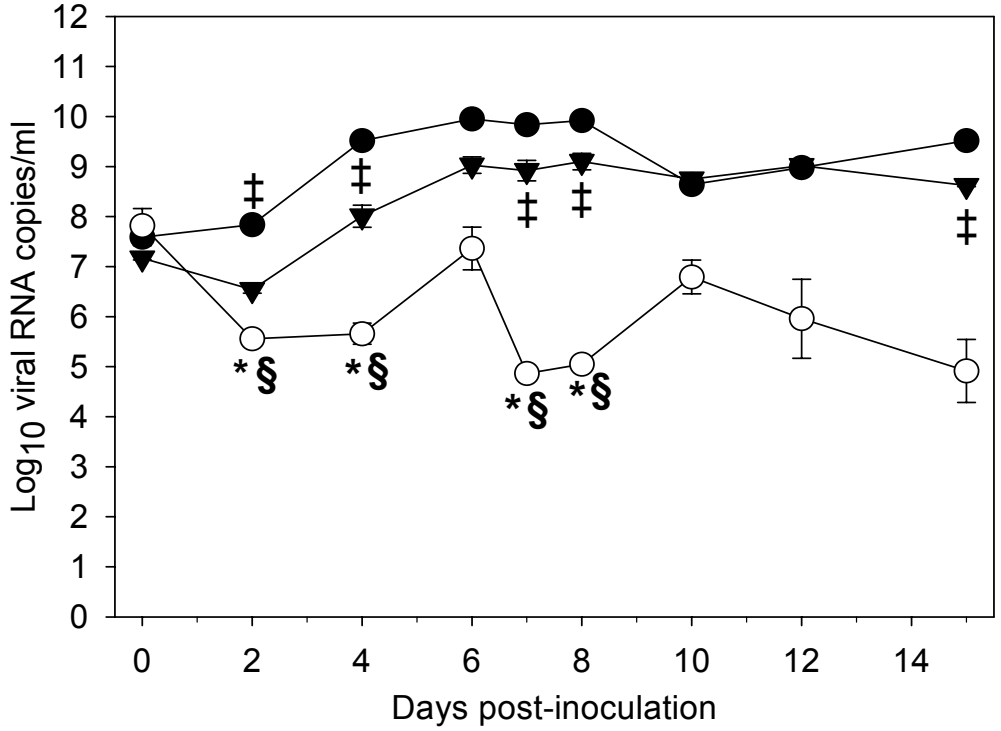


Figure 2.5. West Nile virus (WNV) RNA concentration in equine peripheral blood mononuclear cells (PBMCs). Horse 1. Non-adherent (○), adherent (●) or total peripheral blood mononuclear cells (▼) were inoculated with WNV at an m.o.i. of 0.2 and samples were harvested at the indicated days. Values shown are the mean viral RNA concentrations ± SEM (panel E and F). *****, significant difference ($p < 0.05$) between non-adherent and adherent cells; §, significant difference ($p < 0.05$) between non-adherent and PBMCs; ‡, significant difference ($p < 0.05$) between adherent and PBMCs. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

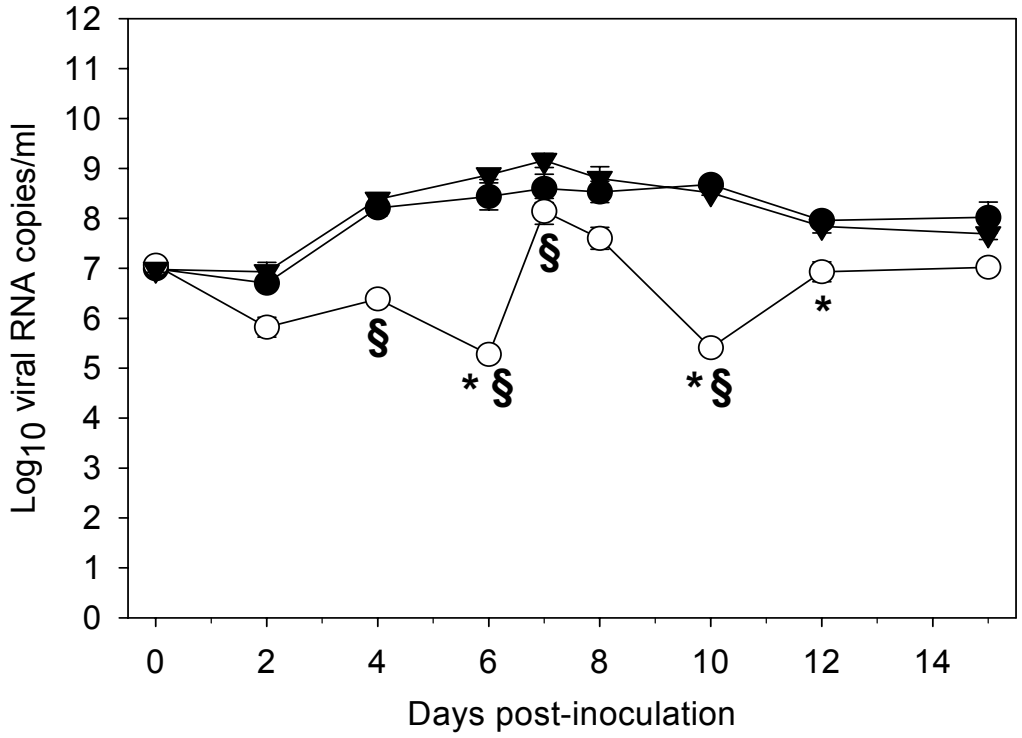


Figure 2.6. West Nile virus (WNV) RNA concentration in equine peripheral blood mononuclear cells (PBMCs). Horse 2. Non-adherent (○), adherent (●) or total peripheral blood mononuclear cells (▼) were inoculated with WNV at an m.o.i. of 0.2 and samples were harvested at the indicated days. Values shown are the mean viral RNA concentrations ± SEM (panel E and F). *****, significant difference ($p < 0.05$) between non-adherent and adherent cells; §, significant difference ($p < 0.05$) between non-adherent and PBMCs. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

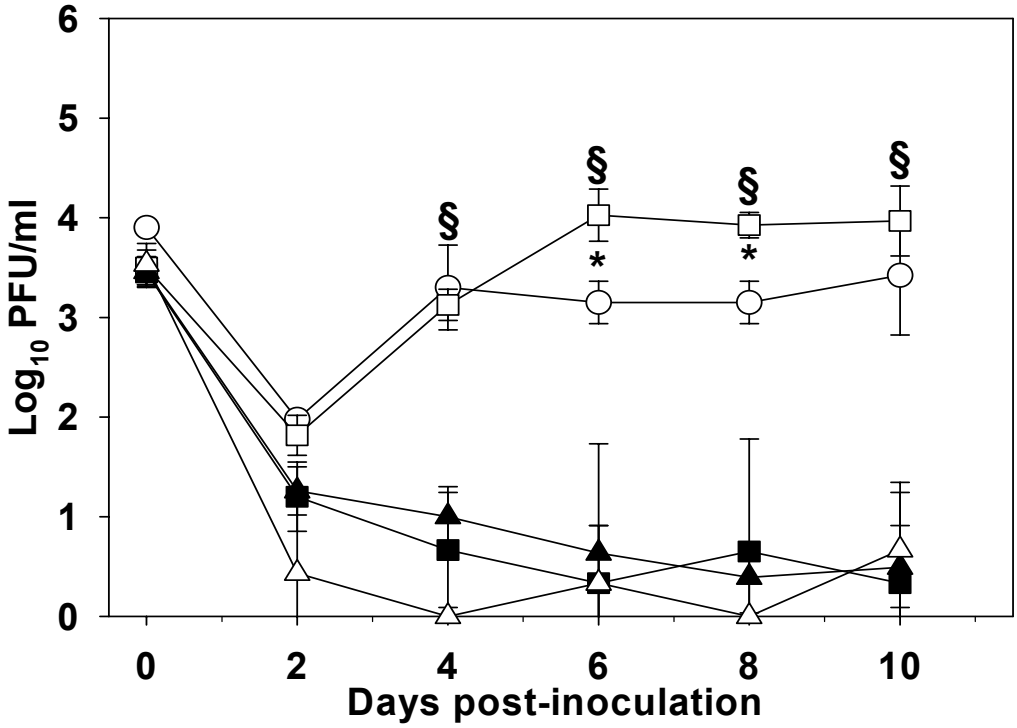


Figure 2.7. Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 1. Sorted monocytes (○), CD4+ cells (Δ), CD8+ cells (▲), B cells (■) and total PBMCs (□), were inoculated in vitro with WNV at an m.o.i of 0.02, and samples were harvested at the indicate days. Titration values from cells purified from horse no. 1 are shown. Values are the mean viral titers (± SEM) of triplicates from two independent experiments. *, significant difference ($p<0.05$) in viral titers between monocytes and PBMCs compared to CD4+, CD8+ or B lymphocytes; §, significant difference ($p<0.05$) between monocytes and PBMCs or CD4+ lymphocytes. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

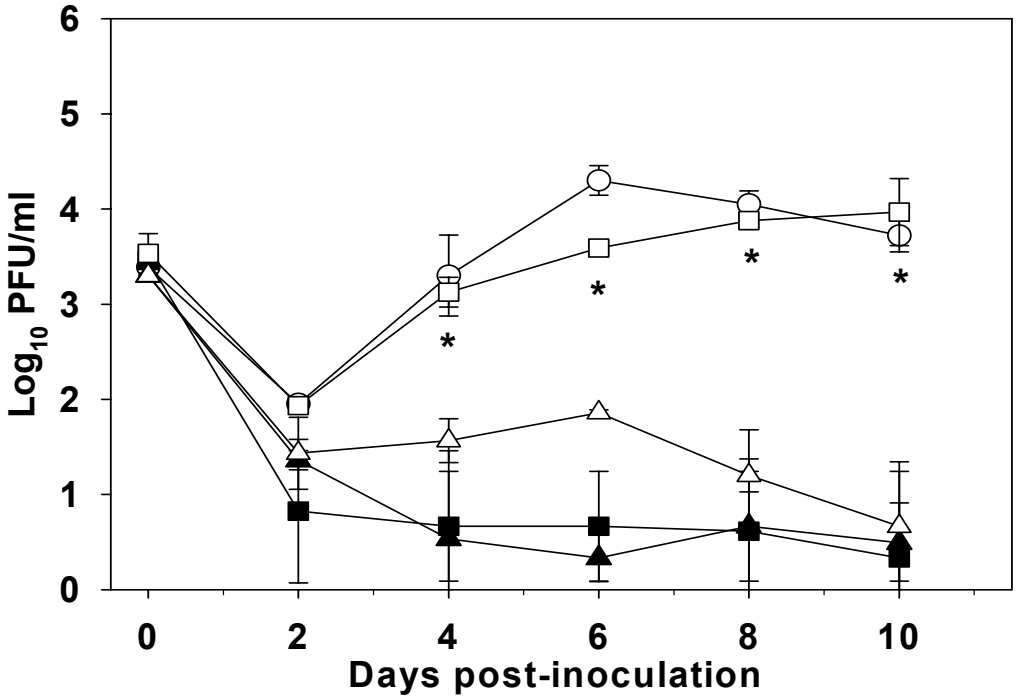


Figure 2.8. Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 2. Sorted monocytes (○), CD4+ cells (Δ), CD8+ cells (▲), B cells (■) and total PBMCs (□), were inoculated in vitro with WNV at an m.o.i of 0.02, and samples were harvested at the indicate days. Titration values from cells purified from horse no. 1 are shown. Values are the mean viral titers (± SEM) of triplicates from two independent experiments. *, significant difference ($p<0.05$) in viral titers between monocytes and PBMCs compared to CD4+, CD8+ or B lymphocytes. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

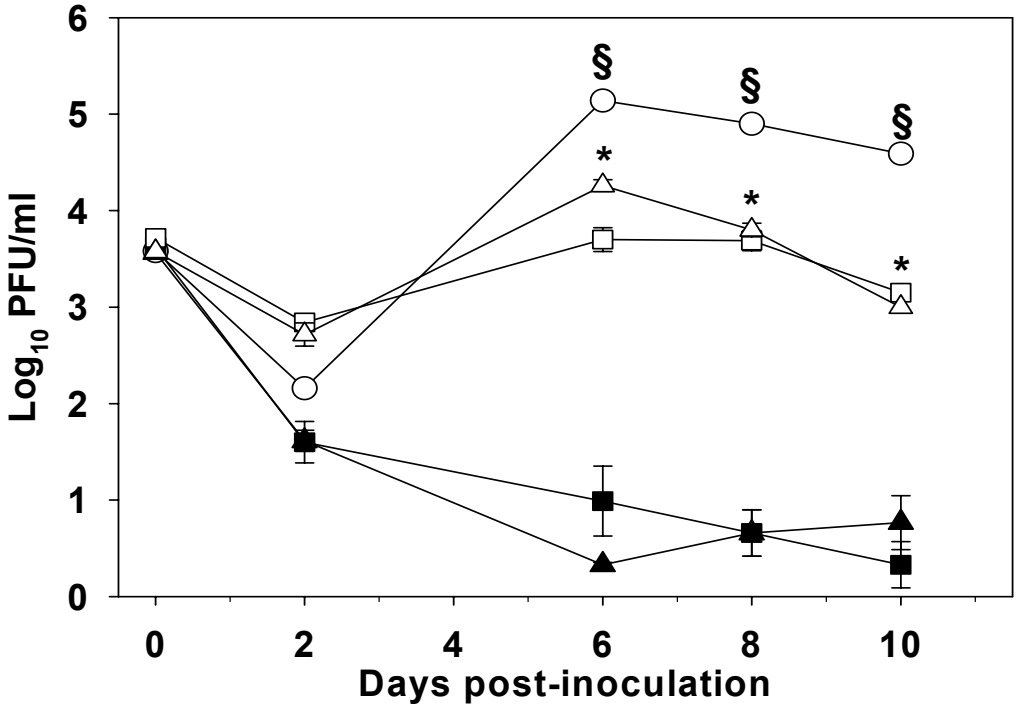


Figure 2.9. Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 3. Sorted monocytes (○), CD4+ cells (Δ), CD8+ cells (▲), B cells (■) and total PBMCs (□), 3 were inoculated in vitro with WNV at an m.o.i of 0.02, and samples were harvested at the indicate days. Titration values from cells purified from horse no. 1 are shown. Values are the mean viral titers (± SEM) of triplicates from two independent experiments. *, significant difference ($p<0.05$) in viral titers between monocytes and PBMCs compared to CD4+, CD8+ or B lymphocytes. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

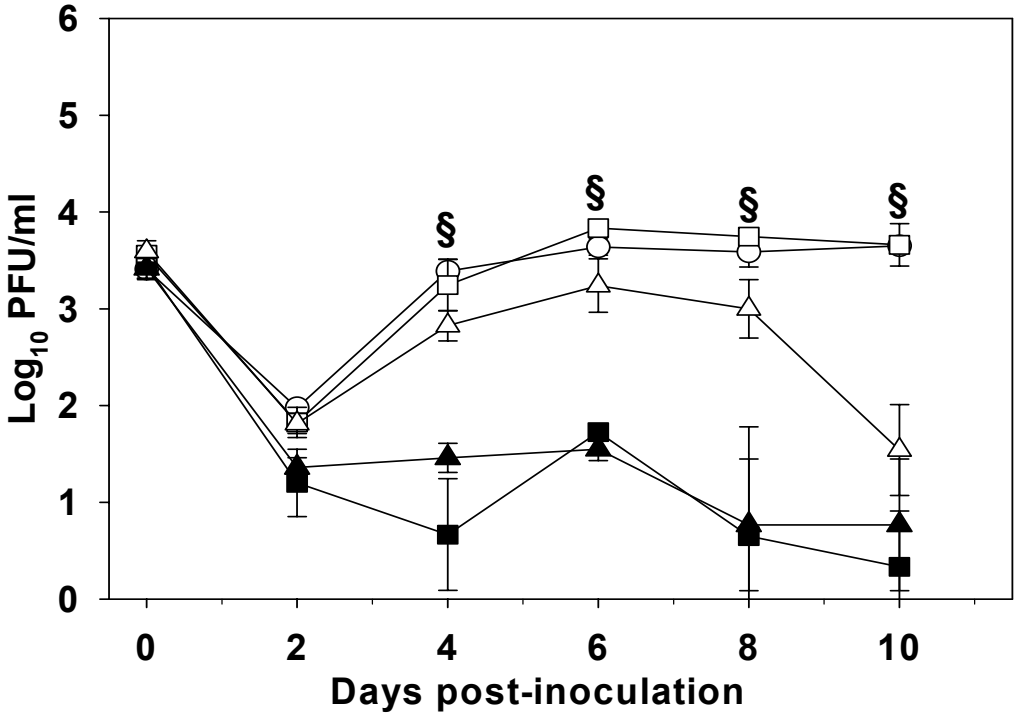


Figure 2.10. Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 4. Sorted monocytes (○), CD4+ cells (Δ), CD8+ cells (▲), B cells (■) and total PBMCs (□), were inoculated in vitro with WNV at an m.o.i of 0.02, and samples were harvested at the indicate days. Titration values from cells purified from horse no. 1 are shown. Values are the mean viral titers (± SEM) of triplicates from two independent experiments. *, significant difference ($p<0.05$) in viral titers between monocytes and PBMCs compared to CD4+, CD8+ or B lymphocytes; §, significant difference ($p<0.05$) between monocytes and PBMCs or CD4+ lymphocytes. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

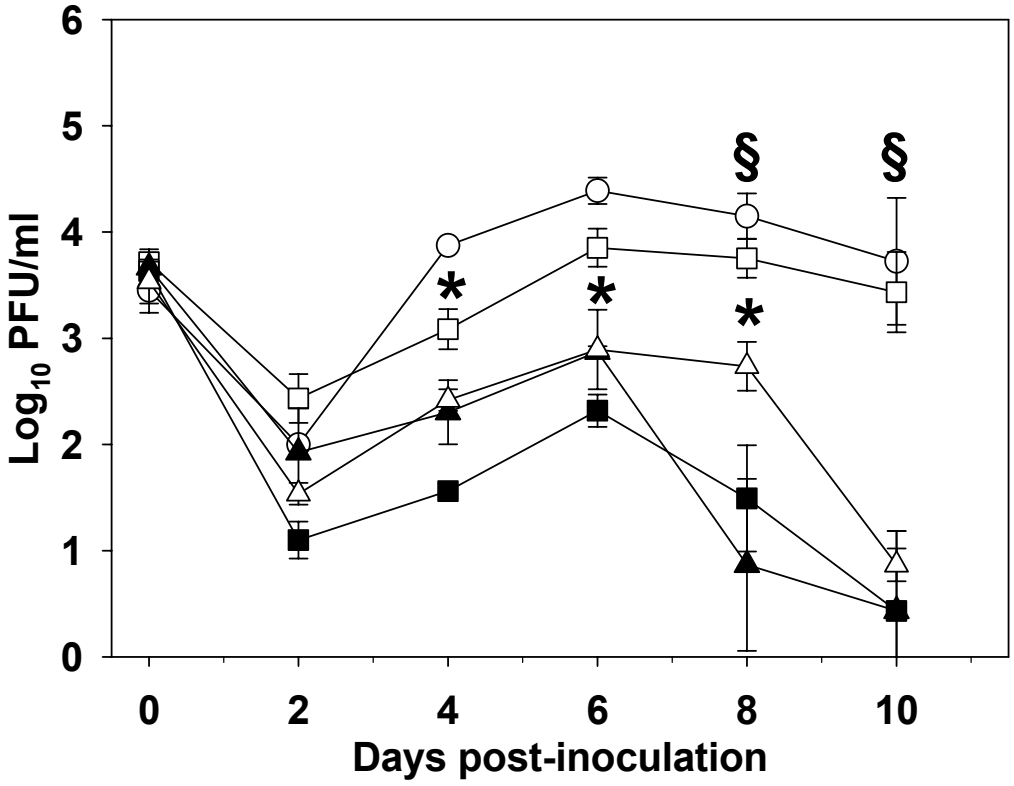


Figure 2.11. Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 5. Sorted monocytes (○), CD4+ cells (Δ), CD8+ cells (▲), B cells (■) and total PBMCs (□), were inoculated in vitro with WNV at an m.o.i of 0.02, and samples were harvested at the indicate days. Titration values from cells purified from horse no. 1 are shown. Values are the mean viral titers (± SEM) of triplicates from two independent experiments. *, significant difference ($p<0.05$) in viral titers between monocytes and PBMCs compared to CD4+, CD8+ or B lymphocytes; §, significant difference ($p<0.05$) between monocytes and PBMCs or CD4+ lymphocytes. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

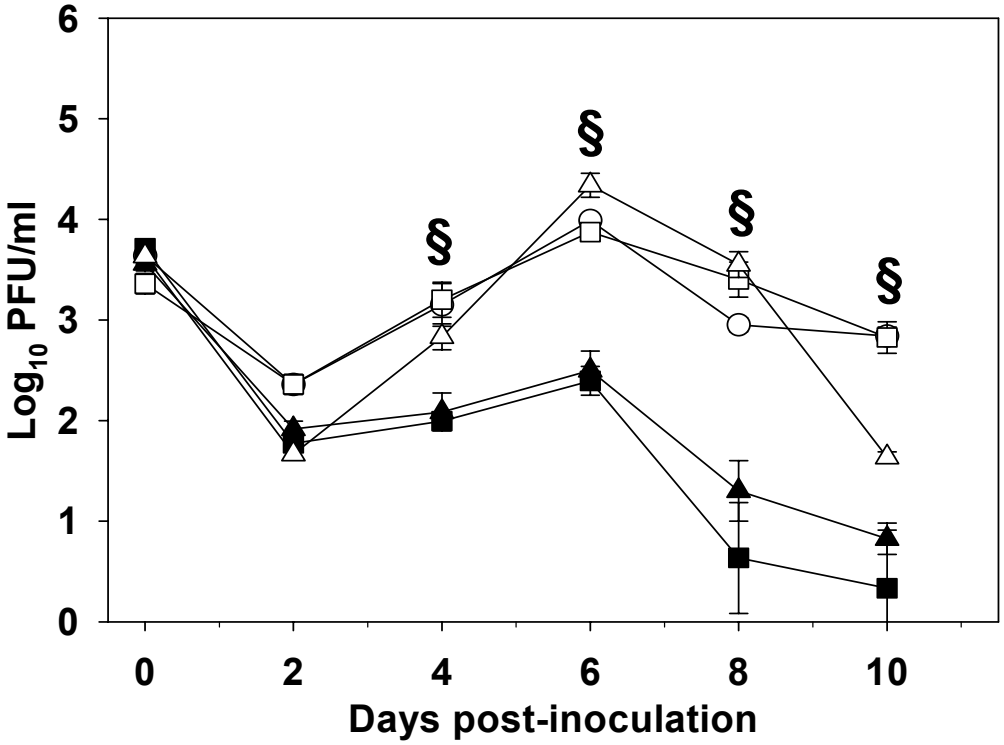


Figure 2.12. Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 6. Sorted monocytes (○), CD4+ cells (Δ), CD8+ cells (▲), B cells (■) and total PBMCs (□), were inoculated in vitro with WNV at an m.o.i of 0.02, and samples were harvested at the indicate days. Titration values from cells purified from horse no. 1 are shown. Values are the mean viral titers (± SEM) of triplicates from two independent experiments. *, significant difference ($p<0.05$) in viral titers between monocytes and PBMCs compared to CD4+, CD8+ or B lymphocytes; §, significant difference ($p<0.05$) between monocytes and PBMCs or CD4+ lymphocytes. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

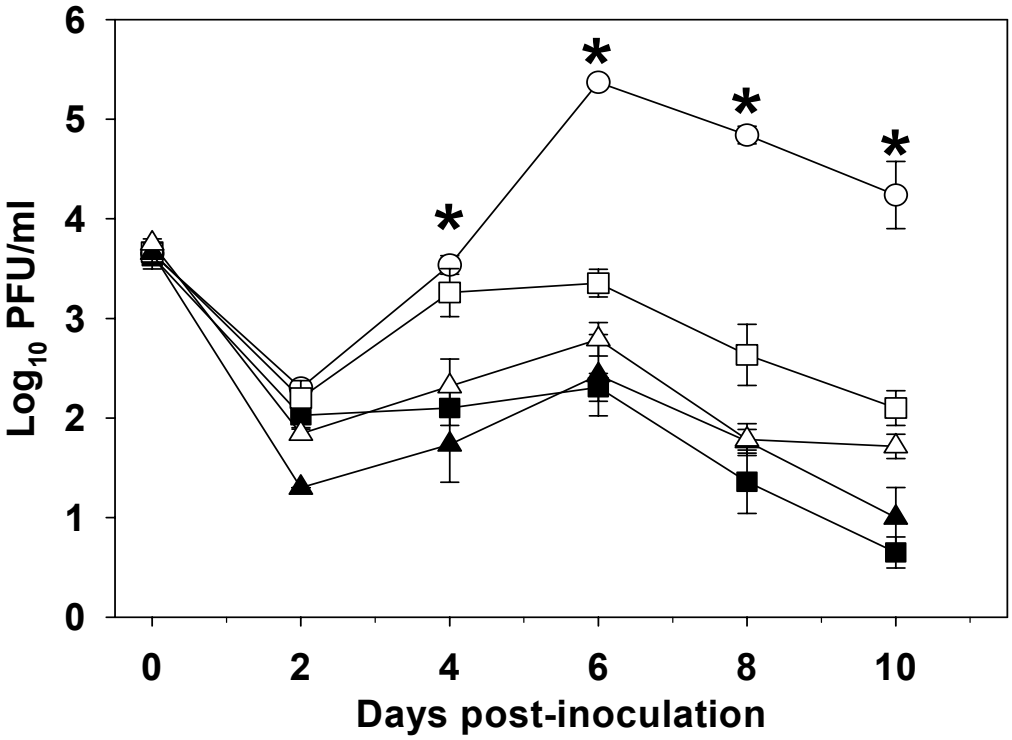


Figure 2.13. Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 7. Sorted monocytes (○), CD4+ cells (Δ), CD8+ cells (▲), B cells (■) and total PBMCs (□), were inoculated in vitro with WNV at an m.o.i of 0.02, and samples were harvested at the indicate days. Titration values from cells purified from horse no. 1 are shown. Values are the mean viral titers (± SEM) of triplicates from two independent experiments. *, significant difference ($p<0.05$) in viral titers between monocytes and PBMCs compared to CD4+, CD8+ or B lymphocytes; §, significant difference ($p<0.05$) between monocytes and PBMCs or CD4+ lymphocytes. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

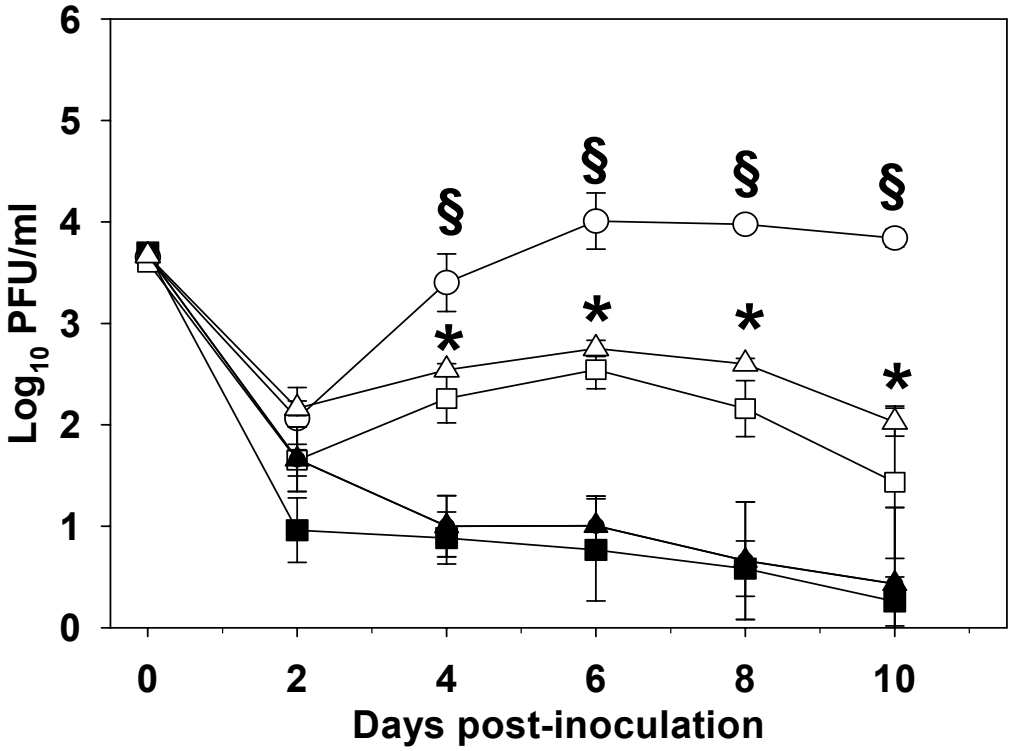


Figure 2.14. Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs) Sorted populations. Horse 8. Sorted monocytes (○), CD4+ cells (Δ), CD8+ cells (▲), B cells (■) and total PBMCs (□), were inoculated in vitro with WNV at an m.o.i of 0.02, and samples were harvested at the indicate days. Titration values from cells purified from horse no. 1 are shown. Values are the mean viral titers (± SEM) of triplicates from two independent experiments. *, significant difference ($p<0.05$) in viral titers between monocytes and PBMCs compared to CD4+, CD8+ or B lymphocytes; §, significant difference ($p<0.05$) between monocytes and PBMCs or CD4+ lymphocytes. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

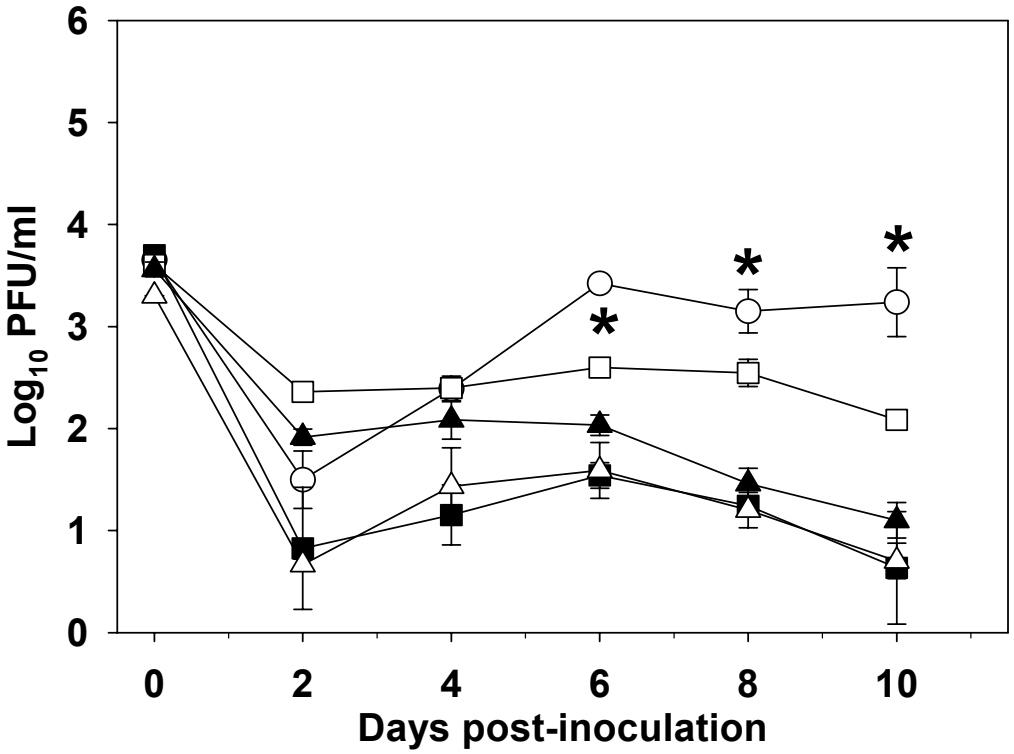


Figure 2.15. Viral growth curves for West Nile virus (WNV) in equine peripheral blood mononuclear cells (PBMCs). Sorted populations. Horse 9. Sorted monocytes (○), CD4+ cells (Δ), CD8+ cells (▲), B cells (■) and total PBMCs (□), were inoculated in vitro with WNV at an m.o.i of 0.02, and samples were harvested at the indicate days. Titration values from cells purified from horse no. 1 are shown. Values are the mean viral titers (± SEM) of triplicates from two independent experiments. *, significant difference ($p<0.05$) in viral titers between monocytes and PBMCs compared to CD4+, CD8+ or B lymphocytes; §, significant difference ($p<0.05$) between monocytes and PBMCs or CD4+ lymphocytes. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

Table 2.2. Purity of cell population following sorting by specific antibody and magnetic beads

Cell type	Range in purity	Mean ± SEM
Monocytes	95.0 – 97.5	96.25 ± 0.44
CD 4 +	96.6 – 98.0	97.22 ± 0.28
CD8 +	87.3 – 90.5	88.91 ± 0.57
B cells	79.0 – 90.4	83.96 ± 2.06

D. WNV replication in LPS-treated monocytes

Adherent cells or sorted monocytes from horses no. 1 – 4 were treated with 5 μg/ml of bacterial lipopolysaccharide (LPS) after 24 hrs in culture. Two hrs after addition of LPS, the cells were inoculated with WNV at an m.o.i. of 0.2. Non-LPS treated cells were also inoculated with WNV at the same m.o.i. Cell viability was assessed by trypan blue staining and both LPS-treated and untreated (control) cultures were found to have cell viabilities greater than 95%. Viral replication was assessed by plaque assay (Fig. 2.17 A and B) and quantitative RT-PCR for WNV RNA (Fig. 2.17 A) for adherent cells and sorted monocytes (Fig. 2.17A and 2.17B, respectively) derived from horse no. 1. By day 4 p.i., LPS-activated cultures were visually different from non-activated control cells when viewed by microscopy. Characteristics of LPS-treated adherent cells and monocytes included high granularity, clumping and less vacuolization than non-stimulated cells. Viral titers and WNV genomic RNA concentrations from untreated cells increased by 2 $\log_{10}$ PFU/ml between days 2 and 8 p.i. Maximum viral titers for cells from the four horses used in this experiment were 3.5 – 5.9 and 5.3 – 6.3 $\log_{10}$ PFU/ml

for adherent and sorted monocytes, respectively, while the maximum WNV RNA concentration was approximately 9 $\log_{10}$ viral RNA copies/ml. In contrast, WNV titers and RNA concentrations in LPS-treated cells did not increase significantly above the 2 day p.i. values for cells derived from any of the four horses tested.

To characterize the effect of LPS on adherent cells and sorted monocytes, nitric oxide (NO) and type I interferon (IFN) levels plus NF-κB translocation were assessed following LPS treatment and WNV-inoculation. A significant increase in NO concentration was observed at 26 hrs post LPS treatment (24 hrs post WNV-inoculation) compared to cells that were inoculated with WNV but untreated with LPS (Fig. 2.18 A). The NO concentration of LPS-treated cells decreased after day 2 p.i. At day 5 p.i. and through the remaining time points, WNV-inoculated cells had higher NO concentrations than those treated with LPS prior to WNV inoculation. The pattern of type I IFN production is similar to that observed for the production of NO. By 24 hrs p.i., type I IFN levels were significantly higher in LPS-treated cells compare to that were only inoculated with WNV (Fig. 2.18B). At day 2 p.i., LPS-treated cells and WNV-inoculated cells had equal type I IFN levels to those that were only inoculated with WNV. At the remaining time points after day 2 p.i., non-LPS treated cells had significantly higher type I IFN levels than those treated with LPS. At early times p.i., WNV inoculation alone did not result in translocation of NF-κB to the nucleus of inoculated cells (Fig. 2.18C). However, as early as 4 hours after treatment with LPS, WNV-inoculated cells nuclear translocation of NF-κB was clearly detected (Fig. 2.18D).

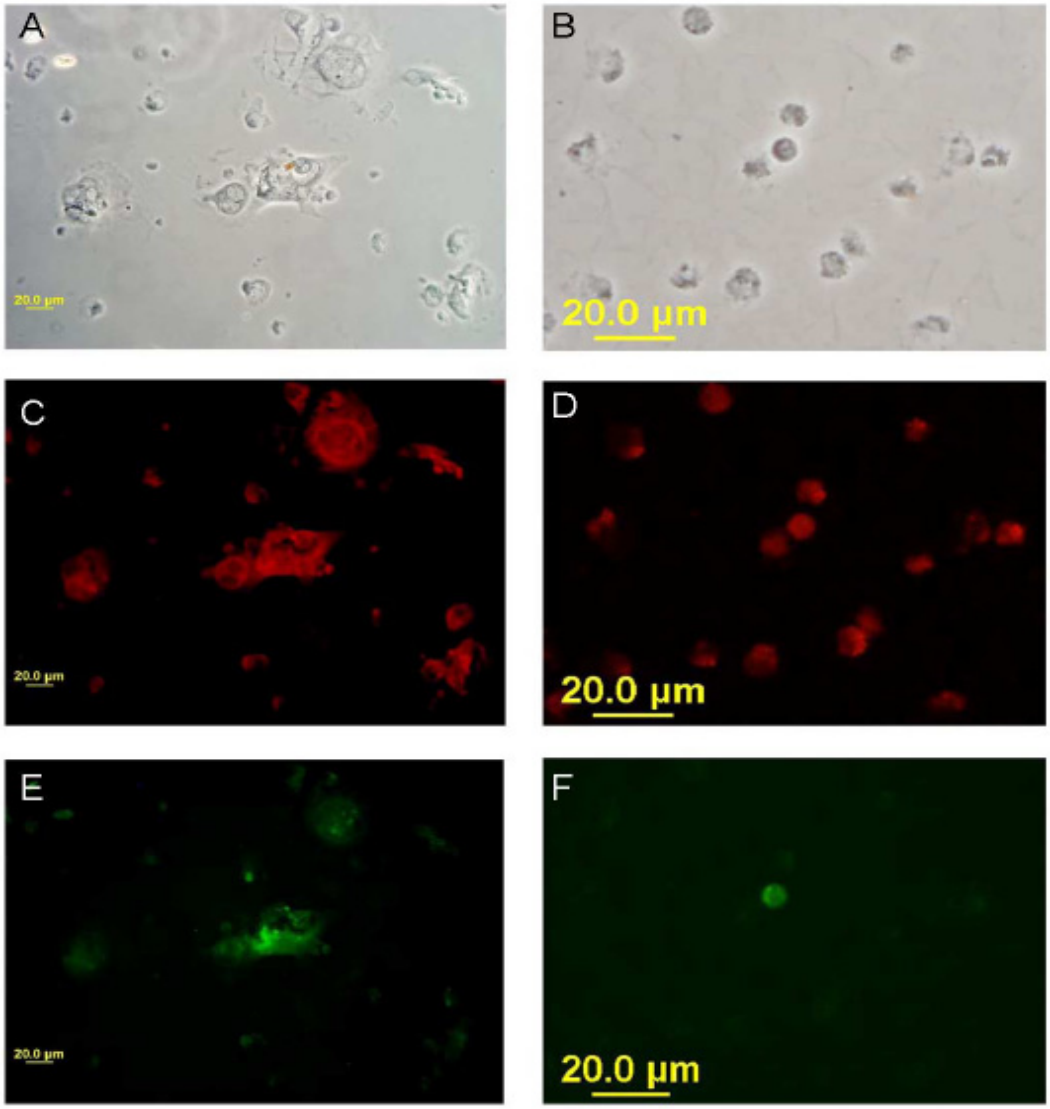


Figure 2.16. Dual fluorescent antibody detection of West Nile virus (WNV) non-structural protein 1 (NS-1) and CD-antigen of infected cells. Sorted horse monocytes (panels A, C, E) or CD4+ lymphocytes (panels B, D, F) were cultured in chamber slides. After one day in culture, the cells were infected with WNV at an m.o.i. of 1. On day 6 post-inoculation the slides were fixed and double-stained to detect expression of the WNV NS-1 and the expression of CD2 (T lymphocyte cell marker) or CD13 (monocyte marker). Reactions were developed using FITC conjugated anti-mouse IgM and rhodamine conjugated anti-mouse IgG1 as secondary antibodies for WNV NS-1 detection and the cell surface markers, respectively. The stained cells were then sequentially visualized with white light (panels A and B), or for rhodamine staining (panels C and D) and then FITC staining (panels E and F). Bars represent 20µM

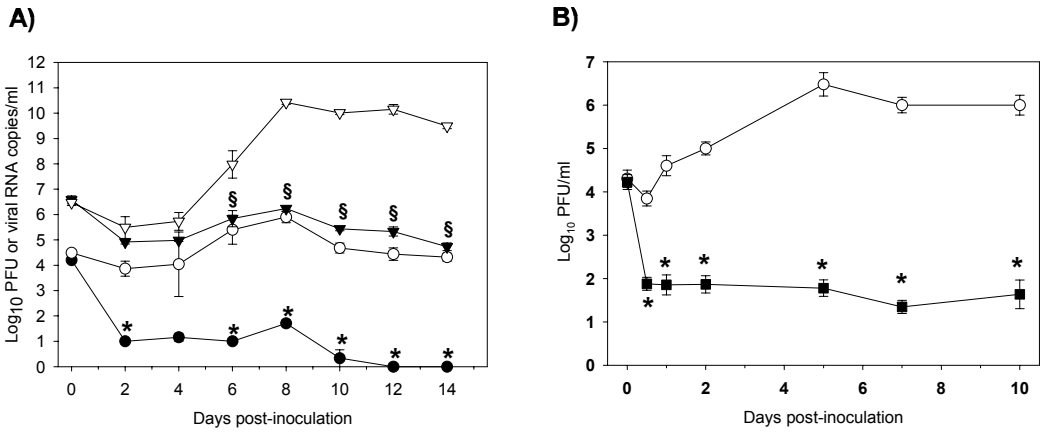


Figure 2.17. Effect of bacterial lipopolysaccharide (LPS) on West Nile virus replication in monocytes. Adherent cells (A) or sorted monocytes (B) purified from peripheral blood mononuclear cells of horse no. 1 were stimulated with LPS (closed symbols) or left resting (open symbols) prior to inoculation with WNV at an m.o.i. of 0.2. This experiment was repeated in three additional horses (no. 2 – 4) with identical results. Values shown are the mean viral titers ± SEM (circles) or mean viral RNA concentrations ± SEM (triangles). **(*)** significant difference in viral titer ($p < 0.05$) between LPS-stimulated and resting cells; (§) significant difference in viral RNA concentration ($p < 0.05$) between LPS-stimulated and resting cells. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

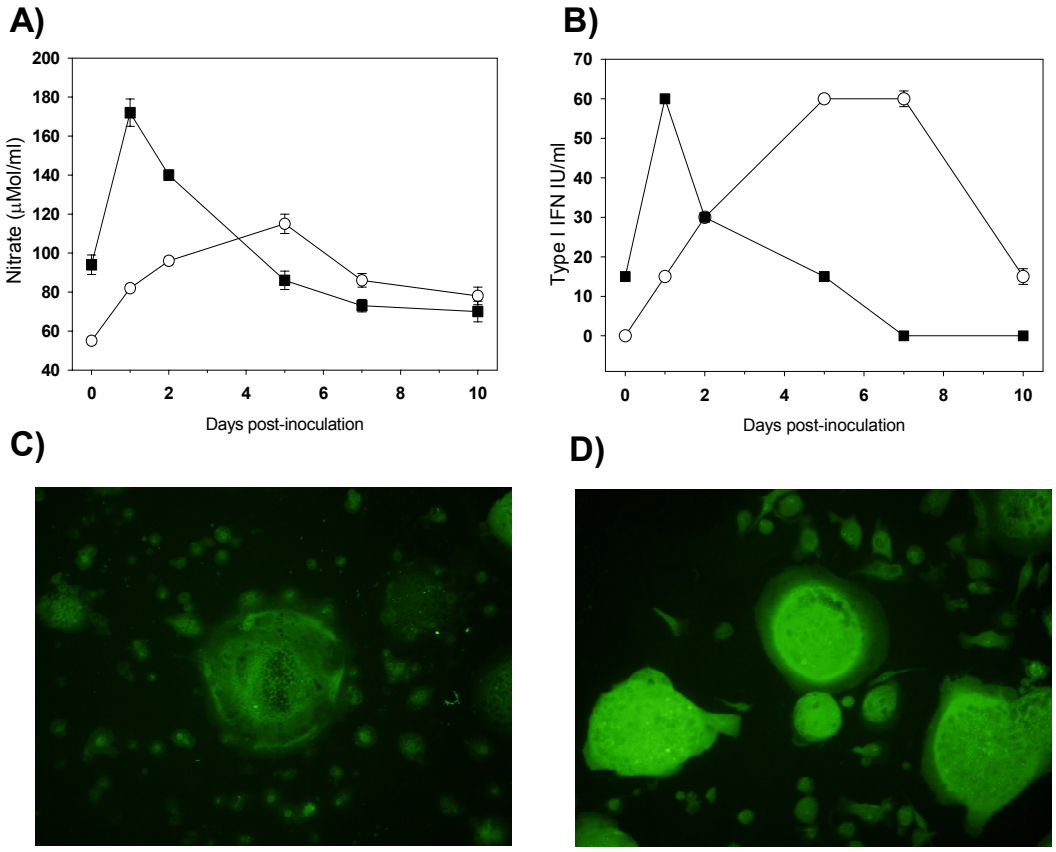


Figure 2.18. Nitric oxide (NO) and type I interferon (IFN) production plus NF-κB translocation in LPS-treated or untreated cells inoculated with WNV. Shown are the production of NO (panel A) and type I IFN (panel B) in LPS-treated (■) or untreated (o) sorted monocytes. LPS-treatment was performed 2 hrs prior to WNV inoculation, which was performed at an m.o.i. of 0.2. Values shown are the mean of triplicate samples (± SEM). Indirect fluorescent antibody detection of NF-κB was performed at 2 hrs p.i. in sorted monocytes that were either untreated (C) or LPS-treated (D) 2 hrs prior to inoculation with WNV. For all panels, data shown are from one representative experiment out of three performed.

IV. Discussion

In this study the kinetics of WNV replication in primary equine peripheral blood mononuclear cells was evaluated. Viral growth curves were performed in primary cultures of mixed cell populations, adherent cells, or non-adherent cells, as well as sorted monocytes, CD4+, CD8+, or B lymphocytes. Productive WNV replication was observed primarily in sorted monocytes, mixed cell populations, or adherent cells from blood of all horses evaluated. Productive WNV replication was also detected in CD4+ lymphocyte cultures, but only in a subset of the horses tested and only a small percentage of the cells were infected as determined by IFA staining for WNV antigen. WNV replication was not detected in any sorted CD8+ or B lymphocyte cultures. The results of quantitative RT-PCR detection of viral genomic RNA paralleled the titration results of the viral plaque assays, although absolute values for WNV RNA were typically >100-fold higher than viral titers. This difference is likely due to the presence of defective, non-infectious progeny virions (Wang et al., 2002). Indirect immunofluorescence double-staining of infected monocytes and CD4+ lymphocytes supported the results of viral growth curves by demonstrating the presence of WNV NS-1 antigen expression in both CD13 stained (monocytes) and CD2 stained (CD4+ lymphocytes) cells. However, at the cellular level replication in CD4+ cells appeared to be a rare event given the low percentage of infected lymphocytes observed by dual antibody staining. Detection of a WNV non-structural protein in infected cells was significant since NS-1 plays an important role in RNA replication, particularly in the initiation of minus strand synthesis (Lindenbach and Rice, 2003). Thus the identification of NS-1 protein from WNV by fluorescent antibody

staining supports the findings from viral growth curves which indicate productive viral replication in monocytes and CD4+ lymphocytes.

Activation of adherent or sorted monocytes cultures with bacterial LPS significantly reduced WNV replication. To investigate potential mechanisms of the antiviral activity observed, NF-κB activation was assessed along with levels of NO and type I IFN. Treatment of monocytes with LPS induced nuclear translocation NF-κB at early times post-treatment. NF-κB is an inducible transcription factor that plays a key role in inflammation, innate immune responses, the regulation of cell proliferation and cell survival (Caamano et al., 2002; Li et al., 2002). Activation of NF-κB by viral infection is a key trigger to inducing type I interferon (IFN) transcription and other immune responses, including pro-inflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases (MMPs), cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS) (Caamano et al., 2002; Santoro et al., 2003). In our experiments, elevated levels of NO were observed at the first time point measured, indicating that up-regulation of inducible nitric oxide synthase (iNOS) had occurred. The role of iNOS as an important molecule in the antiviral response against Japanese encephalitis virus, a member of the Flaviviridae along with WNV, has been demonstrated by others (Lin, et al., 1997). Similarly, type I IFN concentrations peaked rapidly following LPS treatment of cells. The production of these molecules reached its peak production later in the infection course; this observation is coincident with previous results reported by Fredericksen, who observed a late production of type I interferon in human cells infected with WNV (Fredericksen, et al., 2004). Type I IFNs are key molecules in innate host

defenses against viral infection. Type I IFN has been shown to activate or induce the synthesis of several proteins including 2,5-oligoadenylate synthase (OAS), double-stranded RNA-dependent protein kinase (PKR), Mx and ribonuclease L (RNase L) (Samuel, 2001; Vilcek and Sen, 1996), all of which result in induction of an antiviral state. Together, these data demonstrate that a number of potential antiviral pathways may have contributed to the antiviral state observed in LPS-treated monocytes.

Eight of nine donor horses used in this study were seropositive to WNV, either due to vaccination or natural exposure. For the seropositive horses, PRNT values ranged from 1:80 – 1:5,120. However, no correlation was established between the level of humoral immunity to WNV at the time of blood collection and WNV replication, or lack thereof, in any of the immune cells. Antibody-dependent enhancement of WNV infection has been previously reported for infection of a continuous mouse peritoneal macrophage line by WNV (Peiris and Porterfield, 1979; Peiris et al., 1981). However, it is very unlikely that this mechanism of viral entry played any role for the results reported herein since all cell populations (especially sorted cells) were extensively washed prior to inoculation with virus and thus essentially no plasma from the original blood sample was present. Furthermore, all serum added to cell culture media as a supplement was tested by PRNT and IFA for antibody to WNV and found to be negative. Thus in the experiments performed in this study, viral entry into equine mononuclear cells occurs by mechanisms other than those related to antibody binding of viral proteins followed by internalization.

The ability of other flaviviruses within the Japanese Encephalitis virus serocomplex to infect monocytes, macrophages or lymphocytes has been widely reported. Monocytes and macrophages are considered to be primary targets of dengue virus infection both in vitro and in vivo, and the importance of these cells is related particularly to the antibody dependent enhancement phenomenon and to the immune response against the virus (Bosch et al., 2002; Chaturvedi etal., 1999; Cologna and Rico-Hesse, 2003; Diamond et al., 2000; Hase et al., 1989; Ho et al., 2001; Hotta et al., 1984; Kurane and Ennis, 1987; Kurane and Ennis, 1988; Libraty et al., 2001; Mangada et al., 2002; Moreno-Altamirano et al., 2002; Pryor et al., 2001; Sydow et al., 2000; Tassaneetrithep et al., 2003; Wu et al., 2000). Additionally, it has also been reported that Japanese encephalitis virus will specifically infect T lymphocytes (Mathur et al., 1989; Sharma et al., 1991) and that dengue virus can infect both T and B lymphocytes (King et al., 1999; Mentor and Kurane, 1997).

In contrast to other flaviviruses, WNV replication in monocytes, macrophages and lymphocytes has previously been investigated only tangentially. As part of a study on the importance of adherence status, WNV infection of murine macrophages was inferred by enhanced cellular gene expression, although the time course and quantification of viral replication were not assessed by this study (Shen et al., 1995). In another report, Concanavalin A (ConA) stimulated adherent human PBMCs were inoculated with WNV. In these experiments the initial viral titer of 7.4 $\log_{10}$ $TCID_{50}$/ml declined by over 500,000-fold to 1.95 $\log_{10}$ $TCID_{50}$/ml after two weeks (which was the first sample taken) and remained at this level for two additional weeks (Mather et al., 2003). Data presented

in the present study demonstrated that LPS-activation of monocytes and adherent cells effectively reduced or perhaps eliminated WNV replication suggesting that activated monocytes may not support productive WNV replication. Our results following LPS treatment were similar to those observed by others in which activation of human monocytes/macrophages with LPS prior to dengue infection blocked viral replication (Chen et al., 1999). Conversely, enhancement of dengue virus infection of monocytes/macrophages was later reported when LPS was added after infection with the virus (Chen and Wang, 2002). Other studies support our finding of an antiviral state in activated macrophages. For example, it has been reported that when polyIC was used to stimulate mouse macrophages derived from Flavivirus-susceptible and congenic resistant mice, the activated macrophages are able to decrease or abolish the active replication of WNV in vitro (Pantelic, et al., 2005). Additionally, infection of macrophages by the alphavirus Ross River Virus using an antibody-dependent mechanism was not influenced by activation of cells with LPS (Mahalingham and Lindbury, 2002).

Characterization of WNV replication in monocytes is potentially significant since this cell population, or cells derived from monocytes, may be early target cells of WNV after the inoculation of a host by a natural route such as mosquito feeding. Our previous studies have demonstrated low levels of WNV viremia in horses near the time of natural infection with WNV (Kleiboeker et al., 2004). Furthermore, it has been reported that Langerhans cells migrate to local lymph nodes following cutaneous infection by WNV (Byrne et al., 2001; Johnston et al., 2000). Lymph nodes to which the infected Langerhans cells migrate are thus a potential site in which monocytes, or other peripheral

blood mononuclear cells, could become infected. Our demonstration of WNV replication in monocytes and lymphocytes suggests a potential role for both cell types in the movement of WNV into the CNS. One possible mechanism is that once the virus reaches peripheral blood mononuclear cells, these cells travel to capillary vessels which are located close to the CNS. From here, the cells migrate from blood to the neural tissues across the choroid plexus or to the subarachnoid space or the parenchymal perivascular space (Ransohoff et al., 2003). Such movement of WNV-infected immune cells could facilitate the transport of the virus to the CNS.

In summary, productive WNV replication was consistently demonstrated in vitro for adherent, non-adherent and mixed immune cell cultures as well as sorted monocytes and CD4+ lymphocytes derived from equine peripheral blood mononuclear cells. Replication was demonstrated in immune cells collected from a seronegative horse and multiple seropositive horses. Based on these data monocytes and CD4+ lymphocytes of the peripheral blood are potential target cells for WNV infection and may play a role in the dissemination of WNV to the CNS. Furthermore, given the ready availability of large quantities of these cells for in vitro culture and manipulation, equine monocytes and CD4+ lymphocytes may offer a model for study of WNV in naturally infected hosts or when testing compounds such as antivirals for use in WNV treatment.

CHAPTER III

INDUCTION OF ANTIBODY-DEPENDENT ENHANCEMENT (ADE) BY SUBNEUTRALIZING DILUTION OF IMMUNE HORSE SERUM IN AN IN VITRO INFECTION OF HORSE MACROPHAGES AND MURINE TIB-186 CELL LINE BY WEST NILE VIRUS.

Abstract

West Nile virus (WNV) is the etiologic agent of an important emerging disease in North America. Infection by WNV has caused human deaths and considerable economic losses in the equine industry since its introduction into the United States in 1999. West Nile virus is an encephalitic flavivirus that affects birds, humans, horses and other mammals. There are two distinct genetic lineages of WNV. Lineage I is the lineage responsible for the recent outbreak in USA, and lineage II is located mainly in the Southern region of Africa and in Madagascar. Like WNV, Dengue virus is a flavivirus that affects humans. It has been demonstrated that when a person is infected a second time with a distinct genetic lineage of Dengue virus, this individual suffers a more severe disease. This effect, termed antibody dependent enhancement (ADE) of infection, has been attributed to the enhancement of the infection by the presence of antibodies against the lineage that was responsible for the first infection. These antibodies enhance the infection by a different isolate in a secondary infection resulting in fatal hemorrhagic disease. Previously it was shown that ADE can be induced in WNV infection *in vitro* using a macrophage-derived mouse cell line (TIB-186) with rabbit anti-WNV immune serum. Neither mice nor rabbits

are natural hosts of the WNV. Horses, however are natural hosts of the virus. Due to the existing differences in the isotypes of immunoglobulins present in horses in comparison to those present in rabbits, the central question addressed in this chapter was if ADE could be induced by anti-WNV horse immune serum for WNV infection of horse monocyte-derived macrophages or a mouse macrophage-like cell line (TIB-186 cells). Additionally, it was determined if the anti-WNV I horse serum could induce ADE in cells infected with WNV lineage II. We found that WNV I-immune horse serum induced ADE for infection of mouse macrophage-like cell line (TIB-186) or horse monocyte-derived macrophages by either WNV I or WNV II. These differences in viral growth kinetics may result in a different type of immune response and therefore this information should be considered when developing WNV vaccination or prevention programs. The risk of introduction of an additional WNV lineage I strain or a WNV lineage II strain into United States territory is present, and this information is potentially important to predict the outcome of secondary infection with a heterologous strain of WNV in horses that are antibody-positive to the current WNV I strain that is present in the United States.

I. Introduction

West Nile virus (WNV) is a member of the Flaviviridae family and the genus *Flavivirus*. Other members of that genus include dengue, tick-borne encephalitis, yellow fever, Japanese encephalitis, and St. Louis encephalitis (SLE) viruses. Smithburn and colleagues first isolated WNV in 1937 from the blood of a 37-year-old Ugandan woman in the West Nile District of Northern Uganda at the headwaters of the White Nile (Hollinger and Kleinman, 2003). Currently West Nile virus (WNV) is found throughout

Africa, Europe, Central Asia, and, most recently, in North America. The first outbreak in the United States was in New York City during the summer of 1999, and in subsequent years the virus spread throughout much of North America. In 2005, there are 2,016 reported cases of human illness and 55 human deaths associated with WNV (Control Disease Center, Oct. 2005). WNV is a flavivirus transmitted primarily by *Culex* mosquitoes to vertebrate hosts. Flaviviruses are positive strand RNA viruses that contain three structural proteins and a host-derived lipid bilayer. Two lineages of WNV (I and II) have been identified. All isolates that cause severe human disease fall into lineage I (Mukhopadhay, 2003). The isolate that was introduced into the United States in 1999 belongs to the genetic lineage I. To date WNV lineage II strains have typically been identified in countries of Southern Africa and in Madagascar (Burt et al. 2002).

As there is no specific antiviral treatment for disease caused by WNV, major research efforts have focused on diagnosis and prevention. At least two commercial companies have initiated the research for development of human vaccines. In an attempt to protect horses against WNV, both a formalin-inactivated vaccine and a viral-vectored vaccine have been developed and are currently in use (Monath, 2001). Understanding the role of the host immune response in disease pathogenesis is required for development of immune protection with the minimal negative effects. Potential collateral affects include the possibility of induce enhancing antibodies that, contrary to protection, may actually enhance the infection of host cells by the virus. ADE of macrophage and monocyte infection has been demonstrated *in vitro* for some of the most deadly RNA viruses known. For example, ADE has been demonstrated both *in vitro* and *in vivo* for dengue

virus and HIV (Morens, 1994; Halstead, 1988) and these findings represent a significant consideration for development of vaccines against these viruses. Recent evidence suggest that ADE- mediated ligation of Fc receptors might promote the production of interleukin 10 (IL-10), leading to suppression of host-cell antiviral gene expression by expression of suppressor of cytokine signaling (SOCS) proteins and a Th-2 profile. These findings suggest mechanisms by which ADE may enhance viral infections and exacerbate clinical disease symptoms (Suhrbier and Linn, 2003). It has been also demonstrated that anti-viral gene expression is blocked by the ADE phenomenon in a Ross River Virus infection of monocytes. This is due to down-regulation of TNF-α and iNOS as well as blocking of NFκB activation (Lidbury and Mahalingam, 2000). However, ADE in dengue virus infection has been shown to induce expression of TNF-α in human monocytes (Anderson et al. 1997), suggesting that the effects may be specific to the virus and/or cell system studied.

Antibody dependent enhancement of the infection is believed to occur through FcγR I (CD64) and FcγR II (CD32), although a second type of ADE that requires complement has also been described (Chambers and Diamond, 2003). Most of the data regarding to ADE are relevant to the pathogenesis of dengue virus infection; for other encephalitic flaviviruses there is some experimental evidence that ADE may be involved. Wallace and colleagues demonstrated that sub-neutralizing concentrations of antibody may enhance flavivirus infection and virulence *in vivo* (Wallace et al., 2003). Neutralizing antibodies to structural proteins of one serotype of dengue virus, typically not only fail to neutralize but also appear to enhance replication of virus in cases of

dengue hemorrhagic fever, a condition that is generally seen upon re-infection by a different serotype of dengue virus (Morens, 1994). The ADE phenomenon for WNV was demonstrated *in vitro* by Peiris and Porterfield, using a macrophage-like cell line and specific rabbit antibodies against WNV (Peiris and Porterfield et al., 1979). It is known that there are variations in the types of immunoglobulins in horses in comparison to those observed in other mammals (rabbits for instance) (Pastoret et al., 1998; Tizard, 2004a). The differences in antibody isotypes may be reflected with a different avidity of those horse antibodies for Fc receptors found in the mouse cells. This chapter addresses the question of whether the ADE phenomenon can be induced by anti-WNV horse immune serum in a WNV infection of a mouse macrophage-like cell line (TIB-186) or horse monocyte-derived macrophages. The other question addressed is whether anti-WNV I horse serum is able to induce the ADE in cells infected with WNV lineage II.

II. Materials and Methods

A. Virus and cell culture.

West Nile virus was isolated from the brain of an American Kestrel that died in Missouri (United States) with neurological signs in 2002. The initial virus isolation and subsequent propagation were performed in Vero cell cultures using standard techniques (Burleson et al., 1992). Vero cell cultures were grown and maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine, 0.25μg/ml fungizone, and 0.5 mg/ml gentamycin (cell culture reagents supplied by Mediatech, Inc., Herndon, VA). The cells were

maintained at 37°C in a humidified 5% CO_2 incubator. Approximately 4 days post-inoculation (p.i.) with the brain homogenate, a diffuse cytopathic effect was identified in inoculated Vero cells. Identification of the cytopathic agent as WNV was confirmed by indirect fluorescent antibody staining of inoculated cells using hyperimmune anti-WNV ascitic fluid (VR1267 AF, American Type Culture Collection, Manassas, VA) and an FITC labeled anti-mouse IgG conjugate (Sigma, Inc., St. Louis, MO). Additionally, identification of the isolate as WNV was confirmed by WNV-specific RT-PCR (Johnson et al., 2001) followed by sequencing of 441 bp of the amplified viral envelope glycoprotein (E) gene. Comparison of the sequenced region of the isolate demonstrated 99.8% identity to the prototype U.S. strain NY99-98 flamingo382-99 (Lanciotti et al., 1999; GenBank accession no. AF196835) from nucleotide 1401 to 1841. The sequenced region was 100% identical over the 441 bp sequenced to a number of WNV isolates from 2002 (Beasley et al., 2003; GenBank accession no. AY185906, AY185907, 101 AY185908, AY185909, AY185911). The WNV lineage II isolate was obtained from an infectious clone (Yamshchikov et al., 2001) supplied as a gift by Dr. V. Yamshchikov.

Quantification of WNV stocks and experimental samples was performed using a viral plaque assay. Ten-fold serial dilutions of samples were adsorbed for two hrs onto confluent Vero cell monolayers in six-well plates (10 cm^2/well). Cell monolayers were washed once with cell culture media. Overlay media, which consisted of maintenance media plus 0.5% (w/v) agarose, was then added to each well. Cultures were maintained at 37°C in a humidified 5% CO_2 incubator for three days. Overlays were then removed

and cell monolayers were stained with 0.5% (w/v) crystal violet/70% (v/v) methanol and plaques were counted and viral titers were calculated (Burleson et al., 1992).

TIB-186 cultures were grown and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) , 2 mM L-glutamine, 0.25µg/ml fungizone, and 0.5 mg/ml gentamycin (cell culture reagents supplied by Mediatech, Inc., Herndon, VA), 0.15% sodium bicarbonate, 1M HEPES, 1mM Sodium pyruvate. Sorted equine monocytes (see below for details of preparation) were maintained Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% heat-inactivated horse serum (HS), 2 mM L-glutamine, 0.25µg/ml fungizone, and 0.5 mg/ml gentamycin (cell culture reagents supplied by Mediatech, Inc., Herndon, VA). All cells were maintained at 37°C in a humidified 5% CO_2 incubator.

B. Purification of equine monocytes by cell sorting.

Blood (120 – 200 ml) was collected by aseptic technique from a total of two horses. Sodium heparin was present at a final concentration of approximately 50 unit/ml blood. All horses were clinically normal with no known health problems at the times of blood collection and all horses had been previously immunized with a minimum of three doses of killed West Nile virus vaccine (West Nile Innovator, Ft. Dodge Laboratories, Ft. Dodge, IA) in the 12 months preceding blood collection. Heparinized blood was centrifuged at 785 x *g* for 25 min, and the interface (buffy coat) layer was removed and diluted 1:1 with phosphate buffered saline, pH 7.4 (PBS). The diluted buffy coat sample was layered onto a Ficoll-Hypaque gradient (Sigma Chemical Co., St Louis, MO) and

centrifuged at 785 x g for 25 min. The interface was collected, washed three times with PBS by centrifuging at 50 x g for 10 min to eliminate the platelets. After the last wash, the cells were resuspended in sorting buffer (0.5% BSA, 2mM EDTA in phosphate buffered saline pH 7.4) to proceed with the cell sorting.

Positive selection of monocytes was performed using aseptically obtained horse blood (200 ml) following purification of PBMCs as described above. Once PBMC's were separated from whole blood, 10^8 cells were incubated with 100 µg of specific monoclonal antibody against equine CD13 (MCA 1084 Serotec Inc., Raleigh, NC). The antibodies were incubated with cells for 20 min at room temperature in 500 µl of sorting buffer. After incubation with antibody, cells were washed twice with sorting buffer and then incubated with 80 µl of anti-isotype antibody conjugated to magnetic microbeads (rat anti-Mouse IgG1 microbeads catalog no. 130-047-101 Miltenyi Biotech Inc, Auburn, CA) for 20 min at 4°C in with gentle shaking. This incubation was followed by two washes with sorting buffer. Cells were sorted by positive selection, using magnetic cell sorting equipment (Auto-MACS, Miltenyi Biotech, Auburn, CA). After sorting, cells were washed twice with DMEM supplemented with 10% normal horse serum. Cells then were counted and 1.5 x 10^5 cells were seeded on to six-well plates (Corning Inc., Acton, MA) and maintained for five days at 37°C in a humidified 5% CO_2 incubator prior to being infected.

C. Assessment of antibody-dependent enhancement of infection.

2 x 10^5 purified equine monocytes or Vero cells or macrophage-like mouse cells (cell line TIB-186) were infected at a multiplicity of infection (m.o.i.) of 0.001 with WNV lineage I or WNV lineage II. The 90% plaque-reduction neutralization titer (PRNT) in the equine serum had previously been determined to be approximately 1:80. Prior to inoculation of cells with WNV I or WNV II, the virus was incubated with different dilutions of horse serum for 1 hour at room temperature followed by 1 hour at 37°C. Four different dilutions of serum were used, from a neutralizing dilution (1:50) through subneutralizing dilutions (1:500; 1:2,500; 1:10000). Controls using non-immune horse serum (diluted 1:50) were included in all the experiments as were controls to which no horse serum had been added. Following pre-incubation, the virus was added to cells and held at 37C for 2 hours. The cells were then rinsed two times with non supplemented Dulbecco's media. Combined cellular and supernatant samples were collected at days 0, 1, 2, 3, 5, and 7 post-inoculation. The samples were held at -80°C until processed for virus quantification by plaque assay.

D. Statistical analysis.

The results were compared by the Student t-test to their respective control (without serum treatment or treated with normal horse serum) to determine if a significant statistical difference of the means was present. Significance was set at $P < 0.05$.

III. Results

A. The effect of anti-WNV horse serum on WNV I and II viral yields following inoculation of Vero, TIB-186 or equine monocyte cultures.

Prior to experiments with Fc-bearing cell populations, the effect of incubating virus with anti-WNV horse serum dilutions prior to inoculation was assessed for infection of Vero cells with WNV lineage I or II viral stocks (Fig. 3.1). A significant reduction of viral titers was observed at all times post-inoculation for both WNV lineage I and II following pre-incubation with a serum dilution of 1:50. Peak viral titers following incubation with this dilution were 1,000 – 10,000 lower when compared to non-immune horse serum controls or controls to which no horse serum had been added during the pre-incubation step. For WNV I infection of Vero cells, pre-incubation with a serum dilution of 1:500 also led to a significant reduction of viral yield. All other serum dilutions had no significant effect on WNV yields from Vero cells.

Similar to results with Vero cells, pre-incubation with an anti-WNV serum dilution of 1:50 resulted in a significant reduction of viral titers following inoculation of TIB-186 cells, an Fc receptor bearing murine macrophage-derived cell line (Fig. 3.2). However, pre-incubation with higher dilutions of serum resulted in significant increases in viral yields from these cells for both lineages of WNV. For WNV I, increased viral yields were most consistently detected at day 2 p.i. following incubation with a 1:2,500 serum dilution, which led to viral titers nearly 10,000-fold higher than those of control wells. Serum dilutions of 1:500 and 1:10,000 also led to elevated WNV I yields which

were present through day 5 p.i. For WNV II, the increase in viral titers was not as great as those observed for WNV I, but were significantly elevated compared to controls at 1, 5 and 7 days p.i. for serum dilutions ranging from 1:500 to 1:10,000.

The effect of incubating virus with anti-WNV serum prior to inoculation was also evaluated for infection of equine monocytes purified from the peripheral blood of two different horses (Figs. 3.3 and 3.4). As observed for both Vero and TIB-186 cultures, pre-incubation with a 1:50 serum dilution prior to inoculation resulted in significant reduction of viral yields at a majority of time points for both WNV I and II. At higher dilutions of serum, particularly 1:2,500, both WNV I and II viral yields were significantly elevated above control values with peak titers occurring at 1 – 3 days p.i. This dilution of serum resulted in titer values ranging from 10 to 100-fold higher than those detected in control cultures. For WNV II inoculated monocytes, the observed titer differences were maintained from day 3 to 7 p.i.

IV. Discussion

Antibody dependent enhancement in a WNV infection of a murine macrophage-like cell line was previously reported. Peiris and Porterfield (1979) demonstrated enhancement of WNV infection when the virus was incubated with hyperimmune rabbit serum prior inoculation of TIB-186 cells. Although the phenomenon was reported and the importance of the Fc gamma receptor II was evaluated (Peiris and Porterfield et al 1979; Peiris et al., 1981), there are no reports documenting the ability of anti-WNV immune horse serum inducing this effect. Additionally, ADE has not been assessed in cells

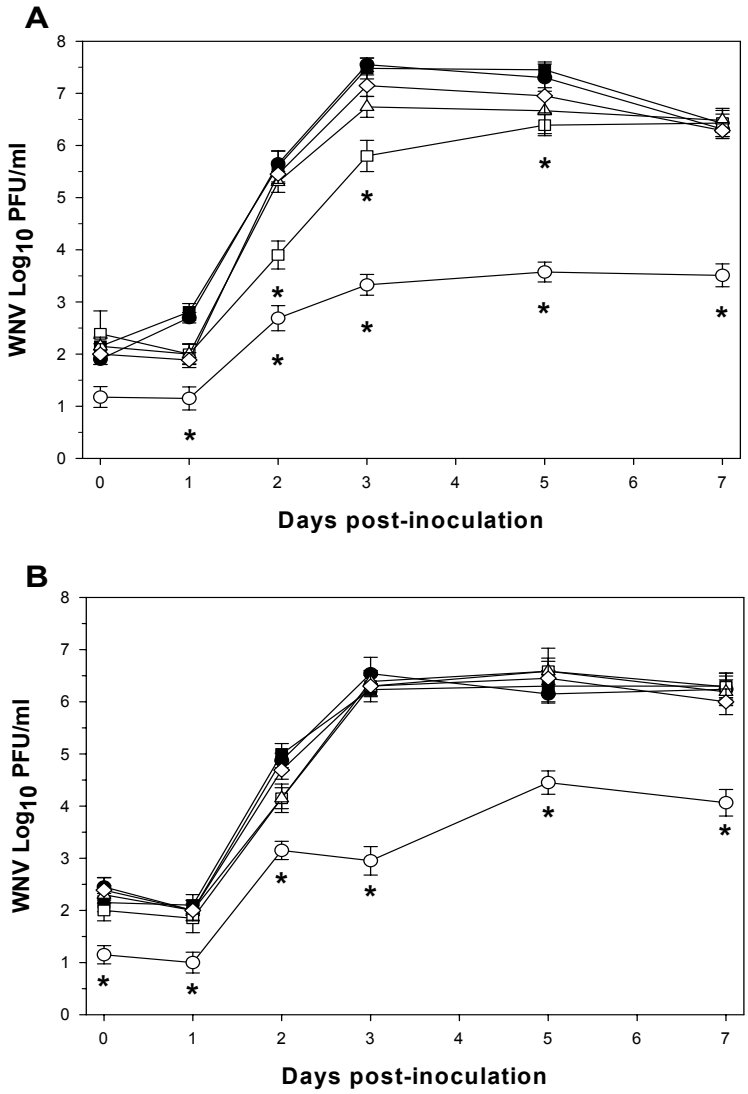


Figure 3.1. **ADE in Vero's cells infected with WNV**. Growth curves of WNV I (panel A) or WNV II (panel B) in Vero's cells are shown. Cells were inoculated at an m.o.i of 0.001 with WNV lineage I or lineage II previously incubated with: (●) culture media; or (■) normal horse serum; or WNV I-immune horse serum diluted at 1:50 (○); 1:500 (◊); 1:2,500 (Δ); 1:10,000 (□). Values are the mean viral titers ± SEM of triplicates from three independent experiments. *, significant difference ($p<0.05$) in viral titers between the different immune horse serum treatments and the normal horse serum and no serum incubation treatments. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

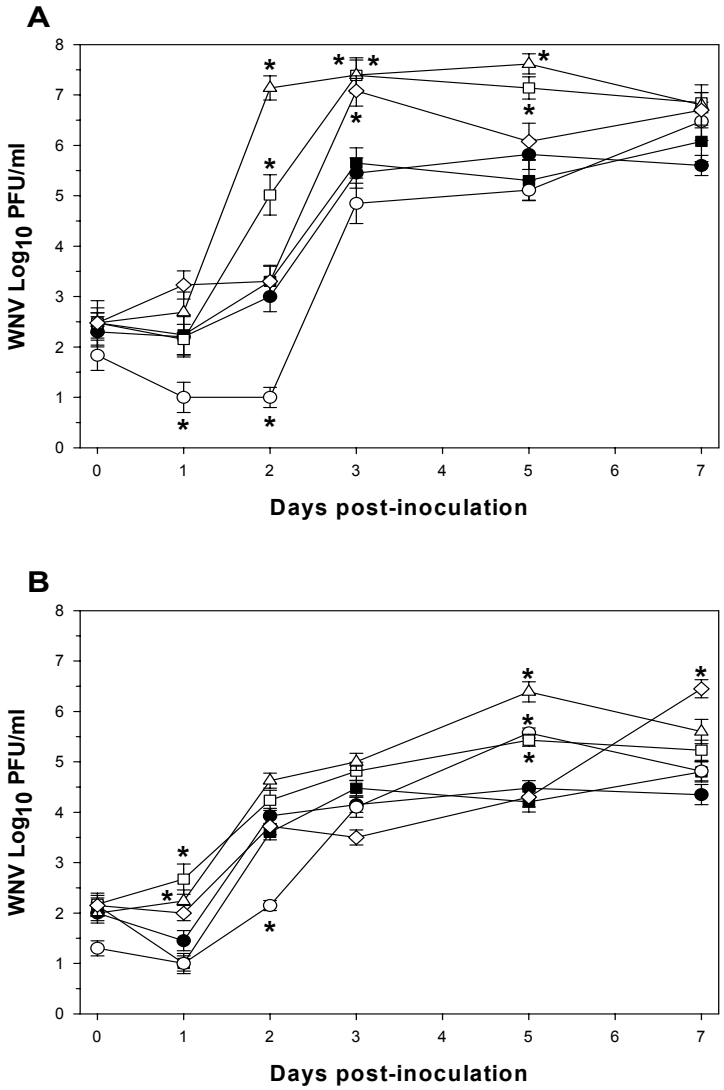


Figure 3. 2. **ADE in TIB-186 cells infected with WNV**. Growth curves of WNV I (panel A) or WNV II (panel B) in TIB-186 cells are shown. Cells were inoculated at an m.o.i of 0.001 with WNV lineage I or lineage II previously incubated with: (●) culture media; or (■) normal horse serum; or WNV I-immune horse serum diluted at 1:50 (○); 1:500 (◊); 1:2,500 (Δ); 1:10,000 (□). Values are the mean viral titers ± SEM of triplicates from three independent experiments. *, significant difference ($p<0.05$) in viral titers between the different immune horse serum treatments and the normal horse serum and no serum incubation treatments. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

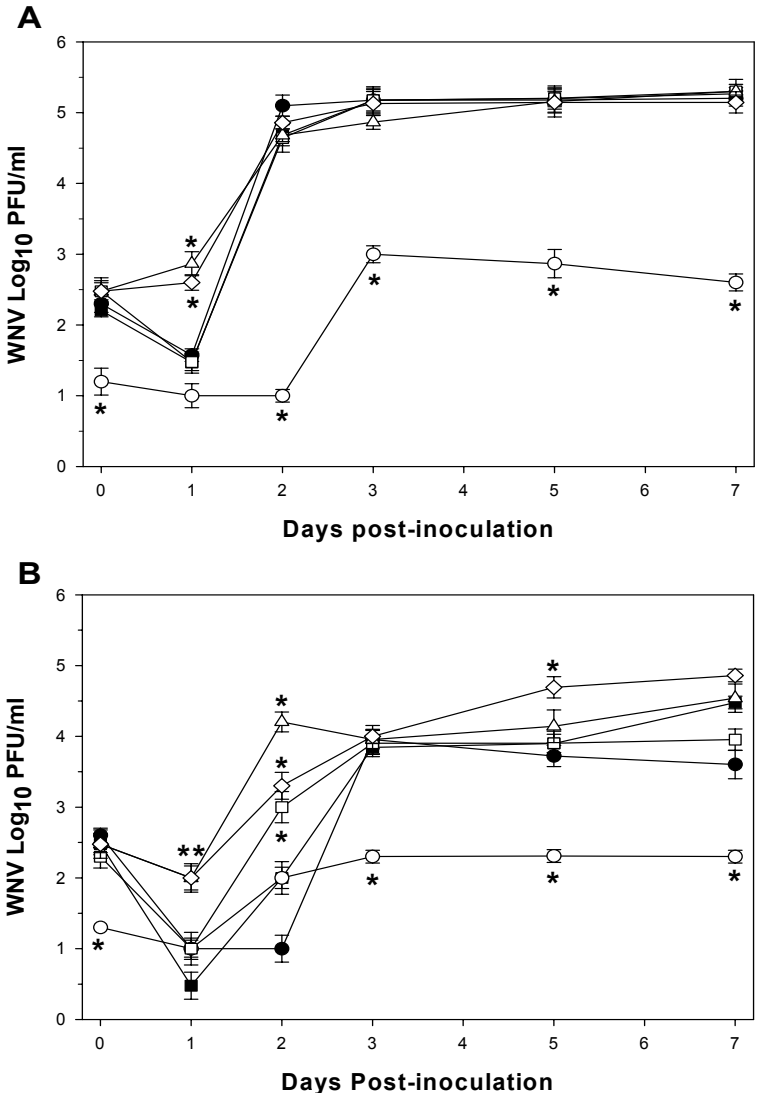


Figure 3.3. **ADE in horse monocyte-derived macrophages infected with WNV**. Growth curves of WNV I in cells from horse 1 (panel A) or cells from horse 2 (panel B) are shown. Cells were inoculated at an m.o.i of 0.001 with WNV lineage I previously incubated with: (●) culture media; or (■) normal horse serum; or WNV I-immune horse serum diluted at 1:50 (○); 1:500 (◊); 1:2,500 (Δ); 1:10,000 (□). Values are the mean viral titers ± SEM of triplicates from three independent experiments. *, significant difference ($p<0.05$) in viral titers between the different immune horse serum treatments and the normal horse serum and no serum incubation treatments. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

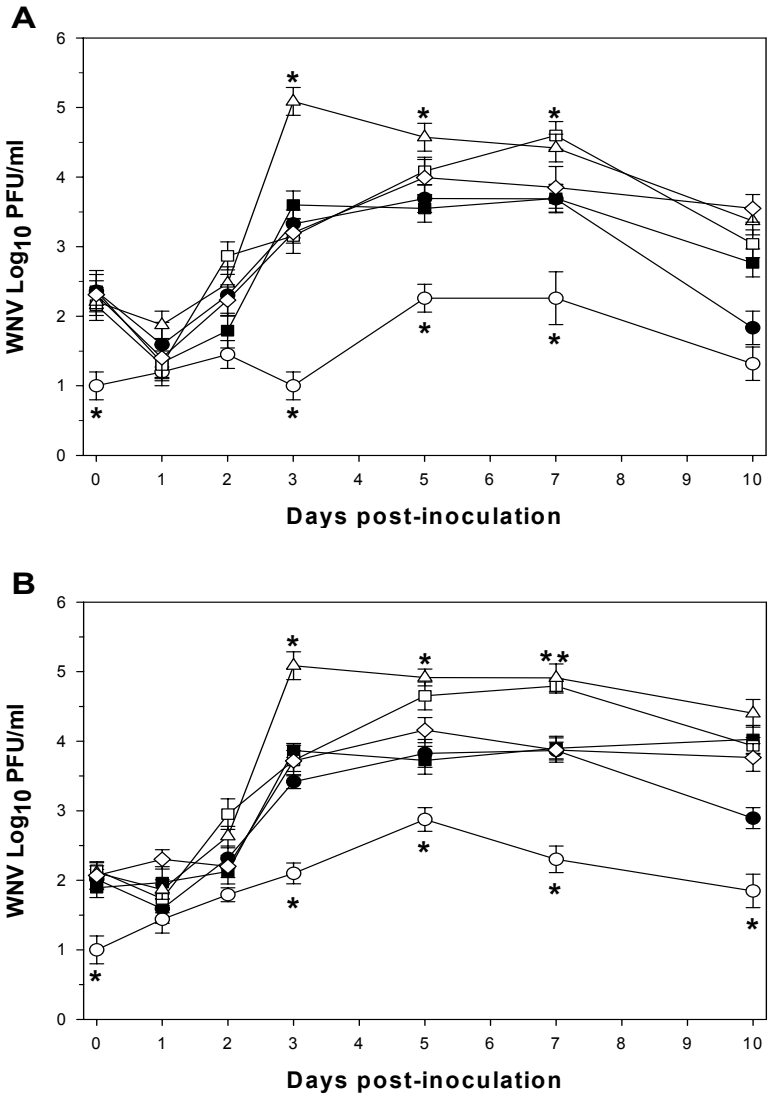


Figure 3.4. ADE in horse monocyte-derived macrophages infected with WNV. Growth curves of WNV II in cells from horse 1 (panel A) or cells from horse 2 (panel B) are shown. Cells were inoculated at an m.o.i of 0.001 with WNV lineage II previously incubated with: (●) culture media; or (■) normal horse serum; or WNV I-immune horse serum diluted at 1:50 (○); 1:500 (◊); 1:2,500 (Δ); 1:10,000 (□). Values are the mean viral titers ± SEM of triplicates from three independent experiments. *, significant difference ($p<0.05$) in viral titers between the different immune horse serum treatments and the normal horse serum and no serum incubation treatments. Time 0 represents the time of the virus addition, prior to rinsing of the cells.

derived from a natural host such as the horse. In the experiments presented herein, an ADE phenomenon was induced by horse serum in an *in vitro* WNV infection of horse monocyte-derived macrophages and mouse macrophage-like cell line (TIB-86), but not in Vero's cells, a cell line that does not express Fc receptors.

Viral infection of Fc bearing cells, which are an important part of the immune system, leads to the activation of certain genes which code for specific important molecules such as cytokines, chemokines and cellular receptors. It has previously been demonstrated that ligation of phagocytic receptors on macrophages can influence cytokine production. Activation of human macrophages with Lipopolysaccharide results in proinflamatory stimuli that are characterized by the production of high levels of IL-12 and a low production of IL-10. This pattern was altered when the Fcγ receptor of LPS-activated macrophages were crosslinked (Gerber and Mosser, 2001). As mentioned earlier activation of macrophages and the use of these cells as antigen presenting cells (APC's) leads to a strongly polarized Th-1 response that is characterized by production of IFN-γ and IL12. This effect was abrogated *in vivo* when mice were vaccinated with IgG-opsonized ovalbumin. These antibodies crosslink the Fcγ receptors leading to a Th-2 type response that was manifested by production of high levels of IgG 1 isotype (Anderson and Mosser, 2002). It has been recently demonstrated that Ross River virus was able to suppress transcription and translation of key antiviral genes such as TNF and iNOS in LPS-stimulated macrophages by disrupting transcription of the genes coding for the associated transcription factors IRF-1 and NF-kB (Lidbury and Mahalingam, 2000).

In the work presented herein, ADE was observed for the infection of a WNV lineage II isolate that was pre-incubated with anti-WNV lineage I antibodies prior to the inoculation of mouse macrophage-like cell line (TIB-186) or horse monocyte-derived macrophages. The ADE phenomenon has been extensively demonstrated for *in vitro* infection of Fc receptor bearing cells by different serotypes of dengue virus. Antibody dependent enhancement has been associated with outbreaks of epidemic dengue hemorrhagic fever. However, the mechanism of ADE is not completely understood *in vivo*. Yang and collaborators demonstrated that serum against dengue virus serotype 1 at a 1:250 dilution enhanced infection of human mononuclear leukocytes by dengue-2. The enhancement was characterized by a 10 – 100-fold increase in viral titers compared to cells infected with virus without previous incubation with antibodies. Additionally, decreased production of IFN-γ by the ADE-infected cells was observed. These findings suggest suppression of the Th-1 response in a heterotypic dengue infection (Yang et al., 2001).

Demonstration of ADE induced by immune horse serum against WNV lineage I in an *in vitro* infection of horse macrophages or TIB-186 cells infected by WNV lineage II indicates the possibility of ADE *in vivo* if animals with antibodies against WNV lineage I obtain a secondary infection by a heterologous strain of WNV I or WNV lineage II. WNV I and II cross-reacted significantly using serum from a horse that was either naturally infected or vaccinated. Induction of ADE by immune horse serum in horse macrophages suggests the possibility of enhancement of WNV lineage I or lineage II *in vivo* in horses with low titers of specific antibodies against WNV lineage I. This

could eventually have importance in the evaluation and design of new vaccines and in the development of vaccination schedules for WNV prevention in horses. Further studies are needed to evaluate the potential effect of ADE *in vivo* in horses or other natural hosts.

CHAPTER IV

EVALUATION OF WNV PATHOGENESIS AND EFFECT OF TRANSFERENCE OF A SUBNEUTRALIZING DILUTION OF IMMUNE HORSE SERUM IN AN *IN VIVO* INFECTION BY WEST NILE VIRUS

Abstract

West Nile virus has emerged as an important cause of encephalitis in humans and horses in North America. Although there is significant knowledge about the pathogenesis of disease caused by this flavivirus and about the immunity against it, no reports exist about the possibility of antibody dependent enhancement (ADE) in WNV infections *in vivo*. In previous experiments, we demonstrated the ability of sub-neutralizing dilutions of immune horse serum to induce ADE in an *in vitro* WNV infection of horse and mouse macrophages. In the present report the ability of sub-neutralizing dilutions of immune horse serum to induce ADE in a murine *in vivo* model of WNV infection was evaluated. Immune horse serum (or appropriate control serum) was passively transferred to mice by intraperitoneal injection 3 days prior to inoculation with WNV. The sub-neutralizing dilution of immune horse serum used in these experiments did not induce ADE, but instead provided protection against WNV infection that was characterized by no mortality, no clinical signs and lower viral titers compared to mice that were not treated with serum. A similar protective effect was observed in non-immune serum treated controls. This protection was correlated with early up-regulation of IL-12, which abolished the down-regulation of IFN-γ and iNOS induced by WNV in non-serum treated

mice. During the normal course of infection the skin, spleen and kidney were all sites of viral replication before virus reached the brain. In brain, increased expression of the chemokines IP-10 and MCP-5, along with other chemokines and cytokines correlated with encephalitis caused by WNV.

I. Introduction

West Nile virus (WNV) is a member of the Flaviviridae family, genus *Flavivirus*. Other members of this genus include dengue, tick-borne encephalitis, yellow fever, Japanese encephalitis (JEV), Murray Valley encephalitis (MVEV), and St. Louis encephalitis (SLE) viruses. Smithburn and colleagues first isolated WNV in 1937 from the blood of a 37-year-old Ugandan woman in the West Nile District of Northern Uganda at the headwaters of the White Nile (Hollinger and Kleinman, 2003). Currently WNV is found throughout Africa, Europe, Central Asia, and, most recently, in North America. The first outbreak in the United States was in New York City during the summer of 1999, and the virus subsequently spread across the United States. In 2002, there were 4,156 reported cases of human illness associated with WNV and 284 deaths. WNV is transmitted primarily by *Culex* mosquitoes to vertebrate hosts. WNV, like other Flaviviruses, has a positive-stranded RNA genome that codes for three structural proteins and a host-derived lipid envelope. Two lineages of WNV (I and II) have been identified, and recently Bakonayi et al. (2005) have suggested that a third lineage exists in Eastern Europe. All isolates that cause severe human disease fall into lineage I (Bakonayi et al., 2005; Suchetana et al., 2003).

Since there is no specific treatment for disease caused by WNV, major research efforts have focused on diagnosis and prevention. At least two commercial companies have initiated research for development of human vaccines. In an attempt to protect horses against WNV, both a formalin-inactivated vaccine and a viral-vectored vaccine have been developed and are currently in use (Monath, 2001). Understanding the role of the host immune response in disease pathogenesis is required for development of immune protection with the minimal negative effects. Potential collateral affects include the possibility to induce non-neutralizing antibodies that, contrary to protection, may actually enhance infection of host cells by the virus. Antibody dependent enhancement (ADE) of macrophage and monocyte infection has been demonstrated *in vitro* for some of the most deadly RNA viruses known. For example, ADE has been demonstrated both *in vitro* and *in vivo* for dengue virus and HIV (Morens, 1994; Halstead, 1988) and these findings represent a significant consideration for development of vaccines against these viruses.

Recent evidence suggests that ADE-mediated ligation of Fc receptors may promote production of interleukin 10 (IL-10), leading to suppression of host-cell antiviral gene expression by means of the expression of suppressor of cytokine signaling (SOCS) proteins and a Th-2 cytokine profile. These findings suggest that ADE might enhance viral infections and exacerbate clinical disease (Suhrbier, and Linn, 2003). ADE of infection is believed to occur through FcγR I (CD64) and FcγR II (CD32), although a second type of ADE that requires complement has also been described (Chambers and Diamond, 2003). For encephalitic flaviviruses there is some experimental evidence indicating that ADE may be involved *in vivo*. Wallace and collages demonstrated that

subneutralizing concentrations of antibody may enhance infection and virulence *in vivo* in murine infection by Murray Valley encephalitis virus (Wallace, et.al., 2003). Peiris and Porterfield demonstrated ADE of WNV infection *in vitro* using a macrophage-like cell line and specific rabbit-derived antibodies against the virus (Peiris and Porterfiled, 1979). It has been observed that neutralizing antibodies to structural proteins of one serotype of dengue virus, typically not only fail to control infection, but actually cause enhanced replication of virus and lead to dengue hemorrhagic fever, a condition that is generally seen upon re-infection by dengue virus of a different serotype (Morens, 1994).

To date, no reports exist to either support or refute the possibility of ADE for *in vivo* WNV infections. Since vaccination of horses against this virus is commonly practiced in the United States, it is important to explore the possibility of ADE for WNV equine infections. However, there are certain limitations that make development of experimental equine infections with WNV difficult. One of the limitations is the fact that WNV is a virus that infects humans. Therefore, it requires special research facilities with biosafety level 3 (BSL-3) laboratories for its manipulation, a requirement that is difficult to meet given the large size of horses relative to other experimental animals. Alternatively, a mouse model is widely accepted for WNV *in vivo* experiments. Although the mouse has been used as a reliable model of the *in vivo* infection of WNV, no correlation has been examined the relationship between histopathological changes, viral titers and the immune response in brain during the course of disease. For the experiment described herein, we use the C57BL/6 strain of mice, which has showed a partial

resistance to the infection by WNV, having similar rates of mortality to those observed in horses and humans (Charlier, et al., 2004; Oliphant, et al., 2005).

To date there is little information about pathological changes observed during the course of WNV infection in mice. Only two reports have provided detailed descriptive information (Chambers and Diamond, 2003; Shrestha, et al., 2003). These reports described the kinetics of the viral replication, reporting that between 24 and 48 hours p.i. virus was detectible in blood. By day 4 p.i. virus was isolated in some visceral organs, including spleen and kidney, but no virus was found in liver. At the same time on day 4 p.i. the maximum titers of virus were observed in spleen and kidney, as well as lymph nodes (Chambers and Diamond, 2003). By day 4 p.i. virus was found in brain and spinal cord. Day 9 and 10 p.i. were associated with the highest viral titers in the CNS (Shrestha et al., 2003). There are several reports describing pathological changes found in tissue from infected humans and horses. Most of the reports describe the main features of inflammation in CNS as the presence of perivascular lympho-monocytic infiltrates. The presence of infected neurons in hippocampus, cerebellum, cerebral cortex, brainstem and ventral horns of spinal cord, as well as the presence of multifocal nodular gliosis, neuronal degeneration and rare neuronophagia have all been described in WNV infection (Agamanolis et.al., 2003; Cantile et.al., 2001; Doron et.al., 2003; Hayes et.al., 2005; Kelley et.al., 2003).

II. Material and methods:

A. Virus and cell culture

West Nile virus was isolated from the brain of an American Kestrel that died in Missouri (United States) with neurological signs in 2002. The initial virus isolation and subsequent propagations were performed in Vero cell cultures using standard techniques (Burleson et al., 1992). Vero cell cultures were maintained in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM L-glutamine, 0.25 µg/ml fungizone, and 0.5 mg/ml gentamycin (cell culture reagents supplied by Mediatech, Inc., Herndon, VA). The cells were maintained at 37°C in a humidified 5% CO_2 incubator. Approximately 4 days post-inoculation (p.i.) with the brain homogenate, a diffuse cytopathic effect was identified in inoculated Vero cells. Identification of the cytopathic agent as WNV was confirmed by indirect fluorescent antibody staining of inoculated cells using hyperimmune anti-WNV ascitic fluid (VR1267 AF, American Type Culture Collection, Manassas, VA) and an FITC-labeled anti-mouse IgG conjugate (Sigma, Inc., St. Louis, MO). Additionally, identification of the isolate as WNV was confirmed by WNV-specific RT-PCR (Johnson et al., 2001) followed by sequencing of 441 bp of the amplified viral envelope glycoprotein (E) gene. Comparison of the sequenced region of the isolate demonstrated 99.8% identity to the prototype U.S. strain NY99-flamingo382-99 (Lanciotti et al., 1999; GenBank accession no. AF196835) from nucleotide 1401 to 1841. The sequenced region was 100% identical over the 441 bp sequenced to a number of WNV isolates from 2002 (Beasley et al., 2003; GenBank accession no. AY185906, AY185907, AY185908, AY185909, AY185911).

Quantification of WNV stocks and experimental samples (homogenized tissues from infected mice) was performed using a viral plaque assay. Ten-fold serial dilutions of samples were adsorbed for two hrs onto confluent Vero cell monolayers in six-well plates (10 cm^2/well). Cell monolayers were washed once with cell culture media. Overlay media, which consisted of maintenance media plus 0.5% (w/v) agarose, was then added to each well. Cultures were maintained at 37°C in a humidified 5% CO_2 incubator for three days. Overlays were then removed and cell monolayers were stained with 0.5% (w/v) crystal violet/70% (v/v) methanol and plaques were counted and viral titers were calculated (Burleson et al., 1992).

B. Mice.

Specific pathogen free, 7 to 8 week-old C57BL/6 female mice (Jackson Labs, NJ, USA). Were maintained in biosafety level 3 (BSL-3) facilities and housed in microisolator cages. Water and feed were provided *ad libitum*. All experiments were performed in compliance with the Animal Welfare Act and other regulations relating to animals and experiments involving animals and in adherence to the Guide for Care and Handling of Laboratory Animals of the University of Missouri (Protocol number 4038).

C. Experimental design.

Two experiments were conducted. For experiment I, 115 mice were used and randomly distributed into seven different groups as described in Table 1. The animals received different serum treatments by intra-peritoneal injection 3 days prior to inoculation with WNV, except for the naïve group (group 7), which did not receive any

treatment (serum injection or virus inoculation). Group 1 was treated with 250 µl of normal horse serum (NHS) diluted 1:350 in phosphate buffered saline (PBS). Animals from group 2 were injected with 250 µl anti-WNV horse serum (IHS) diluted at 1:7 in PBS; this group is referred as IHS 1:7. Group 3 was injected with 250 µl of IHS diluted 1:350 in PBS; this group is referred as IHS 1:350. The mice in group 4 received an injection of 250 µl PBS. Group 5, composed of 6 animals, was treated with 250 µl of IHS diluted 1:350, and this group was not inoculated with WNV. Group 6 received 250 µl of PBS and at the time of the infection this group was inoculated with supernatant from uninfected Vero cells (100 µl). Three animals from each group were euthanized at day 1, 2, 4, 6, 9, 13 and 21 p.i. On days 1 and 4 p.i. mice from the three control groups (groups 5, 6 and 7) were sacrificed also. Mice that showed clinical signs of encephalitis were sacrificed at the time when severe signs were observed. Samples of blood, spleen, liver, kidney, lymph node and brain were taken for viral titration, RNAse protection assays (RPA) and RT-PCR analyses; these same organs plus skin, pancreas, lung, heart, thymus, adrenal glands and muscle were collected for histopathology (HP) and immunohistochemistry (IHC) studies. The second experiment was performed to corroborate results from experiment I and was conducted with only two groups, which are equivalent to groups 3 and 4 from experiment I.

D. Tissue homogenization.

Blood, spleen, liver, kidney, lymph node and brain were collected at necropsy and placed in previously frozen plastic vials containing zirconia-silica beads (Cat. No. 11079110z, Biospec products, Bartlesville, OK). The vials containing the tissue were

immediately immersed in liquid nitrogen then transferred to -80ºC where they were held until homogenization. To homogenize tissues, 1 ml of DMEM media (supplemented with 10% fetal bovine serum) or TRIzol ® (Invitrogen, 15596-018. Carlsbad, CA) was added to each vial. The vials were then subjected to agitation in a mini bead-beater-8 (Biospec products, Bartlesville, OK) for 2 minutes. The samples were then processed for viral titration by plaque assay or RNA was extracted for the RT-PCR or RNAse protection assay.

E. RNA extraction, quantitative RT-PCR and RPA.

Total RNA was extracted using TRIzol ® reagent (Cat. No. 15596-018; Invitrogen, Inc., Carlsbad, CA) according to the manufacturer's instructions. RNA concentrations were determined by UV spectroscopy at 260 nm.

The RPA was performed by a previously described method (Asensio and Campbell, 1997). For the synthesis of a radiolabeled anti-sense RNA probe set for chemokines and the loading control RPL32, the final reaction mixture (10 ml) contained 120 mCi of [a-32P]UTP (3,000 Ci/mmol; Andotek, Irvine, Calif.), UTP (73 pmol), GTP, ATP, and CTP (2.5 mmol each), DTT (100 nmol), transcription buffer, RNase inhibitor (20 U; Ambion), T7 polymerase (10 U; Promega), and an equimolar pool of *Eco*RI-linearized templates (15 ng each). After 1 h at 37°C, the mixture was treated with DNase I (2 U; Ambion) for 30 min at 37°C and the probe was purified by extraction with phenol-chloroform and precipitated with ethanol. Dried probe was then dissolved (2.6 3 105 dpm/ml) in hybridization buffer [80% formamide–0.4 M NaCl–1 mM EDTA–40

mM piperazine-*N*,*N*9-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4)], and 10 ml of this was mixture was added to tubes containing target RNA dissolved in TE. The samples were overlaid with mineral oil, heated to 95°C, and then incubated at 56°C for 12 to 16 h. Single-stranded RNA was digested for 45 min at 30°C by the addition of a mixture of RNase A (0.2 mg/ml) and RNase T1 (50 U/ml; Promega) in 10 mM Tris (pH 7.5)–300 mM NaCl–5 mM EDTA (pH 8). After incubation, 18 ml of a mixture containing proteinase K (1.5 mg/ml; Boehringer Mannheim, Indianapolis, IN), sodium dodecyl sulfate (3.5%), and yeast tRNA (200 mg/ml; Sigma) was added, and the samples were incubated for 30 more min at 37°C. The RNA duplexes were isolated by extraction and precipitation as described above, dissolved in 80% formamide and dyes, and electrophoresed in a standard 6% acrylamide–7M urea–0.5% Tris-borate-EDTA sequencing gel. Dried gels were placed on XAR film (Kodak, Rochester, NY) with intensifying screens and exposed at 270°C. Probe sets used throughout this study included the cytokine probe sets ML11 and ML26, and the chemokine probe sets 1 and 2 (CS1, CS2) (Asensio and Campbell, 1997). The probes were kindly provided by Dr. Daniel Hassett, University of Missouri-Columbia.

One step real-time (quantitative) RT-PCR was used to detect expression of IFN-γ, IL-12p40, and iNOS in RNA purified from spleen. Total RNA was extracted as described above. Quantitative RT-PCR was performed using the specific probes and primers (Overbergh, et.al., 2003) listed in Table 2. Amplification was performed by using the Qiagen QuantiTect Probe RT-PCR kit (Qiagen Inc., Valencia, CA) with thermocycling and detection performed in a Stratagene Quantitative PCR Mx4000™ PCR system

(Stratagene, La Jolla, CA). Normalization of each sample was performed against cyclophilin expression. For quantification, the Ct method was used (Pfaffl, 2001). The comparison of amplification thresholds (Ct) was performed against Ct's obtained in samples from naïve animals using the formula: 2^(Ct cyclophilin-Ct sample cytokine). The final ratio was obtained by dividing the cytokine value by the cyclophilin value. Results were expressed in arbitrary units obtained from the ratio calculation.

F. Plaque-reduction neutralization assays.

The plaque-reduction neutralization titers (PRNT) of serum from mice were determined by adding 50 plaque-forming units of WNV to serial two-fold dilutions of heat-inactivated (56°C, 30 min) mouse serum. Samples were incubated at 37°C for 1 hour followed by one additional hour at room temperature prior to adsorption to confluent Vero cell monolayers. After adsorption for two hrs, the inoculum was removed and the monolayer was rinsed once with cell culture media and then replaced with cell culture media containing 0.5% (w/v) agarose. Three –four days following inoculation the agarose overlay was removed and cell monolayers were stained as described above. Viral plaques were counted and the PRNT was recorded as the final serum dilution that reduced the number of plaques by ≥90% compared to control wells to which no horse serum had been added.

G. Immunofluorescence Antibody Test.

Vero cells were cultured until 100% cellular confluent in an 8-well glass chamber slide (Lab-Tek II Chamber Slide, Nalge Nunc International, Naperville, IL). Cells were

infected with 5 x 10^3 PFU of WNV per well. On day 2 p.i. cells were fixed with acetone and indirect immunofluorescence was performed using sera from experimental mice as primary antibody. The reactions were developed with fluorescein isothiocyanate labeled anti-horse IgM or IgG (Pharmingen, San Diego, CA), and rhodamine labeled anti-mouse IgM or IgG (Pharmingen, San Diego, CA) as the secondary antibodies. Slides were incubated at 37°C in a humidified chamber for 30 min with a mouse serum diluted 1:10 in PBS. The secondary antibodies were added as a mixture of rhodamine-labeled anti-mouse IgG or IgM and FITC-labeled anti-horse IgG or IgM. Three washes with PBS were performed between incubations. After incubation with the secondary antibodies, staining was visualized with an ultraviolet light epifluorescence microscope and photographs were taken with a digital camera. Non-inoculated cells and normal mouse or horse serum were used as negative controls.

H. Histology and Immunohistochemistry.

Tissue samples were fixed in 10% formalin, and then trimmed and embedded in paraffin, sectioned (5 μm thickness) and mounted on positively charged glass slides. The tissue sections were stained with hematoxilin and eosin for histopathological examination. For IHC, a polyclonal anti-WNV antibody derived from rabbit was used (Cat. No. C-585, Invitrogen-Bioreliance Carlsbad, CA). The antibody was adsorbed against normal mouse tissue for 2 hrs at 37°C and then centrifuged at 16,000 x *g* for 30 minutes at 10ºC and used for IHC. The pre-adsorbed antibody was used as primary antibody at a final dilution of 1:800 in antibody dilution buffer (Dako cytomation, Carpinteria, CA). The DAKO EnVision System (DAKO cytomation Coorporation,

Table 4.1. Experimental design. Group distribution and treatment per group, indicating the times of sacrifice and analysis performed.

GROUP/TX	# Mice exp.	# mice mortality	Total Mice	Days Sampling	Tests	Organs tested
1) NHS 1:2500 + WNV	3/day= 18	5	23	1, 2 ,4, 6, 9, 13, 21 Blood and sacrifice of 3 mice per day.	mRNA PA, RT-PCR, Plaque assay, PRNT, IFAT, IHC	Blood, spleen, liver, kidney, LN, brain. HP: skin, eye, heart, Intest, lung, thymus,
2) IHS 1:50 + WNV (high)	3/day= 18	5	23	1, 2 ,4, 6, 9, 13, 21 Blood and sacrifice of 3 mice per day.	mRNA PA, RT-PCR, Plaque assay, PRNT, IFAT, IHC	IDEM
3) IHS 1:2500 + WNV (low)	3/day= 18	5	23	1, 2 ,4, 6, 9, 13, 21 Blood and sacrifice of 3 mice per day.	mRNA PA, RT-PCR, Plaque assay, PRNT, IFAT, IHC	IDEM
4) NS + WNV	3/day= 18	5	23	1, 2 ,4, 6, 9, 13, 21 Blood and sacrifice of 3 mice per day.	mRNA PA, RT-PCR, Plaque assay, PRNT, IFAT, IHC	IDEM
5) NHS 1:2500 + No virus	2/day= 6		6	1, 2 ,4, 6, 9, 13, 21 Blood and sacrifice of 3 mice per day.	mRNA PA, RT-PCR, Plaque assay, PRNT, IFAT, IHC	IDEM
6) NS + No virus	2/day= 6		6	1, 2 ,4, 6, 9, 13, 21 Blood and sacrifice of 3 mice per day.	mRNA PA, RT-PCR, Plaque assay, PRNT, IFAT, IHC	IDEM
7) Naïve	2/day= 6	5	11	1, 2 ,4, 6, 9, 13, 21 Blood and sacrifice of 3 mice per day.	mRNA PA, RT-PCR, Plaque assay, PRNT, IFAT, IHC	IDEM
Total Groups : 7	90	25	115			

Table 4.2. Sequences of primers and probes used for the detection of the expression of murine cytokines and iNOS by quantitative (real-time) RT-PCR.

Molecule	Sequence (5'-3')	Length (bp)	Reference
iNOS	For: CAGCTGGGCTGTACAAACCTT Rev: CATTGGAAGTGAAGCGTTTCG Prb: GGGCAGCCTGTGAGACCTTTGA	95	U43428 L23806
IFN-γ	For: TCAAGTGGCATAGATGTGGAAGAA Rev: TGGCTCTGCAGGATTTTCATG Prb: TCACCATCCTTTTGCCAGTTCCTCCAG	92	K00083 M74466 M28381
IL-12p40	For: TGGAGTGCCAGGAGGACAGT Rev: TCTTGGGTGGGTCAGGTTTG Prb: ATGGTGGATGCCGTTCACAAGCTCAA	147	AF180563 AY008847

Carpinteria, CA) was used as an amplification and reporter system. The assay was partially performed in a DAKO Autostainer Universal Staining System. Briefly, tissue sections were deparaffinized and rehydrated. To increase staining intensity, an antigen retrieval procedure with steaming of the samples was used. Endogenous peroxidases were blocked by incubation in 0.03% hydrogen peroxide. Tissue sections were incubated with a 1:800 dilution of primary antibody, followed by the secondary antibody (horseradish peroxidase-labeled polymer conjugated to goat-anti-rabbit total immunoglobulin). Staining was completed by adding the substrate-chromogen diaminobenzidine (DAB). All samples were incubated in a humidified chamber at room temperature (25 to 27ºC). Tissues were counterstained with hematoxylin, dehydrated, and cleared then coverslips were applied. Negative controls were performed by substitution of normal rabbit serum for the primary antibody, as well as the incubation of the pre-adsorbed antibody with normal mouse tissues. Sections of confirmed WNV positive mouse tissue were uses as positive controls, as well as WNV positive avian tissue.

III. Results

A. Evaluation of ADE in a WNV *in vivo* infection.

To evaluate the ability of passively transferred anti-WNV horse serum (IHS) to induce ADE in a murine *in vivo* model of WNV infection, sub-neutralizing dilutions of immune serum were inoculated into naïve mice 3 days prior to infection with WNV. Previously we demonstrated that sub-neutralizing dilutions of the immune horse serum used induced ADE in a WNV *in vitro* infection model using horse and murine macrophages (Chapter 3). The dilution of the serum that induced the most significant ADE effect *in vitro* was 1:2,500. In Fig. 4.1 the effect *in vitro* of serum harvested from mice that were passively transferred with different dilutions of immune horse serum is shown. In this pilot experiment, three mice were injected intraperitoneally with 250 µl of immune horse serum diluted at either 1: 350 or 1:1,400. Blood was obtained from the mice on day 3 post-injection and serum was then evaluated for ADE of WNV infection *in vitro*. For the evaluation of ADE, TIB-186 cells were infected at an MOI of 0.001 with WNV, which had been previously incubated for 1 hr at 37ºC and 1 hr at room temperature with undiluted mouse serum. In Fig. 1 a clear enhancement of viral replication is observed from day 2 – 7 p.i. in TIB-186 cells infected with WNV previously incubated with serum from mice injected with the 1:350 dilution of IHS. A similar enhancing effect was observed in cells infected with WNV treated with serum from mice injected with IHS diluted 1:1,400. Based on the *in vitro* induction of ADE, the dilution 1:350 of the IHS was selected for using in the *in vivo* evaluation of ADE in both experiment I and II.

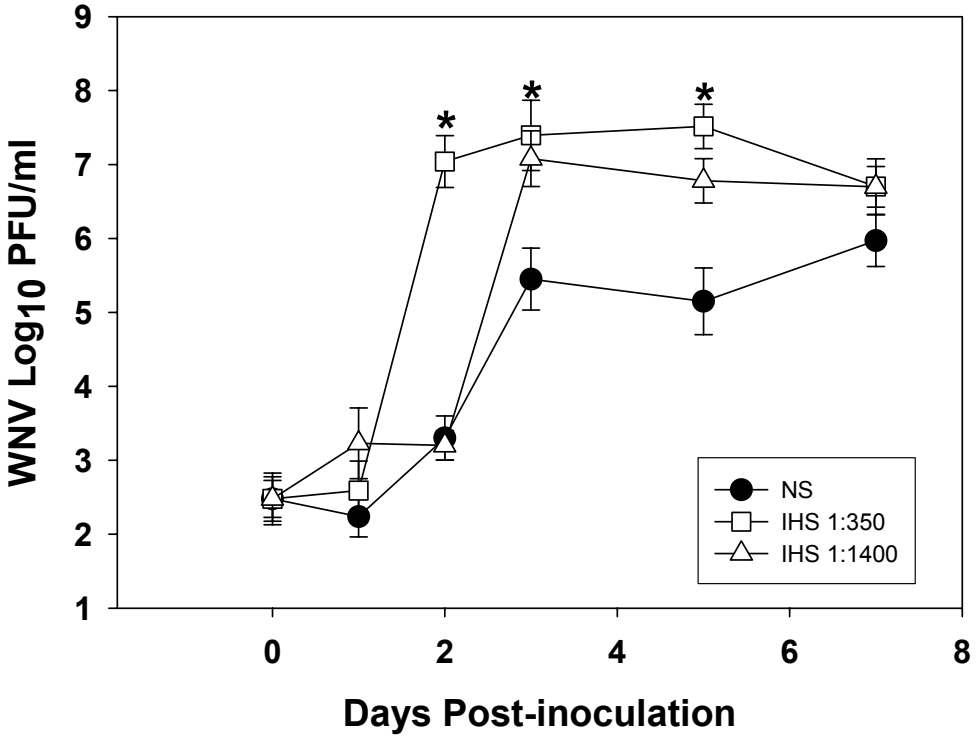


Figure 4.1. **Antibody dependent enhancement in an *in vitro* infection of TIB-186 cells by West Nile virus.** 1.5 x 10^5 cells per ml were inoculated at an MOI of 0.001 with WNV that had been previously treated with undiluted serum from mice that received no serum (closed circles), or mice injected with WNV-immune horse serum diluted 1:350 (open squares) or 1:1,400 (open triangles). Values shown are the mean viral titers ± SEM . *, significant difference ($P < 0.05$) between viral titers from cells infected with serum-treated virus and viral titers from cells infected with non-treated virus.

After determining the dilution of IHS to be used for injection of the mice, different treatments of serum were administered to seven different groups of mice, as described above in Materials and Methods and in Table 1. In Figure 4.2, the WNV titers in four organs are shown. The neutralizing effect of IHS in mice injected with a 1:7 dilution was observed as a clear decline of viral titers in blood and no virus detected in spleen, kidney and brain (Fig. 4.2). Neutralization of the infection was reflected in the 100% (8/8) survival rate observed in this group of mice (Fig. 4.3).

For the Immune horse serum 1:350 (IHS 1:350), normal horse serum (NHS) and no serum (NS) groups, WNV titers were not significantly different in blood from days 1 – 4 p.i., although on day 4 p.i. titers of virus were slightly higher for the IHS 1:350 group. On day 6 p.i. the titer of WNV was >1,000-fold higher in the NS group than in the groups treated with either NHS or IHS. The viremic titers in the NS group decreased on day 9 p.i. but were still 100-fold higher than the other groups. On day 13 p.i. the titers were 10-fold higher in the NS group compared to the other treatment groups. After day 13 p.i the titers were similar, reaching around 10 PFU/ml (Fig. 4.2 A).

When viral titers were evaluated in spleen (Fig. 4.2 B), no virus was detected on days 1 and 2 p.i. in any mice. On day 4 p.i. viral titers reached peak values of approximately 10^5 PFU/g from mice in the NS and NHS groups, while slightly higher titers were detected in mice from the NS group. These values were approximately 100-fold higher than the titers observed in the IHS 1:350 group, a difference that was statistically significant ($P<0.05$). On day 6 p.i. titers were significantly different ($P<0.05$)

when the NS group values (5,000 PFU/g) were compared to those in the NHS group (100 PFU/g). On this day WNV titers in the spleen from the IHS 1:350 group were close to 1,000 PFU/g, a value that was not significantly different from those detected in the other 2 groups. From day 9 to 21 p.i. the titers were not higher than 10 PFU/g in any of the groups.

WNV was also detected by plaque assay in kidney samples from animals in the NS and NHS group. However, no virus was detected in kidney samples from animals treated with either dose of IHS. The maximum viral titers were detected on day 4 p.i. in samples from the NS group. For these mice, the titers were around the 1,000 PFU/g which was 10-fold higher than titers detected in the NHS group. As in spleen, the titers declined on day 6 p.i., although the differences were not significantly different in the NHS and NS groups. From day 6 – 13 p.i., the titers were maintained at similar levels (approximately 100 PFU/g) in the NS group, and no virus was detected in kidney samples from mice in the NHS group. On day 21 p.i. the titers were approximately 10 PFU/g of kidney in the NS group (Fig. 4.2C).

Titers of WNV in brain (Fig. 4.2D) showed a similar pattern to that observed in the other organs, with no virus detected in any of the groups on days 1 or 2 p.i. On day 4 p.i. the first viral titers detected in brain were around the 100 PFU/g in the IHS 1:350 and NHS groups, but no virus was detected from samples collected in the NS group. Viral titers were slightly higher in the animals from the group treated with IHS 1:350, although this difference was not statistically significant. On day 6 p.i. the WNV titer was similar

(100 PFU/g) in the IHS 1:350 group. Approximately 1,000 PFU/g was detected in the brain of mice from the NS group, values that were 5-fold higher than those observed in the brain of mice in the NHS group. On day 9 p.i., peak viral titers were observed in samples of brain from the NHS, NS, and IHS1:350 groups. The maximum titers were detected in the animals from group NS, which on average had approximately 10^6 PFU/g of tissue. This value was significantly different when compared to titers in mice from the NHS group. For the IHS 1:350 group, the maximum titers on average were around 100 PFU/g. Importantly, on this day two out of three mice had only 10 PFU/g and one mouse had more than 10^6PFU/g in the IHS1:350 group.

When the effect of the pre-treatment with serum was evaluated by the mortality (Fig. 4.3), a significant difference ($p<0.05$) was observed when the NS group was compared to the other groups. The survival rate of this group was 50% (4/8) by day 13, while the survival rate was 87.5% (7/8) for the NHS group and 100% (8/8) for both groups treated with immune horse serum (IHS1:7 and IHS1:350).

The course of seroconversion in the infected animals was evaluated by analyzing antibody production using a plaque reduction neutralization assay. In addition, the isotype of immunoglobulin produced was evaluated using an immunofluorescence antibody test. Sera from all mice were evaluated individually and an average of the neutralization titers is shown in Fig. 4.4A. A 90% neutralizing value of 1:40 was observed in serum from animals treated with IHS 1:7 from day 1 – 6 p.i. The neutralizing titer declined on day 9 p.i., then decreased to less than 1:10 on day 21 p.i.. As shown in

Fig. 4.4J and K, these mice had detectible levels of both equine IgM and IgG, which was passively transferred 3 days prior to inoculation with WNV. For the IHS1:350 group, neutralizing activity was first detected on day 4 p.i. with PRNT titers of approximately 1:16, while serum from the mice in the NHS and NS groups had no detectable neutralizing activity on this day. On day 6 p.i. the NS and NHS groups had detectible neutralizing titers reaching 1:60 on average, values that were higher than those observed in the IHS1:350 group. Day 9 p.i. was the first day on which significantly elevated titers of neutralizing antibodies were observed in mice of the NS group. On day 13 p.i. titers in the NHS and NS groups were similar, but the titers in the IHS1:350 were still below 1:80 on average. By day 21 p.i. production of neutralizing antibody was highest in all groups except IHS1:7.

Based on the mortality and viral quantification results, it was clear that ADE of WNV infection was not induced *in vivo* by a sub-neutralizing dilution of immune horse serum. To the contrary, a sub-neutralizing dilution of immune serum induced protection against the infection with WNV, as did passive transfer with normal horse serum (NHS). Quantitative RT-PCR was used determine why NHS and IHS1:350 induced protection against infection with WNV in C57BL/6 mice. We analyzed the differences in expression of genes encoding IL-12, IFN-γ and iNOS in total RNA purified from spleen at the earliest times p.i. evaluated in this experiment. Similarly, RNAse protection assays for detection of cytokine and chemokine expression in brain from the groups IHS1:350 and NS were performed.

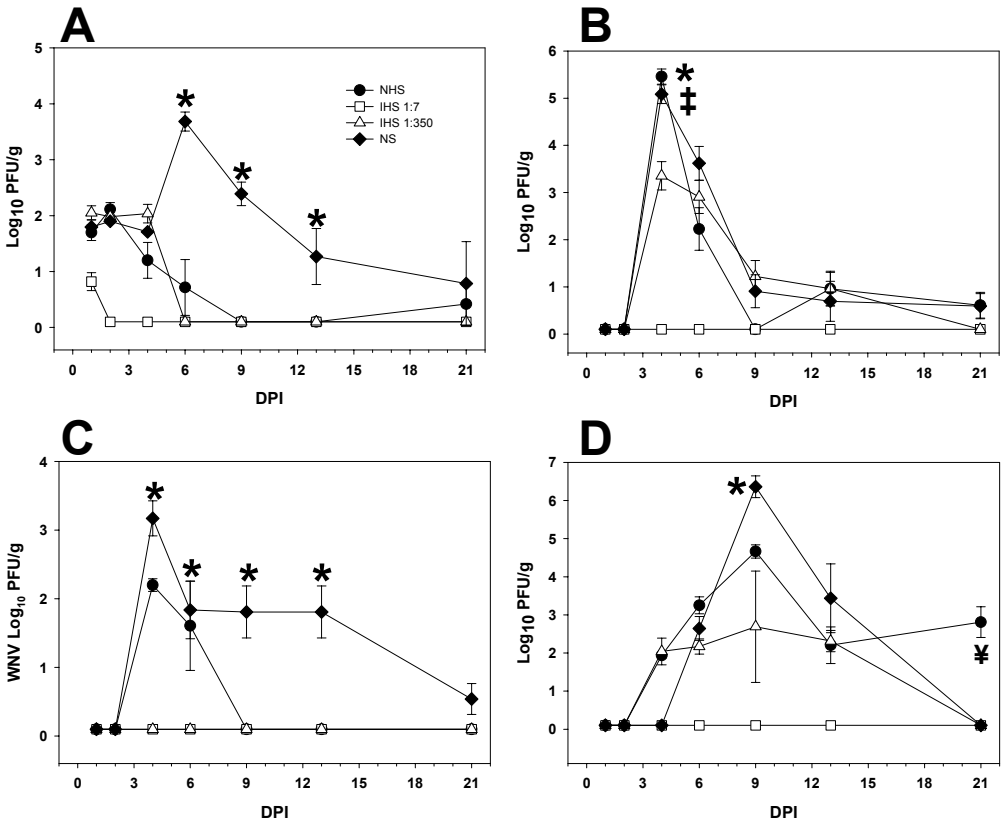


Figure 4.2 Viral growth curves for West Nile virus (WNV) in blood (A), spleen (B), kidney (C) and brain (D) from mice passively transferred with normal horse serum (close circles), with IHS diluted 1:7 (open squares), with IHS diluted 1:350 (open triangles) or with sterile PBS (close diamonds). Intraperitoneal injection of serum was performed 3 days prior to the subcutaneous inoculation of 100 PFU WNV. Values shown are the mean viral titers ± SEM from three mice sacrificed per group at each time point. *, significant difference ($P< 0.05$) between the non-serum treated group and the immune horse serum treated groups. ‡, significant difference ($P< 0.05$) between normal horse serum-treated group and immune horse serum treated groups. ¥, significant difference ($P<0.05$) between normal horse serum trated group and all the other groups.

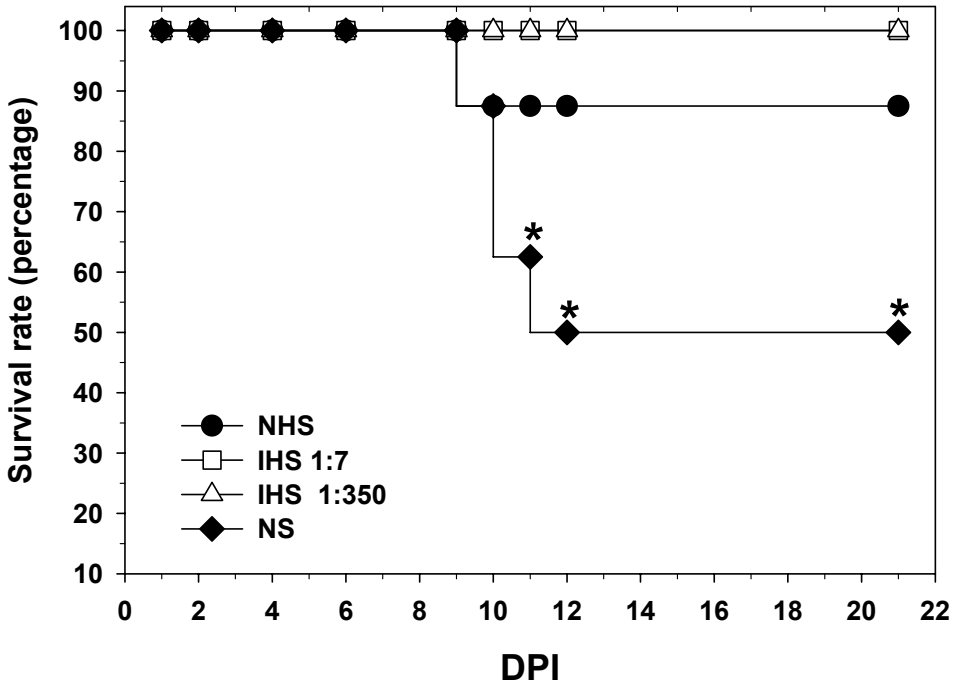


Figure 4.3 Mortality in mice infected with WNV. Mice were passively transferred with normal horse serum (close circles), with immune horse serum diluted 1:7 (open squares), with Immune horse serum diluted 1:350 (open triangles), or with sterile PBS (close diamonds). Transference of serum was performed 3 days prior to the subcutaneous inoculation of 100 PFU WNV. The time points indicate the percentage of surviving animals at the point of reference. *, significant difference ($P< 0.05$) between the non-serum (PBS) treated group and the other three groups.

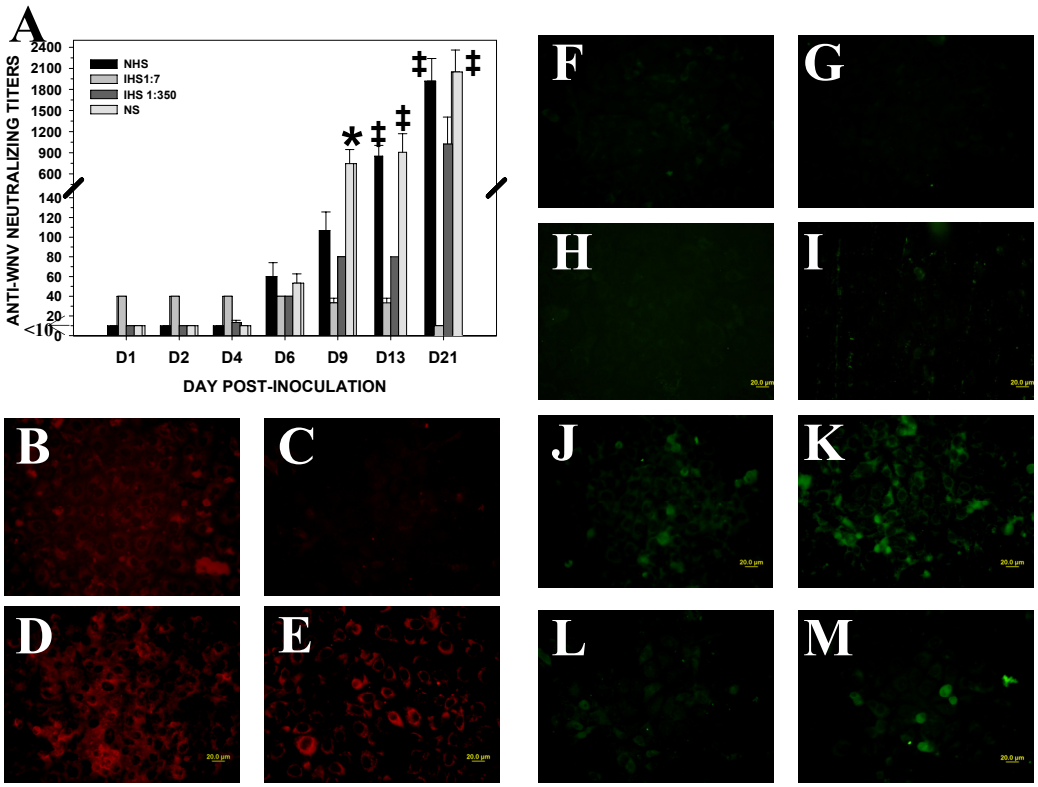


Figure 4.4. Production of anti-WNV antibodies by WNV-inoculated mice. Panel A shows plaque reduction neutralization titers in the four different groups of mice: mice transferred with normal horse serum (black bars), or with immune horse serum diluted 1:7 (gray bars); or with Immune horse serum diluted 1:350 (darker gray bars); or with sterile PBS (white bars). Passive transfer of serum was performed 3 days prior to the subcutaneous inoculation of 100 PFU of WNV. Values shown are mean PRNT titers ± SEM from three mice sacrificed per group at each time point. *, significant difference ($P< 0.05$) between the non-serum treated (PBS) group and the remaining three groups. ‡, significant difference ($P< 0.05$) between normal horse serum-treated group or no serum-treated group and the immune horse serum treated groups**.** Panels B to E show the identification of mouse IgM (Panels B and D) and mouse IgG (Panels C and E) in serum from non-serum (PBS) transferred mice sacrificed on day 1 p.i. (Panels B and C), and on day 9 p.i. (Panels D and E). Panels F to M show identification of anti-WNV horse IgM (Panels F, H, J, and L) or anti-WNV horse IgG (Panels G,I,K, and M) in serum from mice sacrificed on day 1 p.i. Non-serum (PBS) transferred mice (Panels F and G), normal horse serum-transferred mice (Panels H and I), immune horse serum 1:7 treated mice (Panels J and K), and mice transferred with immune horse serum diluted 1:350 (Panels L and M). Identification of antibodies was performed by IFAT using mouse serum diluted 1:10 as primary antibody and goat anti-mouse IgM or IgG conjugated to rhodamine or goat anti-horse IgM or IgG conjugated to FITC as secondary antibodies.

Fig. 4.5 demonstrates the differences in gene expression of IFN-γ between the groups evaluated. On day 1 a reduction in the expression of this molecule was observed in the mice from all the groups infected with WNV compared to IFN-γ expression in the non-serum treated, uninfected (naïve) group. However, the only significant reduction was detected in the NS group. The expression levels in the other two groups were not significantly different from the non-serum treated, uninoculated control group. Interestingly, reduction of the expression of IFN-γ in the NS group became significant ($p<0.05$) up-regulation of gene expression on day 2 p.i. compared to each of the other three groups. The expression levels of IFN-γ in the groups NHS and IHS1:350 were slightly reduced on day 1 and day 2 p.i. but the values were not significantly different compared to the control group.

A similar inhibitory effect was observed on expression of the iNOS in all experimental groups. As observed for IFN-γ, the greatest reduction was observed in the NS group (Fig. 4.6). The levels of iNOS gene expression in this group were significantly lower when compared to iNOS expression in the NHS, IHS 1:350 and naive groups. Although the levels of expression in the NHS and IHS 1:350 groups were lower than those found in the naïve group, the differences were not significant. By day 2 p.i. expression of the mRNA for this enzyme was still lower when compared to the control group. For expression levels of iNOS in the NS group on day 1 p.i., the relative expression was at least 100,000 times lower than the levels detected in the control group. A slight recovery of the expression of this molecule was observed on day 2 in the NS

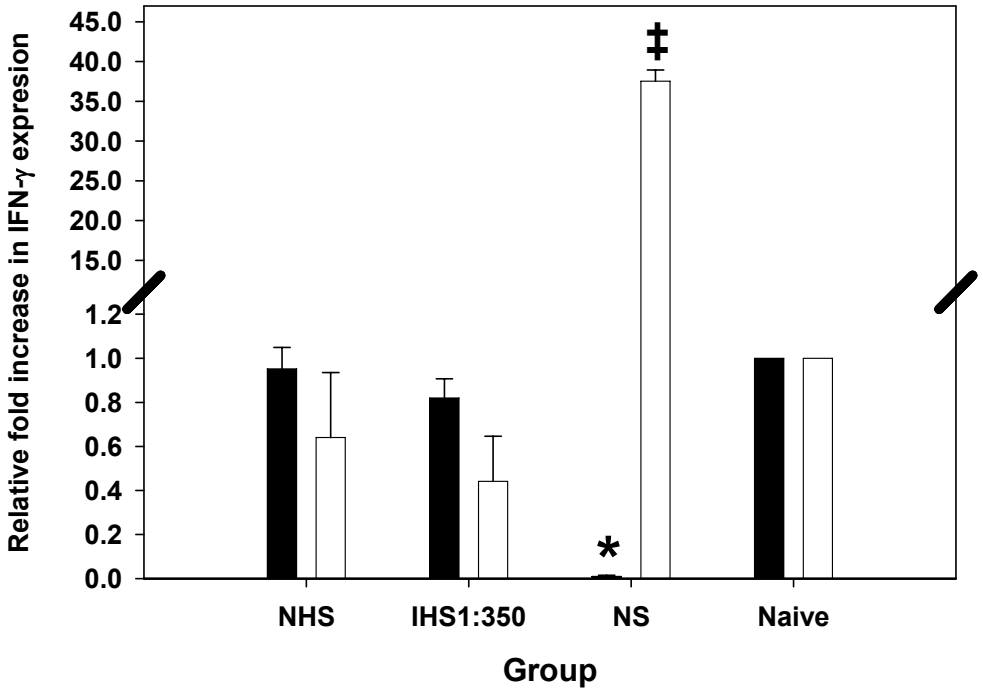


Figure 4.5. **Quantification of IFN-γ expression in RNA purified from spleens of mice infected with WNV**. Mice were passively transferred with normal horse serum, with immune horse serum diluted 1:350, or with sterile PBS and three days after transfer were subcutaneously inoculated with 100 PFU WNV. The naïve group did not receive any treatment. Levels of expression were evaluated by quantitative RT-PCR on days 1 p.i. (black bars) and 2 p.i. (white bars). The bars indicate the average and + SEM of relative fold increase in the expression of IFN-γ in three different mice.*, significant difference ($P< 0.05$) between the non-serum (PBS) treated group and the other three groups. ‡, significant difference ($P< 0.05$) between the non-serum (PBS) treated group and the other three groups on the same day of evaluation and difference between expression levels obtained on day 1 and day 2 p.i. samples from the same group.

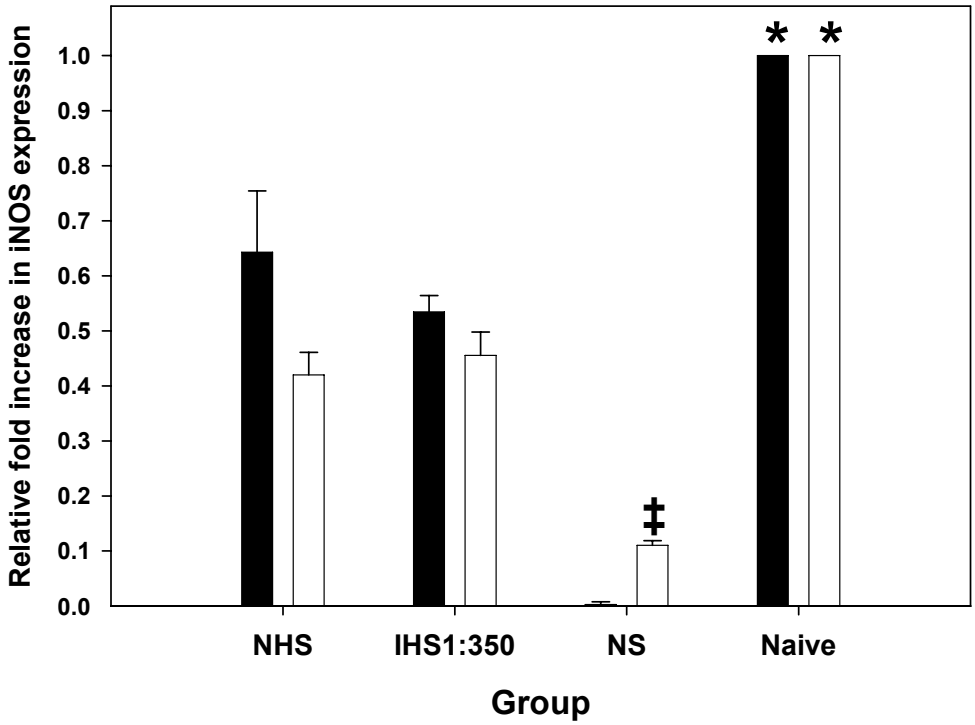


Figure 4.6. **Quantification of iNOS expression in spleens from mice infected with WNV.** Mice were passively transferred with normal horse serum, with immune horse serum diluted 1:350, or with sterile PBS and three days after transfer were subcutaneously inoculated with 100 PFU WNV. The naïve group did not receive any treatment. Levels of expression were evaluated by quantitative RT-PCR on days 1 p.i. (black bars) and 2 p.i. (white bars). The bars indicate the average and + SEM of relative fold increase in the expression of iNOS in three different mice.*, significant difference ($P< 0.05$) between the naive group and the other three groups. ‡, significant difference ($P< 0.05$) between the no serum treated group and the other three groups on the same day of evaluation and difference between expression levels obtained on day 1 and day 2 p.i. in samples from the same group.

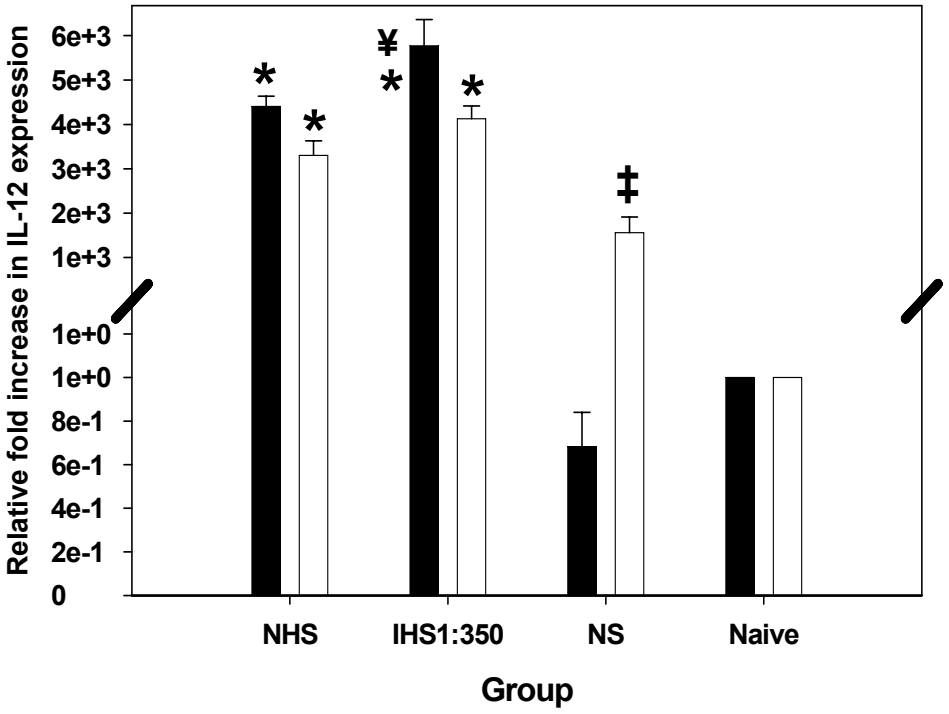


Figure 4.7. **Quantification of IL-12 expression in spleens from mice infected with WNV**. Mice were passively transferred with normal horse serum, with immune horse serum diluted 1:350, or with sterile PBS and three days after transfer were subcutaneously inoculated with 100 PFU WNV. The naïve group did not receive any treatment. Levels of expression were evaluated by quantitative RT-PCR on days 1 p.i. (black bars) and 2 p.i. (white bars). The bars indicate the average and + SEM of relative fold increase in the expression of IL-12 in three different mice.*, significant difference ($P< 0.05$) between the normal horse serum or immune horse serum 1:350 treated groups and the other two groups. ‡, significant difference ($P< 0.05$) between the no serum treated group and the other three groups on the same day of evaluation and difference between expression levels obtained on day 1 and day 2 p.i. in samples from the same group. ¥, significant difference ($P< 0.05$) between the immune horse serum 1:350 treated group and the normal horse serum treated group on day 1 p.i.

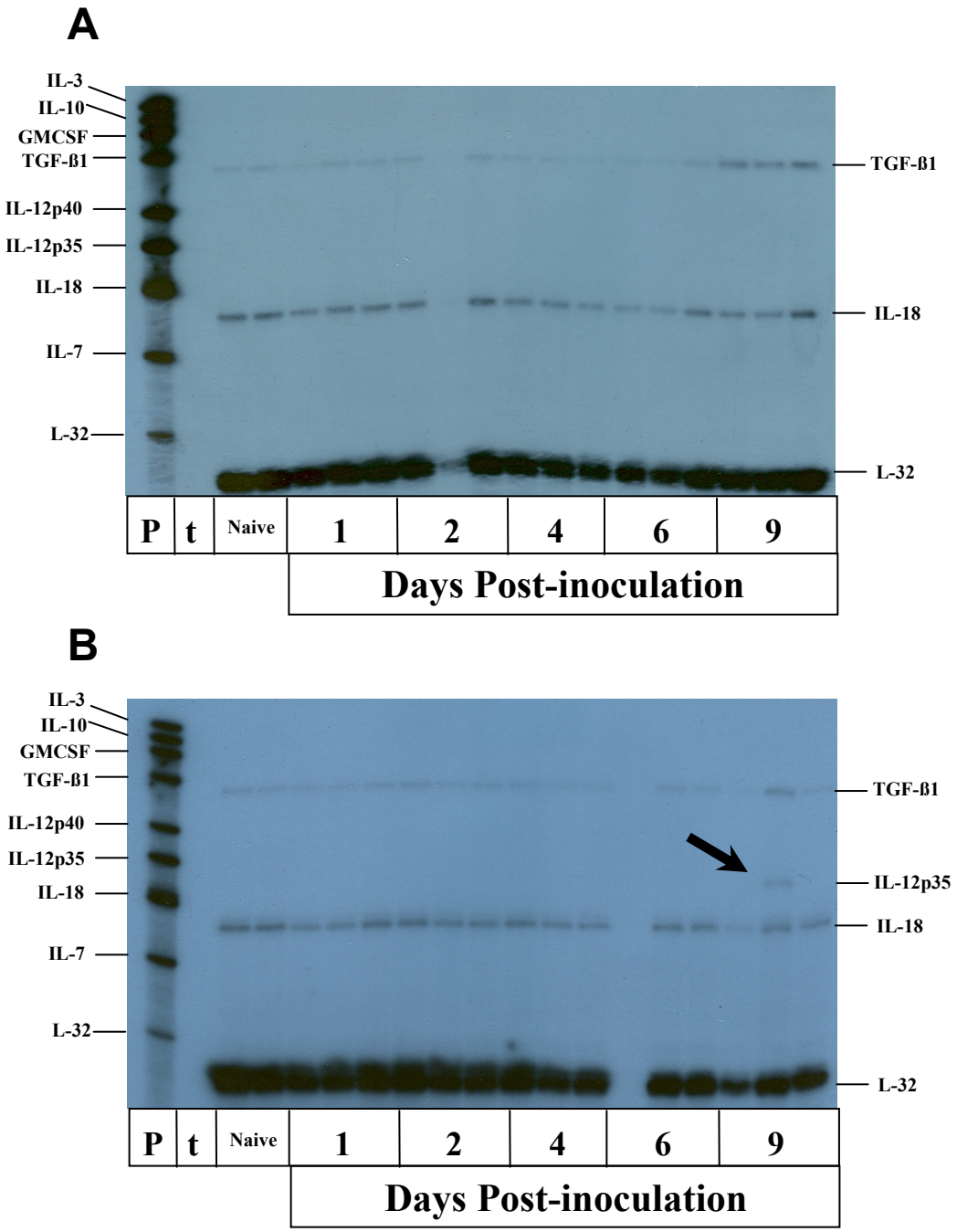


Figure 4.8. **Detection of cytokine expression on brain from infected mice.** RNAse protection assay was performed in RNA samples of brain from no serum transferred mice (Panel A) and from immune horse serum 1:350-transferred mice (panel B). Detection of cytokine expression was performed on days 1,2,4,6, and 9 p.i. Three mice per group were euthanized on the indicated days. (t), indicates a transfer RNA control. Naïve controls were mice sacrificed on day 1 or 4 of the study. P indicates the molecular probe control. In the left side are listed all the cytokines analyzed in each gel. The arrow in panel B shows a differential expression of IL-12 in IHS 1:350-transferred mice.

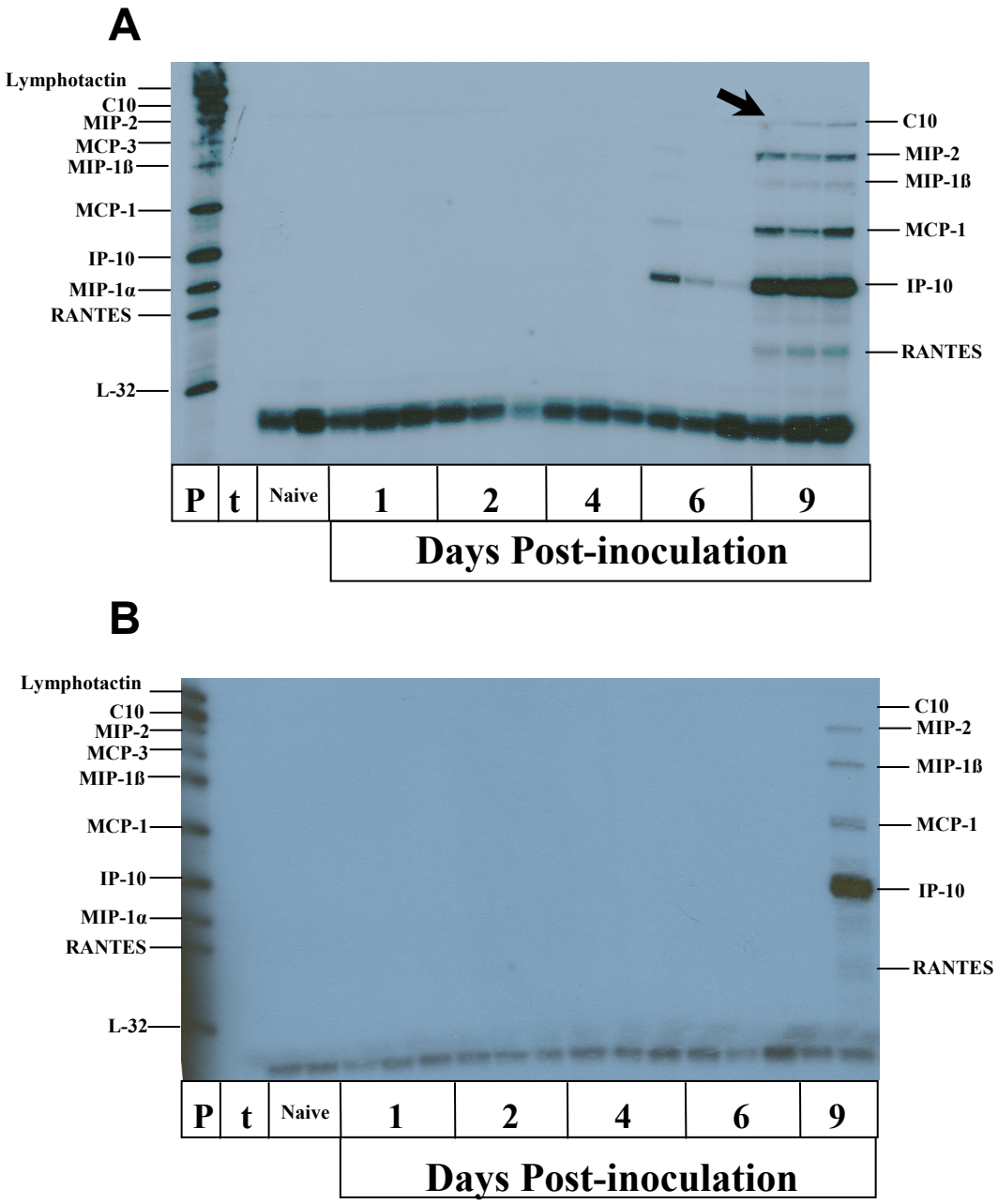


Figure 4.9. **Detection of chemokine expression on brain from infected mice**. RNAse protection assay was performed in RNA samples of brain from no serum transferred mice (Panel A) and from immune horse serum 1:350-transferred mice (panel B). Detection of the chemokine expression was performed on days 1,2,4,6, and nine. Three mice per group were euthanized on the indicated days. (t), indicates a transfer RNA control. Naïve are intact mice sacrificed on day 1 or 4 of the study. P indicates the molecular probe control. In the left side are listed all the chemokines analyzed in each gel. The arrow in panel B shows a differential expression of IL-12 in IHS1:350-transferred mice.

group, but the levels were still significantly reduced in comparison to the levels expressed by the control group.

Another important cytokine in the innate immune response is IL-12. Relative levels of expression of this molecule are presented in Figure 4.7. On day 1 p.i., mice in the NHS and IHS 1:350 groups expressed significantly higher levels of IL-12 than the naïve and NS groups. Mice in the NS group expressed less IL-12 than all other groups, including the naïve controls although the differences were not statistically significant. On day 2 p.i. expression of IL-12 was not significantly different in NHS and IHS 1:350 groups when compared to levels detected on day 1 p.i. Surprisingly, expression levels of this molecule by the NS group increased on day 2 p.i., showing a clear up-regulation in expression of this gene although the relative levels were still lower than those observed in the NHS and IHS 1:350 groups.

As indicated above, one of the mice treated with IHS 1:350 developed high viral titers in brain on day 9 p.i. Although this animal did not show clinical signs of encephalitis, lesions characteristic of WNV infection were observed in the CNS. An observation obtained by the RNAse protection assay performed on RNA extracted from brain of this mouse was expression of IL-12p35 (Fig. 4.8B). This molecule was not expressed by the animals in the NS group (Fig. 4.8A), although these animals developed similar titers of virus in the brain on the same day p.i. Another observation was the marked up-regulation of the chemokine C10 in mice from the NS group on day 9 p.i.

(Fig. 4.9A). This chemokine was not detected in the brain of the mice from the IHS 1:350 group (Fig. 4.9B).

A second experiment, which included only the NS and IHS 1:350 groups, was conducted to corroborate the results obtained in the first experiment. All the results from this second experiment were similar to those documented in the first experiment.

B. Chronology of the pathogenic and immunologic events following infection of C57BL/6 mice with West Nile Virus

Clinical observations, histopathological changes in several tissues, and the expression of cytokines and chemokines in brain were analyzed during the course of infection, starting on day 1 p.i. and continuing through days 2, 4, 6, 9, 13 and 21 p.i. The results are presented as a summary of the events occurred on each of these days in no serum-treated mice inoculated with WNV.

B.1. Day 1 – 2 p.i.

Fig. 4.10 presents the most representative findings from day 1 p.i. Panel A shows the maximum titers found in mice infected with WNV. The only sample in which WNV was detected on this day was blood, with viremic titers reaching up to 100 PFU/ml. Immunodetection of WNV by IHC was performed in subcutaneous tissues as showed in Fig. 4.10B. On this day skin from the injected foot was the only tissue in which the virus was detected by IHC. No apparent pathological changes were detected in any of the tissues analyzed from the three sacrificed mice. Micrographs of different sections of

normal cerebrum, cerebellum and brainstem are presented in Figure 4.10 panels E to H. Also shown are spleen (Fig 4.10C) and thymus (Fig 4.10D). The observations were similar for day 2 p.i. On days 1 and 2 p.i. the brain of both infected and naïve mice expressed detectable amounts of transcripts for the following cytokines and chemokines: IL-6, IL-1ß, IL-3, IL-18, SDF1a, KC and Fractalkine (Figs. 4.17 and 4.18).

B.2. Day 4 p.i.

On day 4 p.i., the virus reached peak titers in spleen with approximately 10^6 PFU/g of tissue (Fig. 4.11A). On this day virus was still detected in blood, with titers of approximately 100 PFU/ml. For the first time in the course of infection, WNV was detected in the kidney of all three mice, and in one of the three animals the virus was isolated from lymph nodes. No virus was detected in any of the three brains analyzed on day 4 p.i. Confirming the data obtained from the plaque assay, WNV was detected by IHC in macrophages from spleen and in tubular epithelial cells in the distal tubules of the renal medulla. Interestingly a higher number of cells in the subcutis stained positive for WNV antigen. The morphology of infected cells was consistent with macrophages and some other positive cells had morphology consistent with fibrocytes. Interestingly, a few cells stained positive for WNV antigen in the muscular layer of the uterus. On day 4 p.i. no apparent lesions were yet detected in the central nervous system and the lymphoid tissues (specifically thymus and spleen) had no apparent changes in any of the three mice analyzed. The expression of cytokines and chemokines in brain was similar to that described for days 1 and 2 p.i.

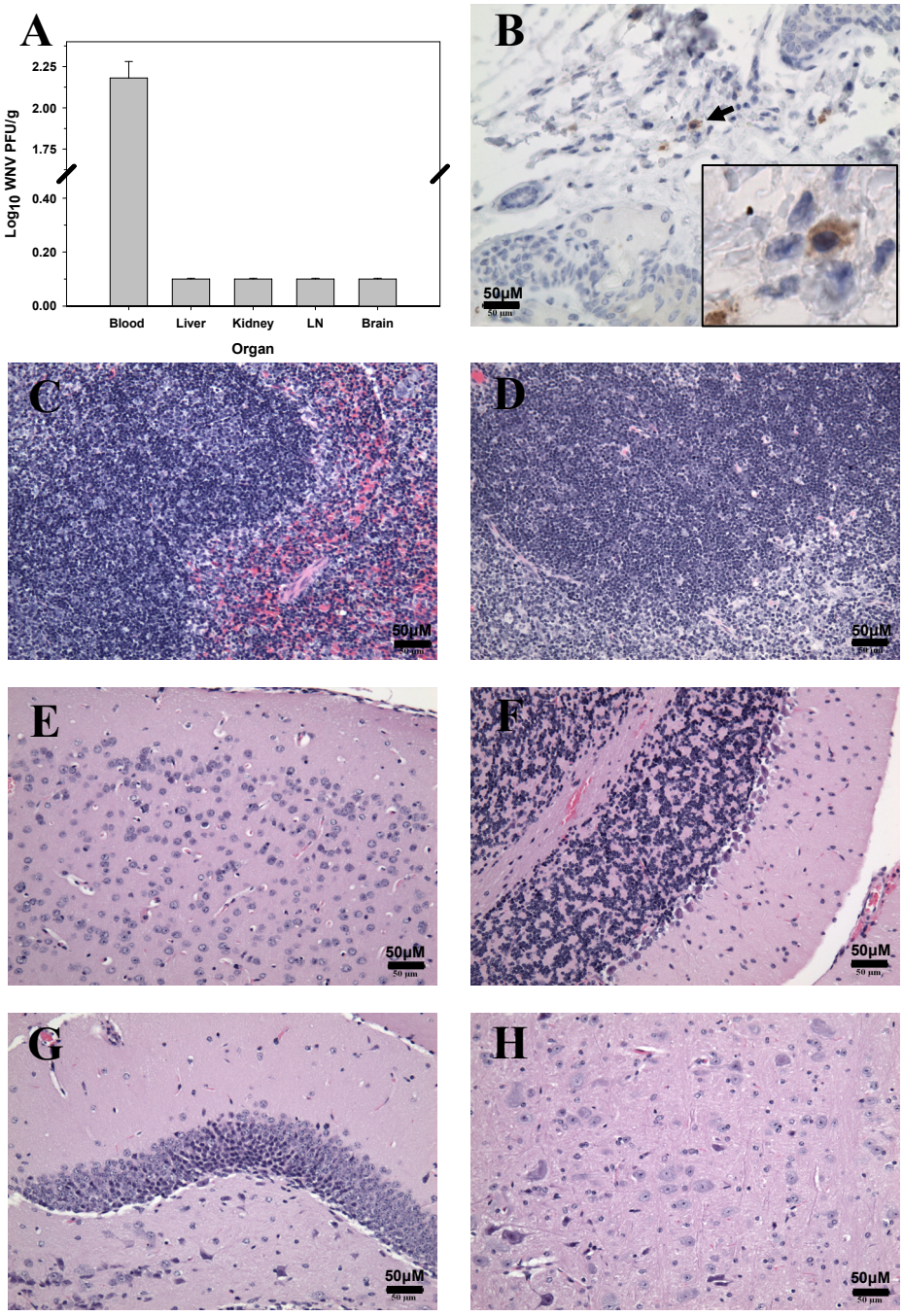


Figure 4.10. **Main features of the pathology of WNV infection in mice on day 1 p.i.** Panel A shows viral titers in five different organs on the indicated day p.i. The bars represent the average ± SEM of the values obtained from two different plaque assays performed in tissue from the mice which presented the highest titers in blood on day 1 p.i. Panel B shows detection of WNV antigen by IHC in the cytoplasm of macrophages or dendritic cells present in dermal tissue (arrow in large frame and 10 times increased magnification in the inset frame). Panels C to H show normal sections of spleen (C), thymus (D), cerebral cortex (E), cerebellum (F), hippocampus (G), and brainstem (H) stained with H&E. The size bar is proportional to the magnification of each photograph.

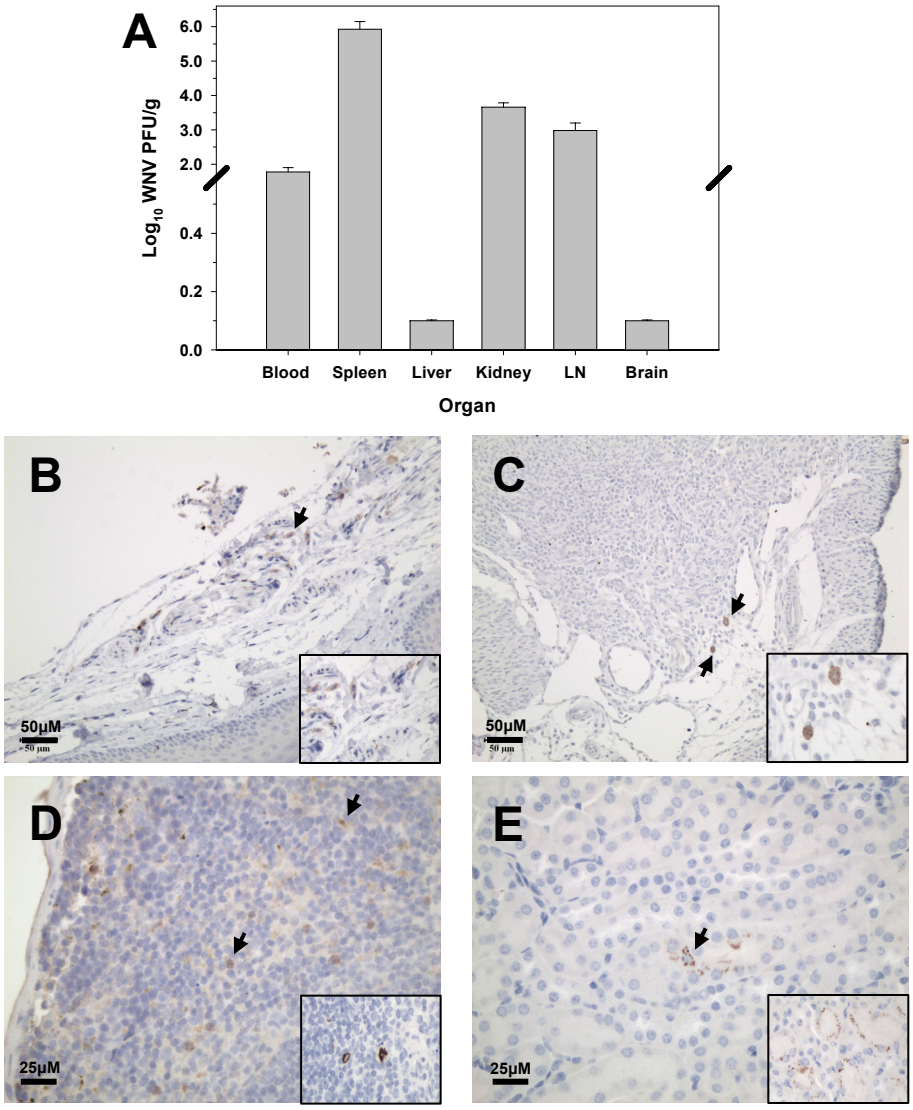


Figure 4.11 Main features of the pathology of WNV infection in mice on day 4 p.i. Panel A shows the viral titers in five different organs on the indicated day p.i. The bars represent the average ± SEM of the values obtained from two different plaque assays performed in tissue from the mice which presented the highest titers in spleen on day 4 p.i. Panel B shows detection of WNV antigen by IHC in the cytoplasm of macrophages or dendritic cells resident in dermal tissue (arrow in large frame and 4 times increased image in the inset frame). Panel C presents the detection of WNV-antigen in muscular cells of uterus (arrows in big frame. Small frame is a 4-fold increased magnification from the larger frame). In panel D detection of WNV-antigen in macrophages of the spleen is shown (arrows in large frame; 4-fold increased image in small frame), Panel E shows detection of WNV-antigen in epithelial cells from renal tubules (arrow in large frame).

B.3. Day 6 p.i.

On day 6 p.i. viremic titers decreased to undetectable levels. However, the titers were still around 10,000 PFU/g of the spleen in all three mice sacrificed on this day. The viral titers also decreased in kidney with the maximum titers of 500 PFU/g of tissue. In two out of three mice, titers of virus were detected in lymph node, and the maximum titers were close to the 10,000 PFU/g. Day 6 p.i. was the first day in which the virus was detectible in brain. The titers obtained from this organ are approximately 4,000 PFU/g (Fig. 4.12 A). Immunohistochemical staining demonstrated that macrophages and fibroblasts of the skin were positive for WNV (Fig. 4.12B). Few macrophages were detected as positive for WNV in spleen (Fig. 4.12C). Clear, multifocal staining was detected in the renal medulla and some epithelial tubular cells (Fig. 4.12D). Although the virus was detected in these three organs, no signs of inflammation were observed. Consistent with result from viral titrations, IHC detection of WNV was positive for the first time in central nervous system, in which there were multifocal areas with some neurons from the olfactory bulb that stained positive for WNV (Fig. 4.12E). In spinal cord, brainstem and cerebral cortex there were small foci of neurons which were positive for WNV by IHC (Fig. 4.12G, H). Moderate multifocal gliosis, neuronal satelitosis and moderate multifocal mononuclear perivascular infiltrates were observed in brainstem and spinal cord (Fig. 4.12G, I and J). At the same time a clear up-regulation of the chemokines IP-10, MIG, and MCP-5, as well as a slight up-regulation of MCP-1 were detected in at least one out of three mice sacrificed on day 6 p.i. (Fig. 4.18A and B).

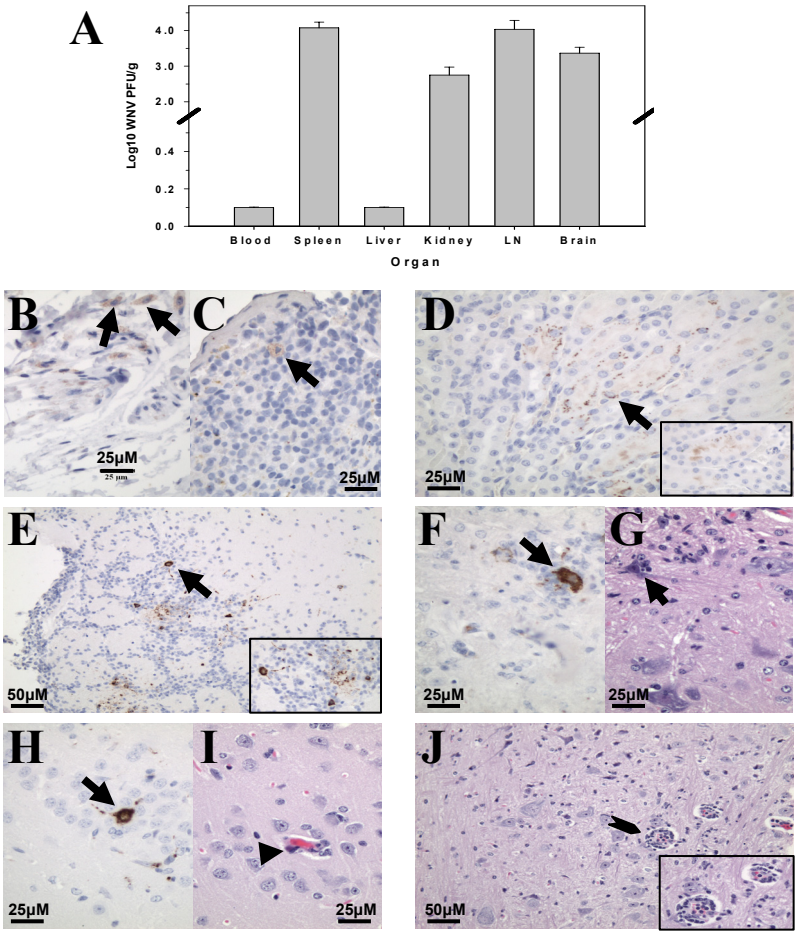


Figure 4.12 Main features of the pathology of WNV infection in mice on day 6 p.i. Panel A shows the viral titers in five different organs on the indicated day p.i. The bars represent the average ± SEM of the values obtained from two different plaque assays performed in tissue from the mice which presented the highest titers in spleen on day 6 p.i. Panel B shows the detection of WNV antigen in the cytoplasm of macrophages or dendritic cells resident in dermal tissue (arrows). Panel C shows the immunolocalization of WNV-antigen in macrophages of spleen (arrow). Panel D shows the detection of WNV-antigen in epithelial cells from renal tubules (arrow). Panel E shows the immunodetection of WNV-antigen in neurons of the olfactory bulb (arrow in large frame; 2-fold increased image in inset frame). Panel F shows a section of spinal cord with the imunolocalization of WNV-antigen in one degenerated neuron surrounded by inflammatory cells (arrow). Panel G shows an H&E stained section from spinal cord with a neuron surrounded by glial cells (satellitosis) (arrow). Panel H shows cerebral cortex with the immunodetection of WNV antigen in cytoplasm of neuron (arrow). Panel I shows an H&E stained section from cerebral cortex with mild perivascular mononuclear infiltrate (arrowhead). Panel J shows an H&E stained section from brainstem, with moderate perivascular mononuclear infiltrate (arrow in large frame, image in inset frame is 2-fold increased in magnification).

B4. Day 9 p.i.

Day 9 p.i. may be considered the most critical day in the course of this experiment given that the most severe lesions and the highest viral titers in brain were observed on this day. In addition, this was the timepoint in which the animals began showing clinical signs and the more severely affected animals died on this day. In the second experiment day 9 p.i. was the day in which all the mortality was observed (Fig. 4.13). Once the clinical signs, characterized by the appearance of a rough hair coat, lethargy, apathy, incoordination, segregation from the group, difficulty in swallowing, and prostration were observed, the animals died in less than 12 hours. Viral titers in brain were the highest observed in all the tissues analyzed, reaching $10^{7.9}$ PFU/g. The virus was still detected in kidney and spleen. Although the viral concentrations were very high in the brain, no virus was detected in blood on day 9 p.i. (Fig. 4.14A).

On day 9 p.i. all the animals presented with inflammatory lesions in the cerebral cortex, hippocampus, brainstem and spinal cord. The inflammation was characterized by multifocal perivascular mononuclear infiltrates, composed mainly of macrophages and lymphocytes with scant neutrophil infiltration (Fig. 4.14C) and neuronal degeneration with multifocal gliosis and satellitosis (Fig. 4.14F). In the spinal cord a severe extensive localization of the virus was detected mainly in the ventral horns (Fig. 4.14D and E), although in one animal, part of the dorsal horns were also affected (Fig. 4.14E). Immunohistochemical staining detected WNV in neurons and astrocytes of the ventral horns of the spinal cord (Fig. 14D and E). A few neurons from hippocampus, cerebral cortex and brainstem stained positive for the virus in a multifocal pattern (Fig. 4.14B). In

the more clinically ill mice, the lesions were more extensive. In these animals, an extensive area of cerebral cortex stained positive by IHC. The virus was identified in neuronal cytoplasm and neuronal projections (Fig. 4.14D and E). Additionally there were areas with multifocal positive staining of neurons and astrocytes in the spinal cord, brainstem (Fig. 4.15E), hippocampus (Fig. 4.15G) and cerebellum in which the affected cells were the granular cells with only a few stellate neurons infected (Fig. 4.15C). In these mice neuronal degeneration was evident in the cerebral cortex and an extensive area of WNV antigen was detected by IHC in the cytoplasm of degenerated neurons (Fig. 4.15A). Those neurons were shrunken and had eosinophilic cytoplasm with pyknotic or karyorrhectic nucleus, and some of the neurons were surrounded by foamy macrophages, glial cells and lymphocytes (Fig. 4.15B and D). Neuronophagia was also observed in the affected areas, and perivascular infiltrates were present (Fig. 4.15D and F) with disruption of the endothelium in some vessels and moderate hemorrhage. In dead mice the spleen was severely depleted, and thymus was also affected by the lymphoid depletion which was characterized by abundant numbers of lymphocytes with karyorrhectic or pyknotic nuclei (Fig. 4.15H).

In the brain of the animals sacrificed on day 9 p.i., a significant up-regulation of several cytokines and chemokines was detected. In all the brains analyzed, TNF-α, IL-1α, IFN-γ, and the immunomodulatory cytokine TGF-ß1 were up-regulated, while the chemokine C10 was detected for first time in the infected mice, as well as MIP-2, MIP-1ß and RANTES. The chemokines up-regulated on day 6 p.i. showed increased

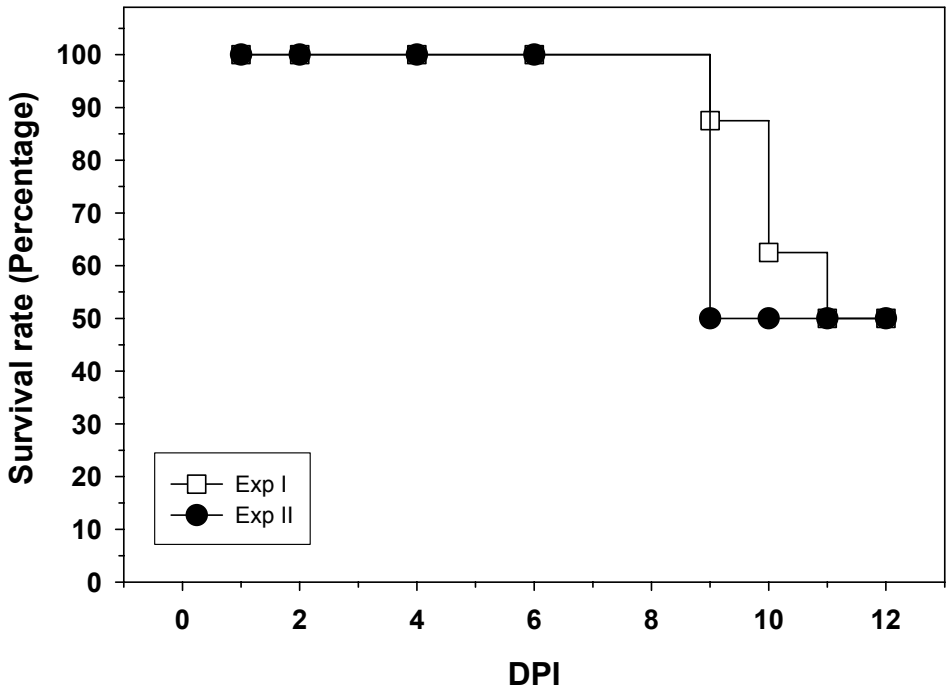


Figure 4.13. **Mortality in mice infected with West Nile virus in experiments I and II**. Percentage of surviving animals during a WNV infection obtained from two different experiments is presented. Represented are the survival percentages in an experiment performed with 8 mice (open squares), or in an experiment performed with 6 animals (closed circles). The mice were inoculated subcutaneously with 100 PFU of WNVnd no serum was passively transferred. The time points indicate the percentage of surviving animals at the point of reference

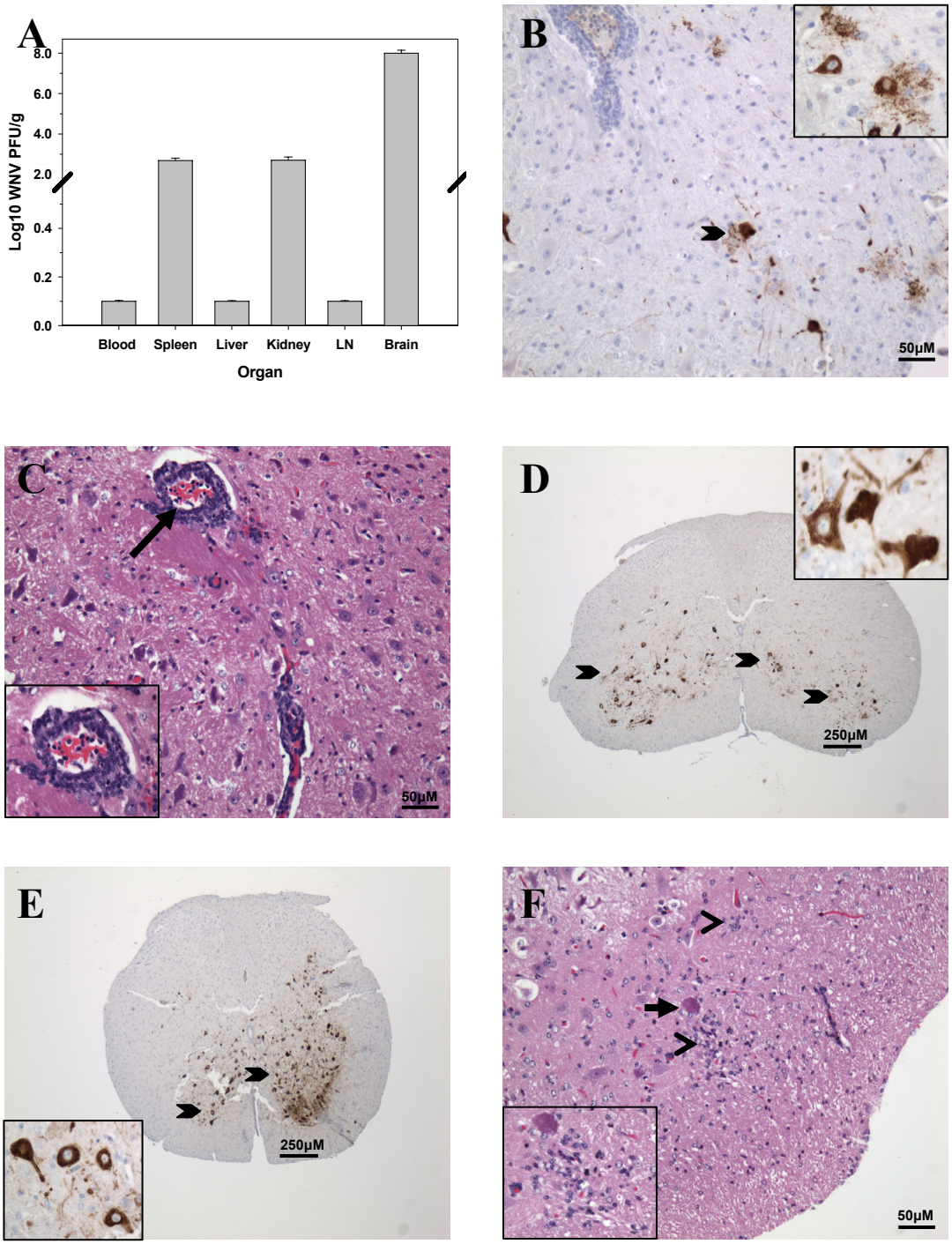

A
Log10 WNV PFU/g
8.0
6.0
4.0
2.0
0.4
0.2
0.0
Blood
Spleen
Liver
Kidney
LN
Brain
Organ
B
50μM
C
50μM
D
250μM
E
250μM
F
50μM

Figure 4.14. Main features of the pathology of WNV infection in mice on day 9 p.i. Panel A shows the viral titers in five different organs on the indicated day p.i. The bars represent the average ± SEM of the values obtained from two different plaque assays performed in tissue from the mice which presented the highest titers in brain on day 9 PI. Panel B shows multifocal detection of WNV-antigen in cytoplasm and neuronal projections of neurons in brainstem (chevron arrow). An adjacent blood vessel has a significant perivascular mononuclear infiltrate. The small frame presents the localization of the virus in neurons from a different area of the brainstem. Panel C shows an H&E stained section from brainstem, in which blood vessels have a severe perivascular mononuclear infiltrate (arrow), the small frame is a 2-fold increase in magnification from large frame. Panel D shows immunolocalization of WNV antigen in neurons of the ventral horns of spinal cord (chevron arrows), the inset frame is a 10 fold increased magnification of the large frame, showing the localization of virus in cytoplasm and neuronal projections. Panel E shows immunolocalization of WNV antigen in the cervical spinal cord, which contains an extensive area with neurons infected with WNV (chevron arrows). The affected area includes not only the ventral horns, but also a section of dorsal horns. The inset frame is a 10 fold increase magnification image of the big frame, showing the cytoplasmic localization of WNV in neurons. Panel F shows an H&E stained section from spinal cord, which contains multifocal nodular gliosis (arrowheads) and neuronal degeneration (arrows), the small frame is a 2 fold increased in magnification image from the big frame, showing a degenerated neuron surrounded by gliosis.

expression on day 9 p.i. leading to clear detection of the transcript for MCP-1, IP-10, MIG and MCP-5 (Figures 4.17 and 4.18).

B. 5. Day 13 – 21 p.i.

Day 11 p.i. was the last day in which mortality was observed. On day 13 p.i. viral titers were still detected in several organs such as the brain, which had titers up to 10^7 PFU/g. Virus was also detected in lymph nodes (10^5 PFU/g) from one out of three mice sacrificed on this day. The kidney and spleen from two mice had viral titers of approximately 1,000 PFU/g and 100 PFU/g, respectively (Fig. 4.16A). No virus was detected in blood on this day.

Interestingly the virus was detected by IHC in ocular muscles, although no clear evidence of virus invasion into the retina was observed (Fig. 4.16B and C). Multifocal staining of infected neurons was observed in the spinal cord, hippocampus and cerebral cortex (Fig 4.16 G; H and J). In addition, there was still evidence of inflammatory lesions in the brainstem, cerebral cortex, hippocampus and spinal cord. At day 13 p.i. three out of three mice had multifocal nodular gliosis, mainly in brainstem and spinal cord (Fig. 4.16I and J). Neuronal degeneration and satellitosis were observed in the cerebral cortex and brainstem (Fig. 4.16F and I), as well as neuronophagia in the ventral horns of spinal cord (Fig. 4.17J). In spleen and thymus, moderate to severe depletion of lymphoid tissue was observed (Fig. 4.16D and E).

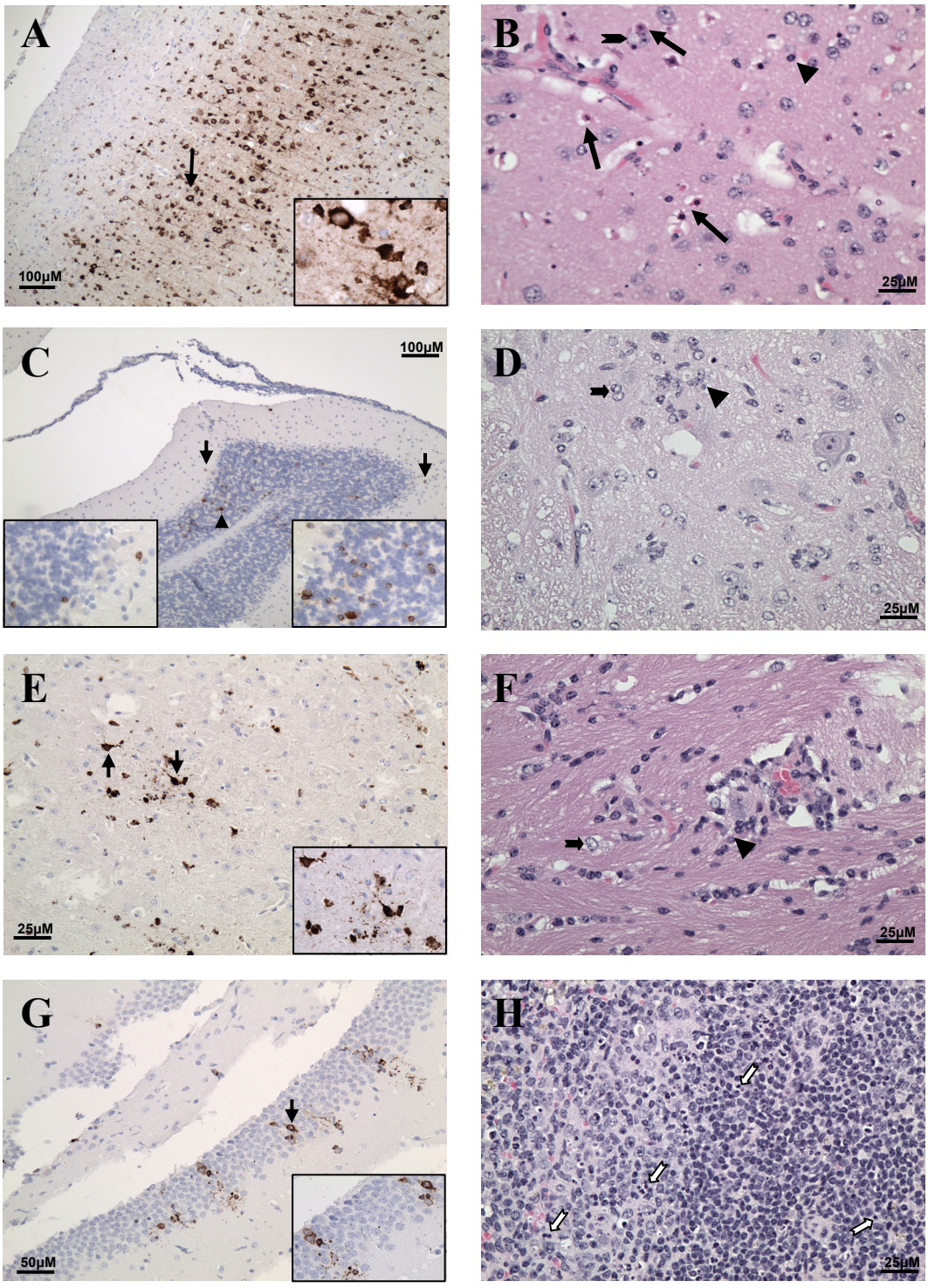

A
100μM
B
25μM
C
100μM
D
25μM
E
25μM
F
25μM
G
50μM
H
25μM

Figure 4.15. **Histopathological changes in a WNV-infected C57BL/6, which succumbed to the disease**. Panel A show a diffuse distribution of intraneuronal WNV antigen in cerebral cortex (arrow), the small frame is 4 fold increaed in magnification image from one of the affected areas, with cytoplasmic localization of WNV antigen. Panel B shows an H&E stained section from the same area of cerebral cortex presented in panel A. A severe diffuse neuronal degeneration is observed, which is characterized by shrunken neurons with highly eosinophilic cytoplasm and pyknotic nucleus (arrows). Foamy macrophages are also observed (chevron arrow) approaching to a degenerated neuron, and gliosis is also observed (arrowhead). Panel C shows the multifocal distribution of WNV infected granular cells (arrowhead) and neurons (arrows), small frames are 4 fold increased in magnification images, which show the areas pointed by the arrowhead and arrows. Panel D shows an H&E stained section from brainstem, in which several multifocal foamy macrophages (arrow) are observed. In some areas those macrophages are surrounding degenerated neurons (satellitosis) (arrowhead). Panel E shows a section of spinal cord with multifocal nodular gliosis, which is close to neurons containing WNV antigen identified by IHC. Panel F, shows an H&E stained section of spinal cord with severe perivascular mononuclear infiltrate. The blood vessel observed seems to have disruption of the endothelium, although no hemorrhage is observed, several monocytes and lymphocytes (arrowhead) are observed in parenchyma. Multifocal foamy macrophages are also observed (arrow). Panel G shows the multifocal immunodetection of WNV antigen by IHC in neurons from hippocampus (arrow), the inset frame is a 2 fold increased in magnification image from the area pointed with the arrow. Panel H shows an H&E stained section from spleen, in which diffuse moderate to severe lymphoid depletion, characterized by the presentation of karyorrhectic and/or pyknotic nuclei in the lymphoid population (white rows).

On day 21 p.i., no virus was detected in any tissue by plaque assay. Scant small nodules of glial cells surrounding degenerated neurons positive for WNV by IHC were observed mainly in cerebral cortex, brainstem and spinal cord in two of four mice examined.

IV. Discussion

A. Evaluation of ADE for WNV *in vivo* infection.

The ability of a sub-neutralizing dilution of WNV-immune horse serum to induce antibody dependent enhancement *in vivo* in a murine model of WNV infection was evaluated. The results obtained showed a significant difference ($P<0.05$) in mortality rates when the different groups were compared, with the non-serum treated, WNV inoculated mice suffering 50% mortality by day 12 p.i. Surprisingly, all mice treated with a sub-neutralizing dilution of immune horse serum (group IHS 1:350) survived infection and none presented with clinical signs of illness. Furthermore only one animal from this group developed high titers of virus in brain. Of the mice pre-treated with non-immune (normal) horse serum, 7 of 8 survived. In contrast to the mice injected with a neutralizing dose (IHS 1:7) prior to WNV inoculation, both the sub-neutralizing and non-immune serum treated mice developed both viremia and tissue titers at multiple days p.i. Use of neutralizing doses of immune serum for protection of mice against challenge with WNV, as well as other flaviviruses, has been previously demonstrated (Kimura and Kuroda, 1988; Agrawal and Petersen, 2003; Ben-Nathan et al., 2003; Engle and Diamond, 2003). This experiment may have failed to induce ADE due to the use of heterologous (horse)

serum instead of using murine serum at sub-neutralizing doses. Wallace et al. reported that infection of mice with Murray Valley encephalitis virus was enhanced when the mice were treated with sub-neutralizing dilutions of serum, but in this case homologous serum was used (Wallace et al., 2003). Due to our observations in the group of animals treated with normal horse serum, we considered the possibility that the heterologous nature of the serum induced a non-specific protection against WNV through activation of innate immune responses prior to inoculation with WNV. The normal horse serum was shown to lack antibodies specific to WNV and was heat inactivated, and therefore had no complement activity. Horse serum, like that from all species, contains high concentrations of proteins and chemical mediators which might induce an immune response in the recipient of the serum, especially if the recipient is a species other than equine.

To identify potential mechanisms by which passive transfer of heterologous serum induced protection against WNV infection in mice, we performed quantitative RT-PCR to determine the relative levels of cytokine expression. We assumed that if there was a difference in the innate immune response, it should be observed in spleen during the earliest time points post-infection since in the first two days there were no differences in viral titers when all the inoculated groups (except for IHS 1:7) were compared (Fig. 4.2). Moreover, the first significant differences were observed on day 4 p.i., particularly in spleen. Therefore, we determined expression levels of IFN-γ, iNOS and IL-12p40 in RNA purified from this organ. We found significant differences in expression of these molecules, especially at the earliest time point tested (day 1 p.i.). It is interesting that the

expression of IFN-γ and iNOs was down-regulated in all inoculated groups. However, the expression levels were more than 1,000-fold lower for mice in the NS group compared to both the naïve controls and the serum pre-treated groups. Mice in the NHS and IHS 1:350 groups had slightly lower expression levels than the naïve control group, but the differences were not significantly different. This result suggests that WNV infection down-regulates expression of IFN-γ and, as consequence of this down-regulation, the suppression of some interferon stimulated genes (ISGs) such as iNOS may follow.

The role of type I interferons has been widely studied, and it is known that these molecules are up-regulated by infection *in vitro* with WNV (Libraty, et al, 2001). Recently Samuel and Diamond reported that type I interferons controlled WNV infection in mice by restricting the tropism and viral burden, as well as preventing the death of infected neurons (Samuel and Diamond, 2005). However, no information is available about the role of IFN-γ in the immune response against other flaviviral infections, although it is known that this molecule has an important role in clearing viral RNA in the CNS (Binder and Griffin, 2001; Griffin, 2003). It has been demonstrated that mice deficient in the production of IFN-γ (IFN-γ-/-) or nitric oxide displayed a marginally increased susceptibility to infection with a neurotropic virus (Lobigs et al., 2003). In addition, other reports have suggested an important role for IFN-γ during infection of mice by WNV (Wang, et.al., 2003). However, no reports exist about the ability of WNV to induce down-regulation of IFN-γ in an *in vivo* infection. On day 2 p.i. in our experiment a significant up-regulation of the IFN-γ was observed in animals from the NS group. However, the elevated levels of IFN-γ expressed on day 2 p.i. failed to control the

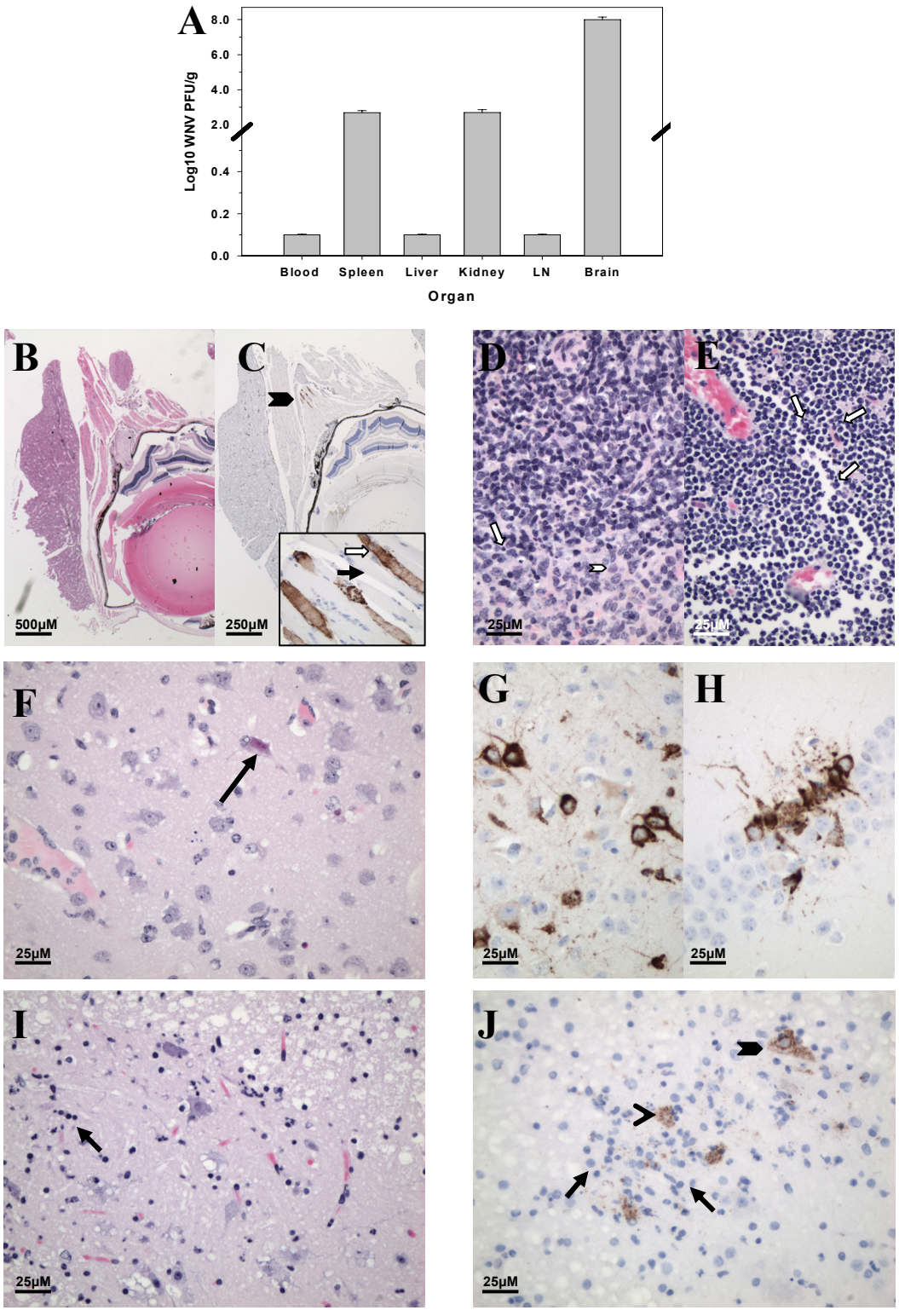

A
Log10 WNV PFU/g
8.0
6.0
4.0
2.0
0.4
0.2
0.0
Blood
Spleen
Liver
Kidney
LN
Brain
Organ
B
500μM
C
250μM
D
25μM
E
25μM
F
25μM
G
25μM
H
25μM
I
25μM
J
25μM

Figure 4.16. Main features of the pathology of WNV infection in mice on day 13 p.i. Panel A shows the viral titers in five different organs on the indicated day p.i. The bars represent the average ± SEM of the values obtained from two different plaque assays performed in tissue from the mice which presented the highest titers in brain on day 13 p.i. Panel B shows a section of eye stained with H&E. The same section of eye is presented in panel C, in this panel the section was subjected to IHC, which shows the localization of WNV antigen in the muscular fibers (chevron arrow in large frame). The small frame shows fibers with WNV antigen in the sarcoplasm and no staining in nucleus (white arrow), and an adjacent muscular fiber which does not stain positive for WNV (black arrow). Panels D and E present H&E stained sections from spleen and thymus respectively. The arrows indicate lymphocytes with pyknotic or karyorrhectic nuclei in both organs. In spleen (Panel D) the absence of lymphoid cells makes easier the observation and identification of macrophages in the organ (chevron arrows). Panel F shows an H&E stained section of cerebral cortex, which have sporadic degenerated neurons (arrow). Panels G and H show the immunodetection of WNV in neurons from the brainstem (G) and hippocampus (H). Panel I shows an H&E stained section from brainstem with moderate nodular gliosis (arrow). Panel J shows a section of spinal cord with neurons containing virus (chevron arrow) and multifocal nodular gliosis (arrow) surrounding accumulations of WNV antigen, which apparently is contained inside degenerated neurons (arrowheads)

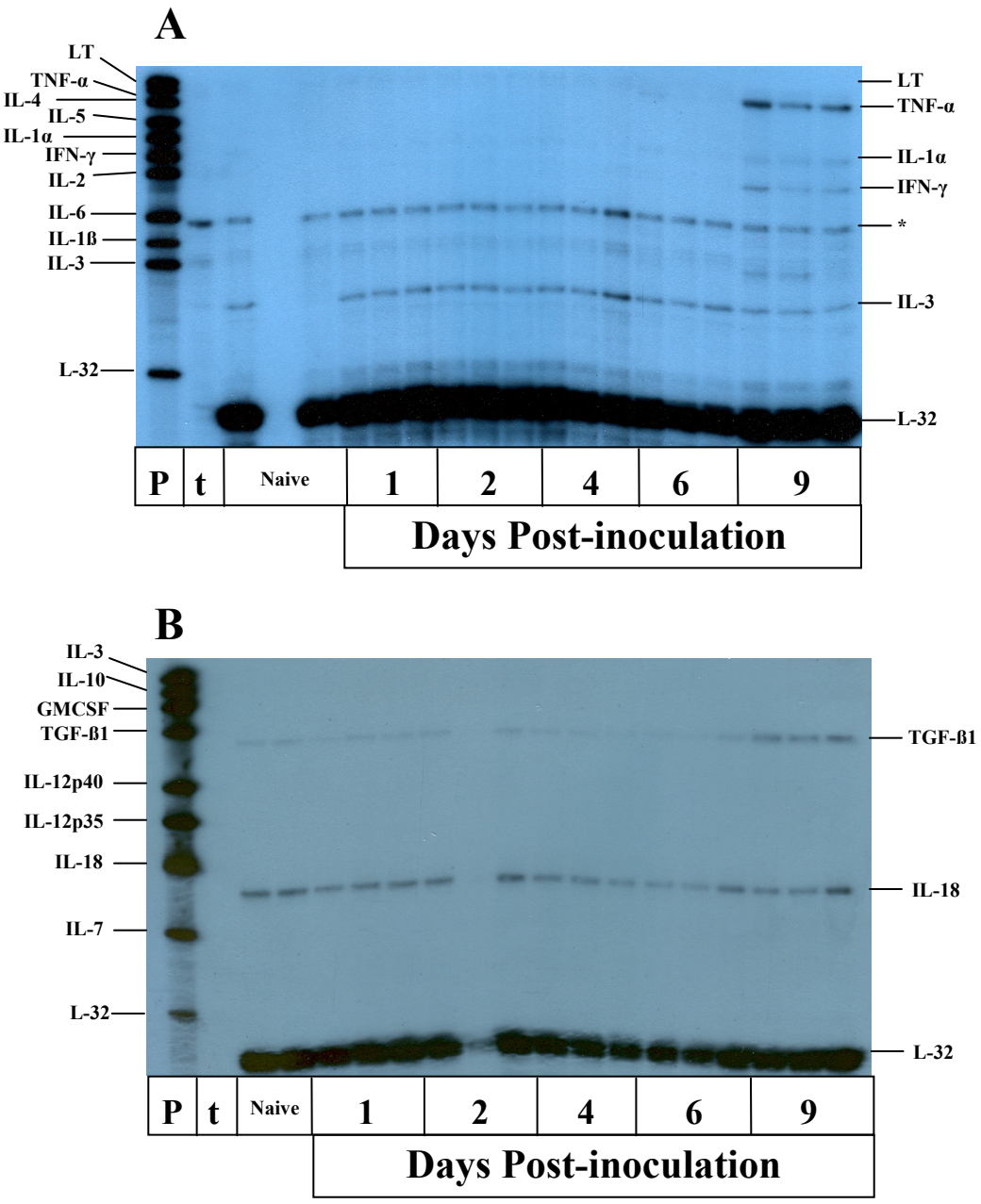


Figure 4.17. Cytokine expression on brain from naive and infected mice. RNAse protection assay was performed in RNA samples of brain from WNV infected mice (both panels). Detection of the cytokine expression was performed on days 1, 2, 4, 6, and 9. Three mice per group were euthanized on the indicated days. (t), indicates a transfer RNA control. Naïve are uninfected, non-serum treated mice sacrificed on day 1 or 4 of the study. P indicates the molecular probe control. In the left side are listed all the cytokines analyzed in each gel.

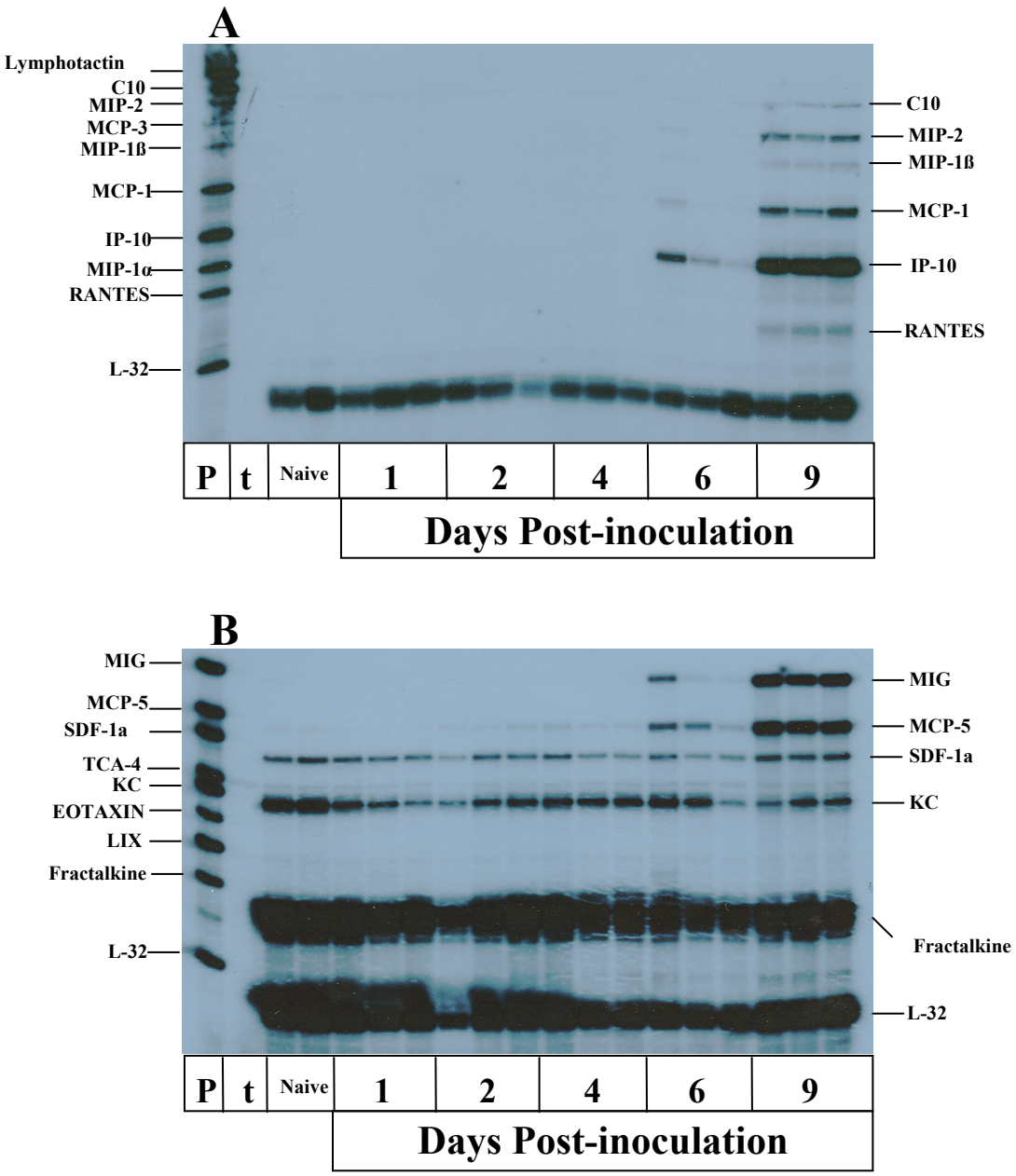


Figure 4.18. **Chemokine expression on brain from naïve and infected mice**. RNAse protection assay was performed in RNA samples of brain from WNV infected mice (both panels). Detection of the chemokine expression was performed on days 1, 2, 4, 6, and 9. Three mice per group were euthanized on the indicated days. (t), indicates a transfer RNA control. Naïve are uninfected, non-serum treated mice sacrificed on day 1 or 4 of the post infection course. P indicates the molecular probe control. In the left side are listed all the chemokines analyzed in each gel.

infection, and therefore the role of this cytokine in the control of WNV infection warrants further investigation.

Expression of iNOS was down-regulated on day 1 p.i., and again the most markedly affected mice were those in the NS group. Interestingly, although down-regulation of this molecule was less severe on day 2 p.i., expression of iNOS was not elevated following the significant increase in IFN-γ production on day 2 p.i. in the NS group. It is known that iNOS is involved in the production of NO. Production of iNOS by macrophages is one of the most important mechanisms of protection against viral pathogens, and it has been demonstrated that NO can inhibit replication *in vitro* of Japanese encephalitis virus (Linn, et.al., 1997). However other reports indicate that NO might be involved in the pathogenesis of flaviviral infections (Kreil and Ebil, 1995; Kreil and Ebil, 1996; Andrews et. Al., 1999). Regardless of the marked reduction in expression of iNOS and IFN-γ, our results indicate that none of these molecules are fully responsible for the protection observed in mice treated with NHS and IHS 1:350.

The observations were different for expression of IL-12p40. This molecule is known to be part of two interleukins: IL-12 and IL-23, which are important for initiation of innate immune responses. It is known that IL-12p35 is constitutively expressed in the spleen of mice, dogs and horses, and expression of IL-12p40 is considered as indicative of IL-12 protein up-regulation (Tizard, 2004). Activated dendritic cells and macrophages produce IL-12 in early stages of the response against infectious agents, including viruses. IL-12 has powerful effects in the induction of NK cell activity and T cell development

(Langrish et.al, 2004). Some of the effects are up-regulation of IFN-γ by NK cells and NO by macrophages (Tizzard, 2004). Our observations indicate that mice treated with either NHS or IHS 1:350 developed a clear up-regulation of IL-12p40 on day 1 and 2 p.i., while the mice only infected with WNV and naïve mice did not express detectable levels of specific IL-12p40 mRNA on day 1 p.i. (Fig. 4.7). We suggest that this is the most important observation for answering the question of why serum-treated animals were protected against WNV infection. Heterologous serum triggered activation of macrophages and dendritic cells (DC) in the spleen of treated mice, inducing up-regulation of IL-12p40 which partially abolished inhibition of the expression of iNOS and IFN-γ caused by infection with WNV. It has been previously demonstrated that treatment of mice with recombinant IL-12 one day prior to the infection with the flavivirus Saint Louis encephalitis (SLEV), eliminated pathology and mortality of infected mice (Phillpotts et al., 2003). These authors reported that although clinical signs, pathology and mortality were ameliorated, the burden of virus was not significantly different when the treated and un-treated animals were compared. We observed a reduced mortality in the NHS group, but still found similar titers of virus in spleen and brain when these animals are compared to the NS group. Interestingly mice from the IHS 1:350 group did not suffer any mortality, and the levels of virus were lower in spleen and brain on day 4 and 6 p.i., respectively. Given that the levels of equine antibody in mice treated with IHS 1:350 were undetectable, we suggest that antibody did not neutralize the infection of WNV, but may have helped the virus to gain entry into dendritic cells, which had been previously activated by the serum. With this the DC were induced to produce more IL-12, and perhaps to be more efficient in antigen presentation to lymphocytes in

the spleen and draining lymph nodes. All this resulted in a more effective cellular immune response against the virus. This response was expressed as no mortality and lower titers of virus in tissue. Although the explanation given is plausible, we still need to know more about the role of IL-12 in the infection of mice by WNV.

As additional evidence for a possible role of IL-12 in protection against WNV or in amelioration of the pathology, it is important to mention that we detected the expression of IL-12 in the brain from the only mouse treated with IHS 1:350 which develop high titers of virus in brain. The role of IL-12 in inflammatory processes in CNS is not completely understood. This cytokine is expressed by astrocytes and microglia after infection of neurons by herpes simplex virus type I as reported by Broberg et al. (2002), and seems to play an important role in stimulation of Th-1 responses for the clearing of RNA viruses from brain (Griffin, 2003). Surprisingly, this same cytokine has been reported to play an important role in the pathogenesis of experimental autoimmune encephalomyelitis. Here we report the expression of IL-12 in the brain of one IHS 1:350-treated mouse which developed high viral burdens in brain yet did not show clinical signs prior to euthanasia, and thus we suggest that IL-12 may play an important role in the amelioration of the encephalitis caused by WNV.

Another observation made during in this experiment was the marked expression of the chemokine C10 in the brain of mice in the NS group (Fig. 4.9A). This chemokine was not detected in the brain of mice from the IHS 1:350 group, even though similar viral titers were present in brain compared to those detected in the animals from NS group.

C10 is a novel chemokine that is chemotactic for mononuclear phagocytes. This molecule is expressed during demyelinating disorders (Asensio et al., 1999). It is known that IL-13, which is a Th2 type cytokine, is a potent inducer of C10 (Belperio et al., 2002). Our observations are consistent with these previous studies since IL-12 is known to be a potent Th-1 cytokine, and thus it is likely that the presence of this cytokine had some effect on the expression of IL-13 and therefore on the expression of the chemokine C10. We suggest that with no C10 expressed, the recruitment of macrophages to the CNS is reduced at least partially and this reduction in recruitment may influence the pathogenesis of WNV in the CNS.

B. Chronology of the pathogenic and immunologic events during the infection of C57BL/6 mice with West Nile Virus.

As described in the results section, on day 1 p.i., WNV was detected in blood by plaque assay. By immunohistochemistry, the only site in which infected cells were detected was the subcutaneous tissue of the injected footpad. These two results are consistent with previous reports in which WNV was detected in serum from inoculated mice 24 to 48 hours after a subcutaneous injection (Diamond et al., 2003a; Kramer and Bernard, 2001). There are no reports documenting replication of WNV in skin. It is believed that after peripheral inoculation, WNV is able to infect Langerhans dendritic cells (Johnson et al., 2000), which migrate to draining lymph nodes, and within 12 to 24 hours viral replication is observed in secondary lymphoid tissue (Diamond et al., 2003a; McMinn et al.,1996). Our findings differ in this regard to these reports due to our observation of no viral replication in any organ other than blood and presence of virus in

skin on days 1 and 2 p.i. On day 4 p.i. we detected the peak of viral replication in spleen and kidney, and this day was the first time point in which WNV was isolated from lymph nodes, kidney, and spleen. Using IHC, WNV was found in epithelial cells of renal tubules, macrophages in spleen and cells from subcutis. In previous reports, day 4 p.i. has been identified as the time of the peak viral replication in spleen and kidney (Diamond et al., 2003a; Kramer and Bernard, 2001; Xiao et al., 2001). No previous reports exist describing detection of WNV in uterus and skin. In 2003, Buckweitz, et.al. detected antigens of WNV in renal tubules from an infected dog (Buckweitz, et.al., 2003), therefore according to this report and our findings the epithelial cells of the renal tubules and subcutis are suggested as a possible site of replication for WNV.

In CNS on day 6 p.i., the virus was identified in neurons of the olfactory bulb, brainstem and spinal cord. The virus was localized in small multifocal nodules in multiple areas of cerebrum, cerebellum, brainstem and spinal cord. The neurons were the main target of WNV in the CNS, and the virus was mainly contained in the cytoplasm of those cells and in neuronal projections. The main features of the inflammation observed in CNS were the presence of perivascular infiltrates composed of monocytes and lymphocytes, multifocal nodular gliosis, mild neuronophagia, and neuronal degeneration, all of which were more severe in animals that succumbed to the disease. The localization of WNV and the lesions mentioned confirm the results obtained in other murine models (Diamond et al., 2003a) and are similar to histopathological observations from WNV-infected humans and horses (Agamanolis et al., 2003; Cantile et al., 2001; Doron et al., 2003; Hayes et al., 2005; Kelley et al., 2003).

On day 13 p.i., WNV was still present in brain. Infected neurons from brainstem, spinal cord and hippocampus are detected by IHC on this day. As mentioned above, mice sacrificed on this day were considered survivors of the infection because no mortality was observed after day 11 p.i. Consequently, the virus observed in these mice could be considered potentially a persistent infection. Persistent viral infection has been reported to occur in other flaviviral infections (Chambers and Diamond, 2003). The host immune response, together with some properties of the neuronal environment allow the possibility of persistence, and both may contribute to the presentation of phenotypic variation in the persistent viruses. An example is the attenuation of WNV which was persistent in monkeys (Pogodina, et.al. 1983), however the persistence of a Sindbis virus infection in mice led to the emergence of neurovirulent strains (Levin and Griffin, 1993).

TNF-α plays a pivotal role in the immunity and pathogenesis of viral encephalitis and it has been demonstrated that in an infection caused by Murray encephalitis virus, this molecule acts in two different ways: (1) activating neurovascular endothelium and increasing adhesion molecule expression, and (2) attracting neutrophils via induction of the neutrophil attracting chemokine N51/KC (CXCL1) (King et al., 2003). Recently with a very elegant experiment, Wang and collaborators demonstrated that in the absence of TLR-3 the WNV loads in blood increased, but the absence of this molecule also reduced the amount of virus in CNS, diminished the neuropathology and also reduced the mortality during an infection by WNV (Wang et al., 2004). From these results, the authors suggested that TLR-3 plays a role in WNV crossing the blood-brain barrier,

especially because crosslinking of this receptor up-regulates expression of TNF-α, which in turn increased capillary permeability by stimulation of the endothelial cells in the brain blood capillaries (Diamond and Klein, 2004). In the same way IFN-γ has been reported to be important in induction of lymphotactic chemokines such as IP-10 (CXCL10) thereby influencing the movement of T cells into the brain parenchyma (Dufour et al., 2002). Our results show an up-regulation of TNF-α and IFN-γ, although these two molecules were not up-regulated until day 9 p.i.. This up-regulation was detected after up-regulation of important chemokines such as IP-10, MIG and MCP-5, which were up-regulated or expressed for first time on day 6 p.i. The production of IP-10 in brain has been attributed to perivascular glial cells, perivascular astrocytes, and endothelial cells (Pratt, et.al., 2001). It has also been reported that IP-10 is produced in early stages of *in vitro* infections of microglial cells and astrocytes by WNV (Cheran, et.al., 2005). MCP-5 has been demonstrated to be a potent monocyte chemoattractant produced in part by endothelial cells (Sarafi et al., 1997). Day 6 p.i. was also the first day in which a perivascular infiltrate was observed in infected mice. Even though other murine models have shown an important role for TNF-α and IFN-γ as important factors in the pathogenesis of West Nile encephalitis, our results demonstrate that those molecules may have a role after some other molecules triggered the inflammatory process in brain. Our hypothesis is that IP-10 and MCP-5 start the recruitment of leukocytes into perivascular spaces. Here antigen presentation occurs, which leads to the activation of circulating leukocytes that produce IFN-γ, IL-1α and TNF-α. These molecules increase the permeability of brain capillaries and facilitate the entry of inflammatory cells into the brain parenchyma, helping eliminate the virus in CNS. It is possible that these events also

facilitate the entry of infected monocytes, increasing the viral burdens and neuronal infection of brain.

Klein and collaborators demonstrated the importance of IP-10 in protection against West Nile encephalitis by using mice deficient in CXCL10 (IP-10). These mice developed higher burdens of virus in brain, as well as presented with more severe pathology and experienced enhanced morbidity and mortality when compared to inoculated wild-type mice (Klein, et.al., 2005). The authors suggest that this molecule may be neuroprotective in response to WNV infection in the CNS. Reports exist about the role of MCP-5 in the protection against WNV. Recent publications reported the up-regulation of other chemokines during West Nile encephalitis. The chemokines which have been reported to be up-regulated during a murine infection by WNV are CXCL10 (discussed above), CXCL9, MIP-2, CCL2, CCL3, CCL4, CCL5 and CCL7 (Glass et al., 2005; Klein et al., 2005). Expression of these chemokines were detected to be expressed on days 6 and 9 p.i. in this study. Differing from previous reports, we found earlier up-regulation (day 6 p.i.) of MCP5 (CCL12) and later up-regulation of C10 (MRP-1 or CCL6) on day 9 p.i. CCL6 is a chemokine that has been identified to be produced by microglial cells. It is though to be important in cell-to-cell communication and is induced by IFN-γ, while TNF-α has no effect on its regulation (Kanno et al., 2005). Asensio et al., described this molecule as a prominent chemokine expressed in CNS during demyelinating disease, inducing the recruitment of macrophages and CD4+ T cells into the brain parenchyma (Asensio et al., 1999).

In summary the conclusions from this study are: (1) Subneutralizing dilutions of WNV-immune horse serum and normal (non-immune) horse serum induce protection against WNV in C57BL/6 mice when the serum is injected three days prior to the inoculation with WNV; (2) The mechanism by which the horse serum induces protection correlates with up-regulation of IL-12 in the spleen in early stages of WNV infection; (3) WNV alone induces a very strong down-regulation of IFN-γ and iNOS in the earliest stages of infection; (4) IP-10 and MCP-5 are likely to be key molecules in triggering of the inflammatory events in CNS as a response to WNV infection; and (5) Up-regulation of C10 (CCL6) and MCP-5 (CCL12) was observed in a murine infection by WNV, suggesting that along with CXCL10 (IP10), CXCL9 (MIG), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1ß), CCL5 (RANTES), and CCL7 (MCP-3) these molecules induce a potent cellular Th-1 type immune response against WNV in the CNS.

CHAPTER V

GENERAL DISCUSSION

I. Discussion

West Nile virus (WNV) is an important pathogen which may cause severe disease and death of birds, humans and horses. Although a growing interest in this pathogen has emerged due to its recent introduction into North America, there are still many aspects of the pathogenesis and immune response that remain to be elucidated. In this study the kinetics of WNV replication in primary equine peripheral blood mononuclear cells, the ability of the immune horse serum to induce antibody-dependent enhancement in an *in vitro* infection of horse macrophages and in an *in vivo* murine infection, as well as the immune and pathologic events in a murine WNV infection were investigated.

In the first set of experiments, the ability of WNV to infect horse peripheral blood mononuclear cells was evaluated. The results indicated that productive replication of WNV was detected mainly in sorted monocytes, mixed populations and adherent cells from blood of all horses that were evaluated. Productive replication was also detected in CD4+ T cells, but only in a subset of horses evaluated. As discussed in Chapter II, the ability of WNV to infect and establish a productive replication in monocytes is greater than the ability of this virus to infect and replicate in CD4+ lymphocytes. Characterization of WNV replication in monocytes is potentially significant since this cell population, or cells derived from monocytes, may be early targets of WNV after

infection of a host by a natural route such as mosquito feeding. Furthermore those cells express Fc receptors, and therefore antibody dependent enhancement of infection may be important in the pathogenesis of WNV infections.

In the second set of experiments, the ability of the immune horse serum to induce ADE in an *in vitro* WNV infection model of horse monocyte-derived macrophages was evaluated. As in our previous studies, we found horse monocytes to be the major target cells in the peripheral blood. Our results indicated that immune horse serum was able to induce ADE of WNV infection *in vitro* when horse monocytes-derived macrophages are used. Interestingly the ADE phenomenon was observed when the host cells were infected with either the enzootic lineage of WNV (lineage I) or a genetically distinct lineage of WNV (lineage II). These observations indicate that both lineage I and lineage II of WNV cross-react significantly using serum from horses that were either naturally infected or vaccinated. Induction of ADE by immune horse serum in horse macrophages suggests the possibility of enhancement of WNV lineage I or lineage II *in vivo* in horses with low titers of antibodies against WNV lineage I.

In order to assess the ability of immune horse serum to induce ADE in an *in vivo* infection by WNV, our third set of experiments focused on evaluation of the effect of passive transfer of a sub-neutralizing dilution of immune horse serum into mice prior to inoculation with WNV. Our findings, reported in Chapter IV, were completely opposite to the expected results. Instead of ADE, which might be observed as a more severe clinical presentation, higher viral titers in tissues or higher mortality, we observed that

immune horse serum-transferred mice were protected against disease caused by WNV. This protection was observed as no mortality, low viral titers and no clinical signs. The serum was transferred in a sub-neutralizing dilution, and therefore the protective effect was judged not to be related to the presence of specific anti-WNV antibodies. Additionally, mice passively transferred with normal horse serum, diluted at the same dilution as the immune horse serum, were also protected. When other protective mechanisms independent of antibodies or complement were explored, we found that treatment of the mice with a 1:350 dilution of immune horse serum or normal horse serum induced a potent up-regulation of IL-12, which apparently prevented the down-regulation of iNOS and IFNγ observed in the spleen of the animals infected with WNV without any previous treatment with serum. In these animals a severe down-regulation of iNOS and IFN-γ were observed, as well as no up-regulation of IL-12. It is interesting that these results are in close correlation to the effects of LPS-activation of horse macrophages *in vitro*, which stimulates the expression of high levels of iNOS and type I IFN as described in Chapter II. In that experiment we observed that activation of horse macrophages by LPS abolished the replication of WNV. In the same experiment we observed that activation of NF-κB was delayed in naïve cells infected with WNV, and up-regulation of iNOS and type I IFN was also delayed in comparison to the LPS-activated cells which showed an immediate activation of NF-κB and an immediate up-regulation of iNOs and type I IFN. Although we did not evaluate expression of IL-12 the LPS-activated horse macrophages, it has been previously reported that activation of macrophages with LPS stimulates a high production of IL-12 and low levels of IL-10 (Gerber and Mosser, 2001), and therefore, we believe it is likely that this interleukin was

involved in the LPS-induced protective process against WNV infection. Although we did not evaluate the mechanism by which treatment with normal horse serum or immune horse serum induced expression of IL-12, we suggest that the mechanism is closely related to that which is involved in stimulation by LPS and which involves activation of transcription factors, including NF-κB. Based on these two observations we suggest that WNV induces a significant down regulation of iNOS and IFN-γ, which can be precluded by prior activation, in this case with either LPS *in vitro* or with heterologous serum *in vivo*. In addition, based on the *in vivo* observations, we also suggest that IL-12 plays an important role in protection against WNV infection, as well as a possible modulator of the immune response in the CNS, which may help to reduce the pathologic effects of this flavivirus in neural tissue.

Another observation in our final set of experiments (Chapter IV) was the kinetics of WNV infection in mice. We found that WNV was detected by plaque assay in blood from day 1 to 9 p.i., and by IHC in macrophages from skin from day 1 to 6 p.i. Similarly, the spleen was observed to be the first target organ for viral replication, showing peak of viral titers by day 4 p.i. All these observations were consistent with our findings in the first set of experiments in which monocytes were identified as sites of WNV replication in peripheral blood. Based on both results, we suggest that monocytes and cells derived from monocytes lineages (e.g. Langerhans cells) are major target host cell in early phases of *in vivo* infection by WNV. Infection of these cells was observed as detectable viral titers in blood, spleen and infected cells identified in skin during the first days of

infection in mice, and suggest that blood serves as a route for the virus to reach the CNS. In addition, skin may be a site of initial viral replication.

We identified WNV in cells from skin, and visceral tissues such as spleen and kidney prior to the detection of the virus in brain, in which WNV was first detected on day 6 p.i. On the same day p.i., the chemokines MCP-1 (CCL2), IP-10 (CXCL10), MIG (CXCL9), and MCP-5 (CCL12) were first detected in brain. By day 9 p.i., the following chemokines and cytokines were also detected: IFN-γ, TNF-α, IL-1α, TGF-ß, C-10 (CCL6), MIP-2 (CXCL1), MIP-1ß (CCL4), and RANTES (CCL5). All of these cytokines and chemokines are characteristic of a Th-1 profile. IFN-γ and TNF-α are widely recognized as major contributors to the pathogenesis of infectious diseases, including viruses. It has been demonstrated that TNF-α induced increased capillary permeability by stimulation of endothelial cells in brain blood capillaries (Diamond and Klein, 2004), contributing to recruitment of leukocytes to the CNS. In the same way IFN-γ has been reported to be important in the induction of major lymphocytic chemokines as IP-10 (CXCL10) and facilitating in the transit of lymphocytes into the brain parenchyma (Dufour et al., 2002). Although the importance of TNF-α and IFN-γ has been suggested in infections by WNV, based in our observations, we suggest that the early expression of the chemokines MCP-5 and IP-10 start the recruitment of leukocytes into the perivascular spaces in brain. In this location antigen presentation occurs and activation of monocytes and lymphocytes is established with the subsequent production of TNF-α and IFN-γ which lead to increased permeability of capillaries. After these events, entry of leukocytes into the neural tissue is facilitated, establishing inflammation and participating

in the pathogenesis of the disease or helping in the elimination of WNV from the CNS. As mentioned in Chapter IV, it is also possible that this inflammatory process may help in the movement of virus from blood stream into the CNS, increasing viral burdens and neuronal infection.

After the analysis of all results, we conclude that: (1) In the horse peripheral blood, monocytes are the main target host cells; (2) CD4+ lymphocytes are also a potential target in the peripheral blood, but the infection rate and replication in these cells by WNV is much more limited than infection in monocytes; (3) Sub-neutralizing dilutions of WNV-immune horse serum induce antibody-dependent enhancement in infections *in vitro* of horse monocytes-derived macrophages by WNV; (4) Sub-neutralizing dilutions of WNV-immune horse serum does not induce ADE in an *in vivo* infection of mice, but instead induced a full protection, in which IL-12 played a significant role; (5) WNV induces down-regulation of IFN-γ and iNOS which may be abolished by up-regulation of IL-12; (6) IP-10 and MCP-5 may be important molecules in triggering of inflammatory events in CNS as a response to WNV infection; and (7) Up-regulation of C10 (CCL6) and MCP-5 (CCL12) occurs in murine infection by WNV, suggesting that along with CXCL10 (IP10), CXCL9 (MIG), CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1ß), CCL5 (RANTES) and CCL7 (MCP-3) a potent cellular Th-1 type immune response is induced against the WNV in CNS.

II. Recommendations for future research:

1. Determine if ADE can be induced in an *in vivo* infection using sub-neutralizing dilution of homologous (murine) WNV-immune serum

2. Explore in more detail the role of IL-12 in protection against WNV in order to evaluate the potential use of this cytokine as therapeutic agent or adjuvant in vaccination against WNV.

3. Evaluate the role of MCP-5 (CCL12) in encephalitis caused by WNV by using genetically modified mice defective for this molecule or its receptor, CCR2.

4. Evaluate the role of skin as an initial site of WNV replication, and evaluate the potential use of this organ as diagnostic sample for equine infections by WNV.

REFERENCES

Agamanolis, D.P., Leslie, M.J., Caveny, E.A., Guarner, J., Shieh W.J.,and Zaki, S.R. (2003). Neuropthological findings in West Nile virus encephalitis. A case report. *Ann Neurol.* 54:547-551.

Agrawal, A.G., and Petersen, L.R. (2003). Human Immunoglobulin as a treatment for West Nile virus infection. *J. Infect. Dis*. 188:1-4.

Aihara, H., Takasaki, T., Matsutani, T., Suzuki, R., and Kurane, I. (1998). Establishment and characterization of Japanese encephalitis virus-specific, human CD4+ T-cell clones: Flavivirus cross-reactivity, protein recognition, and cytotoxic activity. *J. Virol*. 78:8032-8036.

Anderson C.F. and Mosser D.M. (2002). Cutting Edge: Biasing Immune Responses by Directing Antigen to Macrophage Fc_ Receptors. *J. Immunol.* 168: 3697–3701.

Anderson, J.F; Main, J.A.; Andreadis, T.G.; Wikel, S.K.; and Vossbrinck, C.R. (2003) Transstadial transfer of West Nile Virus by three species of ixodid ticks (Acari: Ixodidae). *J. Med. Entomol*. 40:528-533

Anderson, J.F., and Rahal, J.J. (2002). Efficacy of interferon alpha-2b and rivavirin against West Nile virus in vitro. *Emerg. Infect. Dis*. 8:107-108.

Anderson, R.; Wang, S.; Osiowy, C. and Issekutz, A.C. (1997). Activation of endothelial cells via antibody-enhanced dengue virus infection of peripheral blood monocytes. *J. Virol*. 71: 4226-4232.

Andrews, D.M., Matthews, V.B., Sammels, L.M., Carrello, A.C., and Minin, P.C. (1999). The severety of Murray Valley encephalitis in mice is linked to neutrophil infiltration and inducible Nitric Oxide synthase activity in the central nervous system. *J.Virol.* 73:8781-8790.

Anonymous. (1999). Centers for Disease Control. Update: West Nile virus encephalitis-New York, 1999. *Morb. Mort. Weekly Report* 48,944-946.

Arnold, S.J., Osvath, S.R., Hal, R.A., King, N.J.C., and Sedger, L.M. (2004). Regulation of antigen processing and presentation molecules in West Nile virus-infected human skin fibroblasts. *Virology* 324: 286-296.

Asensio, V.C., and Campbell, I.L. (1997) Chemokine gene expression in the brains of mice with lymphocytic choriomeningitis. *J. Virol*. 71:7832-7840.

Asensio, V.C., Lassmann, S., Pagenstecher, A., Steffensen, S.C., Henriksen, S.J., and Campbell, I.L. (1999). C10 is a novel chemokine expressed in experimental inflammatory demyelinating disorders that promotes recruitment of macrophages to the central nervous system. *Am. J. Pathol*. 154(4): 1181-1191).

Atrasheuskaya, A.V., Fredeking, T.M., and Ignatyev, G.M. (2003) Changes in immune parameters and their correction in human cases of tick-borne encephalitis.*Clin Exp Immunol.* 131(1):148-54

Bakonyi, T., Hubalek, Z., Rudolf, I., and Nowotny, N. (2005). Novel Flavivirus or new lineage of West Nile virus, Central Europe. *Emerg. Infect. Dis*. 11(2): 225-231.

Barth, O.M., (1999). Ultrastructural aspects of the dengue virus (flavivirus) particle morphogenesis. *J. Submicrosc. Cytol. Pathol.* 31:404-412

Beasley, D.W., (2004). Genome sequence and attenuating mutations in West Nile virus isolated from Mexico. *Emerging Infect. Dis*. 10: 2221-2224.

Beasley, D.W.C, Davis, C.T., Guzman, H., Vanlandingham, D.L., Travassos da Rosa, A.P.A., Parsons, R.E., Higgs, S., Tesh, R.B., and Barrett, A.D.T., (2003). Limited evolution of West Nile virus has occurred during its southwesterly spread in the United States. *Virology*. 309, 190-195.

Belperio, J.A., Dy, M., Burdick, M.D., Xue, Y.Y., Li, K., Elias, J.A., and Keane, M.P. (2002). Interaction of IL-23 and C10 in the pathogenesis of Bleomycin-induced pulmonary fibrosis. *Cell. Mol. Biol.* 27: 419-427.

Ben-Nathan, D., Huitinga, I., Lustimg, S., van Rooijen, N., and Kobiler, D. (1996). West Nile virus neuroinvasion and encephlitis induced by macrophage depletion in mice. Arch. *Virology*. 141:459-469.

Ben-Nathan, D., Lusting, S., Tam, G., Robinzon, S., Segal, S., and Rager-Zisman, B. (2003). Prophylactic and therapeutic efficacy of human intravenous immunoglobulin in treating West Nile virus infection in mice. *J. Infect. Dis*. 188-5-12.

Berthet. F.X., Zeller, H.G., Drouet, M.T., Rauzier, J., Digoutte, JP., and Deubel, V. (1997). Extensive nucleotide changes and deletions within the envelope glycoprotein gene of Euro-African West Nile viruses. *J Gen Virol.* 78 (9):2293-2297.

Biggerstaff, B.J., and Petersen, L.R. (2003) Estimated risk of transmission of West Nile virus through blood transfusion in the US, 2002. *Transfussion*. 43: 1007-1017

Binder, G.K., and Griffin, D.E. (2001). Interferon-γ-mediated site-specific clearance of alphavirus from CNS neurons. *Science*. 293:303-306.

Bonnevie-Nielsen, V.; Heron, I.; Monath, T.P.; and Calisher, C.H. (1995). . Lymphocytic 2',5'-Oligoadenylate Synthetase Activity Increases prior to the Appearance of Neutralizing Antibodies and Immunoglobulin M and Immunoglobulin G Antibodies after Primary and Secondary Immunization with Yellow Fever Vaccine. Clin. Diag. Lab. Immunol. 2(3):302-306.

Bosch, I., Xhaja, K., Estevez, L., Raines, G., Melichar, H., Warke, R.V., Fournier, M.V., Ennis, F.A., and Rothman, A. (2002). Increased production of interleukin-8 in primary human monocytes and in human epithelial and endothelial cell lines after dengue virus challenge. *J. Virol*. 76:5588-5597.

Brault, A.C., Langevin, S.A., Bowen, R.A., Panella, N.A., Biggerstaff, B.J., Miler, B.R., and Komar, N. (2004). Differential virulence of West Nile strains for American crows. *Emerg. Infect. Dis.* 10(12): 2161-2168.

Broberg, E.K., Setala, N., Eralinna, P.J., Salmi, A.A., Roytta, M., and Hukkanen, V. (2002). Herpes simplex virus type I infection induces up-regulation of interlekin-23 (p19) mRNA expression in trigeminal ganglia of BALB/c mice. *J. Interferon Cytok. Research*. 22:641-651.

Brooks, T.J., and Phillpotts, R.J. (1999). Interpheron-alpha protects mice against lethal infection with St Louis encephalitis virus delivered by the aerosol and subcutaneous routes. *Antiviral Res.* 41:57-64.

Buckweitz, S., Kleiboeker, S., Marioni, K., Ramos-Vara, J., Rottinghaus, A., Schwabenton, B., and Jonson, G. (2003). Serological, reverse transcriptase-polymerase chain reaction, and immunohistochemical detection of West Nile virus in a clinically affected dog. *J Vet Diagn Invest.* 15(4):324-329.

Bunning, M.L., Bowen, R.A., Cropp, B.C., Sullivan, K.G., Davd, B.S., Komar, N., Godsey, M.S., Baker, D., Hettler, D.L., Holmes, D.A., Biggerstaff B.J., and Mitchell, C. J. (2002). Experimental infection of Horses with West Nile virus. *Emerging Infect. Dis.* 8(4):380-386.

Burleson F.G., Chambers, T.M., Wiedbrauk, D.L., (1992). Virology: A Laboratory Manual. pp. 74-84. Academic Press, San Diego, CA.

Burt, F. J., Grobbelaar, A. A., Leman, P. A., Anthony, F. S., Gibson, G. V.F., and Swanepoel. R. (2002). Phylogenetic Relationships of Southern African West Nile Virus Isolates. *Emerg. Infect. Dis*. 8 (8):820-826.

Byrne, S.N., Halliday, G.M., Johnston, L.J., and King, N.J.C. (2001). Interleukin-1ß but not tumor necrosis factor is involved in West Nile virus-induced Langerhans cell migration from the skin in C57BL/6 mice. *J. Invest. Dermatol*. 117:702-709.

Caamano, J., and Hunter, C.A., (2002). NF-kappaB family of transcription factors: central regulators of innate and adaptive immune functions. *Clin. Microbiol. Rev.* 15**,** 414-429.

Cantile, C., Del Piero, F., Di Guardo, G., Arispici M. (2001). Pathologic and immunohistochemical findings in naturally occuring West Nile virus infection in horses. *Vet. Pathol*. 38(4):414-21.

Cardosa, M.J., Porterfield, J.S., and Gordon, S. (1983). Complement receptor mediates enhanced flavivirus replication in macrophages. *J.Exp. Med*. 158:258-263.

Castillo-Olivares, J, and Wood, J. (2004). West Nile virus infection of horses. *Vet. Res*. 35: 467-483.

Chambers, T.J., and Diamond M.S. (2003). Pathogenesis of flavivirus encephalitis. In: Advances in virus research. The flaviviruses: Pathogenesis and immunity. Vol 60:273-342. Elsevier Academic Press 2003.

Charlier, N.; Leyssen, P.; De Clercq, E.; Neyts, J. (2004). Rodent models for the study of therapy against flavivirus infectins. *Antiviral Research* 63: 67-77.

Charlier, N., Leyssen, P., Paeshuyse, J., Drosten, C, Schmitz, H., VanLommel, A., De Clerq, E., and Neyts, J. (2002). Nfection of SCID mice with Montana Myositis leukoencephalitis virus as a model for flavivirus encephalitis. *J. Gen. Virol*. 83:1887-1896.

Charrel, R.N., Brault. A.C., Gallian, P., Lemasson, J.J., Murgue, B., Murri. S., Pastorino, B., Zeller, H., Chesse, R.de., Micco, P.de., and Lamballerie, X.de. (2003). Eolutionary relationship between Old World West Nile virus strains evidence for viral gene flow between Africa, the Middle East, and Europe. *Virology*. 315:381-388.

Chaturvedi, U.C., Elbishbishi, E.A., Agawal, R., Raghuapathy, R., Nagar, R., Tandon, R., Pacsa, A.S., Younis, O.I., and Azizieh, F., (1999). Sequential production of cytokines by dengue virus-infected human peripheral blood leukocyte cultures. *J. Med. Virol*. 59, 335- 340.

Cheeran, M.C., Hu, S., Sheng, W.S., Rashid, A., Peterson, P.K., and Lokensgard, J.R. (2005). Differential responses of human brain cells to West Nile virus infection. *J. Neurovirol*. 11(6):512-524.

Cheng, Y., King, N.J.C., and Kesson, A.M. (2004a). Major histocompatibility complex class I (MHC-I) induction by West Nile virus: Involvement of 2 signaling pathways in MHC-I up-regulation. *J. Infect. Dis*. 189:658-668

Cheng, Y., King, N.J.C., and Kesson, A.M. (2004b) The role of tumor necrosis factor in modulating responses of murine embryo fibroblasts by flavivirus, West Nile.*Virology*. 329(2):361-70.

Chen, Y.C., Wang, S.Y., and King, C.C., (1999). Bacterial lipopolysaccharide inhibits dengue virus infection of primary human monocytes/macrophages by blockade of virus entry via a CD14-dependent mechanism. *J. Virol*. 73, 2650-2657.

Chen, Y.C., and Wang, S.Y. (2002). Activation of terminally differentiated human monocytes/macrophages by dengue virus: Productive infection, hierarchical production of innate cytokines and chemokines, and the synergistic effect of lipopolysaccharide. *J. Virol*. 76:9877-9887.

Chu J.J.; and Ng, M.L. (2003). The mechanism of cell death during West Nile virus infection is dependent on initial infectious dose. *J. Gen. Virol*. 80: 3305-3314.

Chu J.J.; and Ng, M.L. (2004). Interaction of west Nile virus with $\alpha_V ß_3$ integrin mediates virus entry into cells. *J.Biol. Chem*. 279 (52): 54533-54541

Chu, J.J., Rajamanonmani, R., Li, J., Bhuvanakantham, R., Lescar, J., and Ng, M.L. (2005). Inhibition of West Nile virus entry by using a recombinant domain III from the envelope glycoprotein. *J Gen Virol*. 86(Pt 2):405-12

Cologna, R., and Rico-Hesse, R., (2003). American genotype structures decrease dengue virus output from human monocytes and dendritic cells. *J. Virol*. 77, 3929-3938.

Control Diseases Center. 2005. (2005). West Nile Virus Activity in the United States (Reported to CDC as of October 4, 2005). http://www.cdc.gov/ncidod/dvbid/westnile/surv&controlCaseCount05_detailed.htm

Cook, D.N., Pisetsky, D.S., and Schwartz D.A. (2004). Toll-like receptors in the pathogenesis of human disease. *Nat. Immunol*. 5(10):975-979.

Corapi, W.V., Darteil, R.J., Audonnet, J.C., and Chappuis, G.E. (1995). Localization of antigenic sites of the S glycoprotein of feline infectious peritonitis virus involved in neutralization and antibody-dependent enhancement.*J Virol*. 69(5):2858-2862.

Cummings, D.A.T., Schwartz, I.B., Billings, L., Shaw, L.B., and Burke, D.S. (2005). Dynamic effects f antibody-dependent enhancement on the fitness of viruses. *Proc. Natl. Acad. Sci*. 102(42):15259-15264.

Davis, C.T., Beasley, D.W.C., Guzman, H., Raj, P., Anton, M.D., Novak, R.J., Unnasch, T.R., Tesh, R.B., and Barrett, A.D.T. (2003). Genetic variation among temporally and geographically distinct West Nile virus isolates, United States, 2001 and 2002. *Emerg. Infect. Dis*. 9:1423-1429.

Davis, C.T., Ebel, G.D., Lanciotti, R.S., Brault, A.C., Guzman, H., Siirin, M., Lambert, A., Parsons, R.E., Beasley, D.W.C., Novak, R.J., Elizondo-Quiroga, D., Green. E.N., Young, D.S., Stark, L.M., Drebot, M.A., Artsob, H., Tesh, R.B., Kramer, L.D., and Barrett, A.D.T. (2005). Phylogenetic analysis of North American West Nile virus, isolates 2001-2004: evidence for the emergence of a dominant genotype. *Virology* 342:252-265.

Davis, D., Trischmann, H., Stephens, D.M., and Lachmann, P.J. (2001). Antibodies raised to short synthetic peptides with sequences derived from HIV-1 SF2 gp120 can both neutralize and enhance HIV-1 SF13: a later variant isolated from the same host.*J Med Virol*. 64(3):207-216

Despres, P., Frenkiel, M.P., Ceccaldi, P.E., Duarte dos Santos, C., and Deubel, V. (1998) Apoptosis in the mouse central nervous systemin response to infection with mouse-neurovilunt dengu virus. *J. Virol.* 72 (1): 823-829. .

Deubel, V., Fiette, L., Gounon, P., Drouet, MT., Khun, H., Huerre, M., Banet, C., Malkinson, M., Despres, P. (2001). Variations in biological features of West Nile viruses. *Ann N Y Acad Sci*. 951:195-206

Diamond, M.S., Edgil, D., Roberts, T.G., Lu, B., and Harris, E., (2000). Infection of human cells by dengue virus is modulated by different cell types and viral strains. *J. Virol.* 74, 7814-7823.

Diamond, M.S., and Klein, R.S. (2004). West Nile virus: crossing the blood-brain barrier. *Nat. Med.* 10:1294-1295.

Diamond, M.S., Shrestha, B., Marri, A., Mahan, D., and Engle, M. (2003a). B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. *J. Virol*. 77, 2578-2586.

Diamond, M.S., Shrestha, B., Mehlhop, E., Sitati, E., and Engle, M. (2003b). Innate and adaptive immune responses determine protection against disseminated infection by West Nile encephalitis virus. *Viral Immunol*. 16, 259-278.

Diamond, M.S., Sitati, E.M., Friend, L.D., Higgs, S., Shrestha, B., and Engle, M. (2003c). A critical role for induced IgM in the protection against West Nile virus infection. *J. Exp. Med.* 198:1853-1862.

Dufour, J.H., Dziejman, M., Liu, M.T., Leung, J.H., Lane, T.E., and Luster, A.D. (2002). IFN-γ-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *J. Immunol.* 168:3195-3204.

Dupuis AP II, Marra PP, and Kramer LD. (2003). Serologic evidence of West Nile virus transmission, Jamaica, West Indias. *Emerg. Infect. Dis*. 9(7):860-863.

Duron, S.I., Dashe, J.F., Adelman, L.S., Brown, W.F., Werner, B.G., Hadley, S. (2003). Histopathologically proven poliomyelitis with quadriplegia and loss of brainstem function due to West Nile virus infection. *Clin. Infect. Dis*. 37:e74-e77.

Engle, M.J., and Diamond, M.S. (2003). Antibody prophylaxis and therapy against West Nile virus infection in wild-type and immunodeficient mice. *J. Virol*. 77:12941-12949.

Ferguson, N., Anderson, R., and Gupta, S. (1999). The effect of antibody-dependnt enhancement on the transmission dynamics and persistence of multiple-strain pathogens. *Proc. Natl. Acad. Sci*. 96:790-794.

Fernandez-Salas, I., Contreras-Cordero, J.F., Blitvich, B.J., Gonzalez-Rojas, J.I., Cavazos-Alvarez, A., Marlene, N.L., Elizondo-Quiroga, A., Lorono-Pino, M.A., Gubler, D.J., Cropp, B.C., Calisher, C.H., and Beaty, B.J. (2003). Serologic evidence of West Nile virus infection in birds, Tamaulipas State, Mexico. *Vector-Borne and Zoonotic dis.* 3(4): 209-213.

Fredericksen, B.L., Smith, M., Katze, M.G., Shi, P.Y., and Gale, M. (2004). The host response to West Nile virus infection limits viral spread through the activation of the interferon regulatory factor 3 pathway. *J.Virol.* 78(14):7737-7747.

Gerber J. S., and Mosser D.M. (2001). Reversing Lipopolysaccharide Toxicity by Ligating the Macrophage Fcγ Receptors. *J.f Immunol.* 166: 6861–6868.).

Gimenez, H.B., Chisholm, S., Dorman, J., and Cash, P. (1996). Neutralizing and enhancing activities of human respiratory syncytial virus-specific antibodies. Clin Diagn Lab Immunol. 1996 May;3(3):280-286.

Girn, J., Kavoosi, M., and Chantler, J. (2002). Enhancement of coxsackievirus B3 infection by antibody to a different coxsackievirus strain. *J. Gen Virol.* 83(2):351-358.

Glass, W.G., Lim, J.K., Cholera, R., Pletnev, A.G., Gao, J.L., and Murphy, P.M. (2005). Chemokine recptor CCR5 promotes leukocyte trafficking to the brain and survival in West Nile virus infection. *J. Exp. Med.* 202(8):1087-1098.

Gould L.H and Fikrig, E. (2004) West Nile virus: a growing concern? *J. Clin. Invest*. 113(8):1102-1106

Griffin, D.E. (2003). Immune reponses to RNA-virus infection of the CNS. *Nature Rev. Immunol*. 3:493-502.

Guarner, J., Shieh, WJ, Hunter, S., Paddock, C.D., Morken, T., Campbell, G.L, Marfin, A.A., and Zaki, S.R. (2004). Clinicopathologic study and laboratory diagnosis of 23 cases with West Nile virus encephalomyielitis. *Hum. Pathol*. 35:983-990.

Guillon, C., Schutten, M., Boers, P.H., Gruters, R.A., and Osterhaus, A.D. (2002). Antibody-mediated enhancement of human immunodeficiency virus type 1 infectivity is determined by the structure of gp120 and depends on modulation of the gp120-CCR5 interaction.*J Virol.* 76(6):2827-2834

Guo, J.T., Hayashi, Y., and Seeger, C. (2005). West Nile virus inhibits the signal transduction pathway of Alpha interferon. *J. Virol.* 79 (3): 1343-1350.

Guy, B., Chanthavanich, P., Gimenez, S., Sirivichayakul, C., Sabchareon, A., Begue, S., Yoksan, S., Luxemburger, C., and Lang, J. (2004). Evaluation by flow cytometry of antibody-dependent enhancement (ADE) of dengue infection by sera from Thai

children immunized with a live-attenuated tetravalent dengue vaccine. *Vaccine*. 22:3563-3574.

Halstead, SB. (1988). Pathogenesis of Dengue: Challenges to molecular biology. *Science*. 239:476-481

Hase, T., Summers P.L., and Eckles K.H., (1989). Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. *Arch. Virol*. 104, 129-143.

Harrington, T., Kuehnert, M., Kamel, H., Lanciotti, R.S., Hand, S., Currier, M., Chamberland, M.E., Petersen L.R., and Marfin, A. (2003). West Nile virus infection transmitted by blood ransfussion. *Transfussion*. 43: 1018-1022

Hayes, E.B., Komar, N., Nasci, R.S., Montgomery, S.P., O'Leary, D.R., and Campbell, G.L. (2005). Epidemiology and transmission dynamics of West Nile virus disease. *Emerg. Infect. Dis*. 11 (8): 1167-1173

Hayes, E.B., Sejvar, J.J., Zaki, S.R., Lanciotti, R.S., Bode, A.V., and Campbell, G.L. (2005). Virology, patology, and clinical manifestations of West Nile virus disease. *Emerg. Infect. Dis*. 11 (8): 1174-1179

Higgs, S, Schneider B.S., Vanlandingham, D.L., Klingler, K.A., and Gould, E.A. (2005). Nonviremic transmission of West Nile virus. *Proc Natl Acad Sci.* 102 (25): 8871-8874

Ho, L.J., Wang, J.J., Shaio, M.F., Kao, C.L., Chang, D.M., Han, S.W., and Lai, J.H., (2001). Infection of human dendritic cells by dengue virus causes cell maturation and cytokine production. Infection of human dendritic cells by dengue virus causes cell maturation and cytokine production. *J. Immunol*. 166, 1499-1506.

Hober, D., Chehadeh, W., Bouzidi, A., and Wattre, P. (2001). Antibody-dependent enhancement of coxsackievirus B4 infectivity of human peripheral blood mononuclear cells results in increased interferon-alpha synthesis *J Infect Dis*. 184(9):1098-1108. Epub 2001 Oct 12.

Hohdatsu, T., Yamada, M., Tominaga, R., Makino, K., Kida, K., and Koyama, H. (1998). Antibody-dependent enhancement of feline infectious peritonitis virus infection in

feline alveolar macrophages and human monocyte cell line U937 by serum of cats experimentally or naturally infected with feline coronavirus.*J Vet Med Sci*. 60(1):49-55.

Hollinger, F.B., and Kleinman, S. (2003). Transfussion transmission of West Nile virus: a emerging of historical and contemporary perspectives. *Transfussion*. 43:992-997

Hotta, H., Wiharta, A.S., Hotta, S., and Homma, M., (1984). Dengue type 2 virus infection in human peripheral blood monocyte cultures. *Microbiol. Immunol*. 28, 1099-1109.

Huang, C.; Slatter, B.; Rudd, R.; PArchuri, N.; Hull, R.; Dupis, M.; and Hindenburg, A. (2002). First Isolation of West Nile virus from a patient with encephalitis in the United States. *Emerg. Infect. Dis*. 18 (12): 1367-1371.

Iwamoto, M., Jernigan, D.B., Guasch, A., Trepka, M.J., Blackmore, C.G., Hellinger, W.C., Pham, S.M., Zaki, S., Lanciotti, R.S., Lance-Parker, S.E., Diaz-Granados, C.A., Winquist,A.G., Perlino, C.A., Wiersma, S., Hillyer, K.L., Goodman, JL., Marfin, A.A., Chamberland, M.E., and Petersen, L.R. (2003). Transmission of West Nile virus from an organ donor to four transplant recipients. *N. Engl. J. Med.* 348:2196-2203).

Iwasaki, A., and Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune response. *Nat. Immunol.* 5(10):987-995.

Jerzak, G., Bernard, K.A., Kramer, L.D., and Ebel, G.D. Genetic variation in West Nile virus from naturally infected mosquitosand birds suggests quasispecies structure and strong purifying selection. *J.Gen. Virol*. 86:2175-2183.

Johnson, D.J., Ostlund, E.N., Pedersen, D.D., and Schmitt, B.J., (2001). Detection of North American West Nile virus in animal tissue by a reverse transcription-nested polymerase chain reaction assay. *Emerg. Infect. Dis*. 7, 739-741.

Johnston, J., Halliday, G.M., and King, N.J.C., (2000). Langerhans cells migrate to local lymph nodes following cutaneous infection with an arbovirus. *J. Invest. Dermatol*. 114, 560-568.

Joseph, T., Kibenge, M.T., Kibenge, F.S. (2003). Antibody-mediated growth of infectious salmon anaemia virus in macrophage-like fish cell lines. *J Gen Virol*. 84(7):1701-1710.

Kalil, A.C., Devetten, M.P., Singh, S., Lesiak, B., Poage, D.P., Bargenquast, K., Fayad, P., and Freifeld A.G. (2005). Use of Interferon-α in patients with West Nile encephalitis report of 2 cases. *Clin. Infect. Dis*. 40: 764-766.

Kanno, M, Suzuki, S, Fujiwara, T, Yokoyama, A, Skamoto, A., Takahashi, H, Imai, Y., Takanaka, J. (2005). Functional expression of CCL6 by rat microglia: a possible role of CCL6 in cell-cell communication. *J. Neuroimmunol.* 167 (1-2): 72-80.

Kawaguchi, I., Sasaki, A., and Boots, M. (2003). Why are dengue virus serotypes so distantly related? Enhancement and limiting serotype similarity between dengue virus strains. *Proc. R. Soc. Lond. B*. 270:2241-2247.

Kelley, T.W., Prayson, R.A., Ruiz, A.I., Isada, C.M., and Gordon, S.M. (2003). The neuropathology of West Nile virus meningoencephalitis and report of two cases and review of literature. *Am. J. Clin. Pathol*. 119:749-753.

Kesson, A.M., Blanden, R.V., and Mullbacher, A., (1987). The primary in vivo murine cytotoxic T cell response to the flavivirus, West Nile. *J. Gen. Virol*. 68, 2001-2006.

Kesson, A.M., and King, N.J.C. (2001). Transcriptional regulation of major histocompatibility complex class I by flavivirus West Nile is dependent on NFκB activation. *J. Infect. Dis.* 184:947-954.

Kimura-Kuroda, J., and Yasui, K. (1988). Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. *J. Immunol*. 3606-3610.

King, A.D., Nisalak, A., Kayalanrooj, S., Myint, K.S., Pattanapanyasat, K., Nimmannitya, S., and Innis, B.L., (1999). B cells are the principal circulating mononuclear cells infected by dengue virus. S. *Asian J. Trop. Med. Public Health*. 30, 718-728.

King, C.A., Marshall, J.S., Alshurafa, H., and Anderson, R. (2000). Release of vasoactive cytokines by antibody-enhanced dengue virus infection of a human mast cell/basophil line. *J Virol*. 74(15):7146-7150.

King, N.J.C., Shrestha, B., and Kesson, A.M. (2003). Immune modulation by Flaviviruses in: Advances in Virus Research. Vol 60 pp 121-155.*Academic Press.*

Klein R.S., Lin, E., Zhang, B., Luster, A.D., Tollet, J., Samuel, M.A., Engle, M., and Diamond, M.S. (2005). Neuronal CXCL10 directs CD8+ T-cell recruitment and control of West Nile virus encephalitis. *J. Virol*. 79:11457-11466.

Kleiboeker, S.B., (2003). Applications of Competitor RNA in Diagnostic RT-PCR. *J. Clin. Microbiol*. 41, 2055-2061.

Kleiboeker, S.B., Lioacono, C.M., Rottinghaus, A., Pue, H., and Johnson, G.C., (2004). Diagnosis of West Nile Virus Infection in Horses. *J. Vet. Diagn. Invest*. 16, 2-10.

Koh, W.L., and Ng, M.L. (2005). Molecular mechanism of West Nile virus pathogenesis in brain cells. *Emerg. Infect. Dis*. 11(4):629-632.

Komar, N. (2003a). West Nile Virus: epidemiology and ecology in North America. In Advances in Virus Research. 59: 185-234. Elsevier Academic Press 2003

Komar, N., Langevin, S., Hinten, S., Nemeth, N., Edwards, E., Hettler, D., Davis, B., Bowen, R., and Bunning, M. (2003b). Experimental infection of North American birds with the New York 1999 strain of West Nile virus. *Emerg Infect Dis*. 9(3):311-22.

Komar, O., Robbins, M.B., Klenk, K., Blitvich, B.J., Marlenee, N.L., Burkhalter, K.L., Gubler, D.J., Gonzalvez, G., Pena, C.J., Peterson, A.T., and Komar, N. (2003c). West Nile virus transmission in resident birds, Dominican Republic. *Emerg. Infect. Dis*. 9 (10): 1299-1302.

Koschinski, A.; Wengler, G.; Wengler, G.; and Repp H. (2003). The membrane proteins of flaviiruses form ion-permeable pores in the target membrane after fusion: idenfication of the pores and analysis of their possible role in virus infection. *J. Gen. Virol*. 84: 1711-1721

Kramer, L.D., and Bernard, K.A.. (2001) West Nile virus infection in birds and mammals. *Ann*. *New York Acad. Sci*. 951, 84-93.

Kreil, T.R., Berting, A., Kistner, O., and Kindermann, J. (2003). West Nile virus and the safety of plasma derivates: verification of high safety margins, and the validity of predictions based on model virus data. *Transfussion*. 43: 1023-1028

Kreil, T.R., and Ebil, M.M. (1995). Viral infection of macrophages profoundly alters requirements for induction of nitric oxide synthesis. *Virology* 212: 174-178.

Kreil, T.R., and Ebil, M.M. (1996). Nitric oxide and viral infection: NO antiviral activity against a flavivirus in vitro, and evidence for contribution for pathogenesis in experimental infection in vivo. *Virology*. 219:304-306.

Kreil, T.R., and Ebil, M.M. (1997). Pre- and postexposure protection by passive immunoglobulin but no enhancement of infection with a flavivirus in a mouse model. *J.Virol*. 71:2921-2927.

Kulkarni, A.B., Mullbacher, A., and Blanden, R.V., (1991). In vitro T-cell proliferative response to the flavivirus, West Nile. *Viral. Immunol*. 4, 73-82.

Kumar, P., Krishna, V.D., Sulochana, P., Nirmala, G., Haridattatreya, M., and Satchinandam, V. (2004). Cell mediated immune responses in healthy children with a history of subclinocal infection with Japanese encephalitis virus: análisis of CD4+ and CD8+ T cell target specificities by intracellular delivery of viral proteins using the human immunodeficiency virus Tat protein transduction domain. *J.Gen. Virol*. 85:471-482.

Kurane, I., and Ennis, F.A., (1987). Induction of interferon alpha from human lymphocytes by autologous, dengue virus-infected monocytes. *J. Exp. Med*. 166, 999-1010.

Kurane, I, and Ennis, F.A., (1988). Production of Interferon Alpha by dengue virus-infected human monocytes. *J. Gen. Virol*. 69, 445-449.

Lanciotti, R.S., Kerst, A.J., Nasci, R.S., Godsey, M.S., Mitchell, C.J., Savage, H.M., Komar, N., Panella, N.A., Allen, B.C., Volpe, K.E., Davis, B.S., and Roehrig,

J.T., (2000). Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan Reverse Transcriptase-PCR assay. *J. Clin. Microbiol*. 38, 4066–4071.

Lanciotti, R.S., Roehrig, J.T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K.E., Crabtree, M.B., Scherret, J.H., Hall, R.A., MacKenzie, J.S., Cropp, C.B., Panigrahy, B., Ostlund, E., Schmitt, B., Malkinson, M., Banet, C., Weissman, J., Komar, N., Savage, H.M., Stone, W., McNamara, T., Gubler, D.J., (1999). Origin of the West Nile virus responsible for an outbreak of encephalitis in the Northeaster United States. *Science* 286, 2333–2337.

Langrish, C.L., McKenzie, B.S., Wilson, N.J., Malefyt, R.W., Kastelein, R.A., and Cua, D.J. (2004). IL-12 and IL-23: master regulators of innate andaptive immunity. *Immunological Rev.* 202:96-105.

Lee, J.W., Chu, J.J., and Ng, M.L. (2005). Quantifying the specific binding between West Nile virus envelope domain III protein and the cellular receptor alpha Vbeta 3 integrin. *J Biol Chem.* Epub ahead of print.

Levine, B., Griffin, D.E., (1993). Molecular analysis of neurovirulent strains of Sindbis virus that evolve during persistent infection of scid mice. *J.Virol.* 67:6872-6875.

Li, Q., and Verma, I.M., (2002). NF-kappaB regulation in the immune system. *Nat. Rev. Immunol.* 2**,** 725-734.

Liao, C.L., Lin, Y.L., Wang, J.J., Huang, Y.L., Yeh, C.T., Ma, S.H., and Che, L.K. (1997). Effect of enforced expression of human *bcl-2* on Japanese encephalitis virus-induced apoptosis in cultured cells. *J. Virol*. 71 (8): 5963-5971.

Libraty, D.H., Pichyangkul, S., Ajariyakhajorn, C., Endy, T.P., Ennis, F.A., (2001). Human Dendritic cells are activated by dengue virus infection: Enhancement by gamma interferon and implications for disease pathogenesis. *J. Virol*. 75, 3501–3508.

Lidbury, B.A. and Mahalingam, S. (2000). Specific Ablation of Antiviral Gene Expression in Macrophages by Antibody-Dependent Enhancement of Ross River Virus Infection *J. Virol*. 74(18):8376-81.

Lin, R.J., Liao, C.L., Lin. E., Lin, Y.L. (2004). Blocking of the alpha interferon-induced Jak-Stat signaling pathway by Japanese encephalitis virus infection. *J. Virol*. 78 (17): 9285-9294.

Lin, Y.L., Huang Y.L., Ma, S.H., Yeh C.T., Chiou, S.Y. Chen L.K., and Liao, C.L. (1997). Inhibition of Japanese encephalitis virus infection by nitric oxide: Antiviral effect of nitric oxide on RNA virus replication. *J. Virol*. 71, 5227–5235.

Lindenbach, B.D., and Rice, C.M., (2003). Molecular Biology of Flaviviruses. In: Thomas Chambers and Thomas Monath, (Eds.). Advances in virus research.. Vol. 59 pp. 23–61. Elsevier Academic Press 2003

Liu, T., and Chambers, T. (2001). Yellow fever virus encephalitis: properties of the brain associated T-cell response during virus clearance in normal and gamma interferon-deficient mice and requirement for CD4+ lymphocytes. *J. Virol*. 75:2107-2118.

Liu, W.J., Chen, H.B., Wang, X.J., Huang, H., Khromykh, A.A. (2004). Analysis of adaptive mutations in Kunjin virus replicon RNA reveals a novel role for the Flavivirus nonstructural protein NS2A in inhibition of beta Interferon promoter driven transcription. *J. Virol*. 28 (22): 12225-12235.

Lobigs,M., Müllbacher, A., Wang, Y., Pavy, M., and Lee, E. (2003). Role of type I and type II interferon responses in recovery from infection with an encephalitic flavivirus. *J.Gen. Virol*. 85:567-572.

Loke, H., Bethell, D.B., Phuong, C.X.T., Dung, M., Schneider, J., White, N.J., Day, N.P., Ferrar, J., and Hill, A.V.S. (2001). Strong HLA class I--restricted T cell responses in dengue hemorrhagic fever: a double-edged sword?. *J. Infect. Dis*. 184:1369-1373.

Lorono-Pino, M.A., Blitvich, B.J., Farfan-Ale, J.A., Puerto, F.I., Blanco, J.M., Marlenee, N.L., Rosado-Paredes, E.P., Garcia-Rejon, J.E., Gubler, D.J., Calisher, C.H., and Beaty, B.J. (2003). Serologic evidence of West Nile virus infection in horses, Yucatán State, Mexico. *Emerg. Infect. Dis*. 9 (7): 857-859.

Lucas, M., Mashimo, T., Frenkiel, M.P., Simon-Chzottes, D, Montagutelli, X., Cecaldi, P.E., Guenet, J.L., and Despres, P. (2003). Infection of mouse neurons by West

Nile virus is modulated by the interferon-inducible 2'-5' oligoadenylate synthetase 1b protein. *Immunol. Cell. Biol*. 81:230-236.

Mackenzie, J.S., Gubler, D.J., and Petersen, L.R. (2004). Emerging Flaviviruses: the spread and resurgence of Japanese Encephalitis, West Nile and dengue viruses. *Nat. Med*. 10(12):S98-S109

Mahalingham, S., and Lidbury, B.A., (2002). Suppression of lipopolysaccharide-induced antiviral transcription factor (STAT-1 and NFκB) complexes by antibody-dependent enhancement of macrophage infection by Ross River virus. *Proc. Natl Acad. Sci.* 99, 13819–13824.

Mangada, M.M., Endy, T.P., Nisalak, A., Chunsuttiwat, S., Vaughn, D.W., Libraty, D.H., Green, S., Ennis, F.A., and Rothman A., (2002). Dengue specific T cell responses in peripheral blood mononuclear cells obtained prior to secondary dengue virus infections in Thai school children. *J. Infect. Dis*. 185, 1697–1703.

Marfin, A.A., and Gubler, D.J. (2001). West Nile encephalitis: An emerging disease in the United States. *Clinical Infect. Dis*. 33:1713-1719.

Markoff, L. (2003). 5'-and 3'- Noncoding regions in Flavivirus RNA. In: Advances Virus Research Vol 59: pp208-209. Elsevier Acad. Press. 2003.

Mashimo, T., Lucas, M., Simion-Chazottes, D., Frenkiel, M.P., Montagutelli, X., Ceccaldi, P.E., Deubel, V., Guenet, J.L., and Despres, P. (2002). A onsense mutation in the gene encoding 2'-5'oligoanelyte synthetase/L1 isoform is associated with West Nile virus susceptibility in laboratory mice. *Proc. Natl. Acad. Sci.* 99:11311-11316.

Mason, P.W., Rieder, E., and Baxt, B. (1994). RGD sequence of foot-and-mouth disease virus is essential for infecting cells via the natural receptor but can be bypassed by an antibody-dependent enhancement pathway.*Proc Natl Acad Sci.* 1;91(5):1932-1936

Mather T., Takeda, T., Tassello, J., Ohagen, A., Serebryanik, D., Kramer, E., Brown, F., Tesh, R., Alford, B., Chapman, J., and Lazo, A. (2003). West Nile virus in blood: stability, distribution, and susceptibility to PEN110 inactivation. *Transfussion*. 43: 1029-1037.

Mathur, A., Kulshreshtha, R., and Chaturvedi, U.C., (1989). Evidence for latency of Japanese encephalitis virus in T lymphocytes. *J. Gen. Virol.* 70, 461–465.

McMinn, P.C., Dalgrano, L., and Weir, R.C. (1996). A comparison of the spread of Murray Valley encephalitis viruses of high or low neuroninvasiveness in tissues of Suiss mice after peripheral inoculation. *Virology.* 220:414-423.

Mehlhop, E., Whitby, K., Oliphant, T., Marri, A., Engle, M., and Diamond, M.S. (2005). Complement activation is required for induction of a protective antibody response against West Nile virus infection. *J. Virol*. 79(12):7466-7477.

Mentor, N.A., and Kurane, I., (1997). Dengue virus infection of human T lymphocytes. *Acta Virol*. 41, 175–176.

Meyer, K.; Basu,A.; Przysiecki, C.T.; Lagging, L.M.; Di Bisceglie, A.M.; Conley, A.J.; and Ray, R. (2002) Complement-Mediated Enhancement of Antibody Function for Neutralization of Pseudotype Virus Containing Hepatitis C Virus E2 Chimeric Glycoprotein *J.Virol*. 76 (5): 2150-2158.

Momburg, F., Mulbacher, A., and Lobigs, M. (2001). Modulation of transporter associated with processing (TAP)-Mediated peptide import into the endoplasmic reticulum by Flavivirus infection. *J. Virol*. 75 (12): 5663-5671

Monath, T.P., and Heinz, F.X. (1996) Flaviviruses. *In* "Virology" (B.N. Fields ed), pp961-1034. Lippincott-Raven Philadelphia;

Monath, T.P. (2001). Prospects for developing of a vaccine against the West Nile Virus. *Annals New York Acad. Sci.* 951:1-12. Review.

Mongkolsapaya, J., Dejnirattisai, W., Xu, X., Vasanawatana, S., Tangthawornchaikul, N., Chairunsri, A., Sawasdivorn, S., Duangchinda, T., Dong, T., Rowland-Jones, S., Yenchitsomanus, P., McMichael, A., Malasit, P., and Screaton, G. (2003). Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. *Nat. Med*. 9 (7): 921-927.

Moreno-Altamirano, M.M.B. Sanchez-Garcia, F.J., and Munoz, M.L. (2002). Non Fc receptor-mediated infection human macrophages by dengue virus serotype 2. *J. Gen. Virol*. 83:1123-1130.

Moreno-Altamirano, M.M.B., Romano, M., Legorreta-Herrera, M., Sanchez-Garcia, F.J., and Colston, M.J. (2004). Gene expresión in human macrophages infected with dengue virus serotype-2. *Scad. J. Immunol*. 60:631-638.

Morens, D.M. (1994). Antibody-Dependent enhancement of infection and the pathogenesis of viral disease. *Clin. Infect. Dis.* 19:500-12.

Mukhopadhay, S., Kim, B.S., Chipman, P.R., Rossmann, M.G., and Kuhn, R.J. (2003). Structure of West Nile virus. *Science*. 302:248.

Mumcuoglu, K.Y., Banet-Noach, C., Malkinson, M., Shalom, U., and Galun, R. (2005) Argasid ticks as possible vectors of West Nile virus in Israel. *Vector Borne Zoonotic Dis*. 5(1):65-71

Munoz-Jordan, J., Sanchez-Burgos, G.G., Laurent-Rulle, M., Garcia-Sastre, A. (2003). Inhibition of interferon signaling by dengue virus. *Proc. Natl. Acad. Sci*. 100 (24): 14333-14338.

Murphy, F.A., Gibbs, E.P.J., Horzinek, M.C., and Studdert, M.J. (1999). Flaviviridae. In: Veterinary Virology. 3rd. Ed. pp. 555-569. Acad. Press..

Navarro-Sanchez, E., Altmeyer, R., Amara, A., Schwartz, O., Fieschi, F., Virelizier, J.L., Arenzana-Seisdedos, F., and Desprès, P. 2003 Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. EMBO Rep. 4(7):723-8.

Oliphant, T., Engle, M., Nybakken, G.E., Doane, C., Johson, S., Huang, L., Gorlatov, S., Mehlhop. E., Marri, A., Chung, K.M., Ebel, G.D., Kramer, L.D., Fremont, D.H., and Diamond, M.S. (2005). Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. *Nat. Med*. 11:522-530.

Ostlund, E.N., Andresen, J.E., and Andresen, M., (2000). West Nile encephalitis. *Vet. Clin. North Am. Equine Pract*. 16, 427–441.

Ostlund, E.N., Crom, R.L., Pedersen, D.D., Johnson, D.J., Williams, W.O., and Schmidt, B.J., 2001. Equine West Nile encephalitis, United States. *Emerg. Infect. Dis*. 7, 665–669.

Overbergh, L., Giuletti, A., Valckx, D., Decallonne, R., Bouillon, R., and Mathieu, C. (2003). The use of real-time reverse transcriptase PCR for the quantification of cytokine gene expression. *J Biomol Tech*. 14(1):33-43.

Palucka, A.K., (2000). Dengue virus and dendritic cells. *Nat. Med*. 6(7):748-9.

Pantelic, L., Sivakumaran, H., and Urosevic, N., (2005). Differential induction of antiviral effects against West Nile Virus in primary mouse macrophages derived from Flavivirus-susceptible and congenic resistant mice by alpha/beta interferon and Poly(I-C). *J. Virol*. 79, 1753–1764.

Parquet, M.C., Kumatori, A., Hasebe, F., Morita, K., and Igarashi, A. (2001). West Nile virus-induced bax-dependent apoptosis. *FEBS Letters* 500: 17-24.

Pastoret, P.P.; Bazin, PGH; Govaerts, A. Immunology in horses and donkeys. In Handbook of vertebrate immunology. Pp 347-349. Acad Press 1998.

Peiris, J.S.M., and Porterfield, J.S., (1979). Antibody-Dependent Enhancement of Flavivirus replication in macrophages-like cell lines. *Nature* 282, 509-511.

Peiris, J.S., Gordon, S., Unkeless, J.C., and Porterfield. J.S., (1981). Monoclonal anti-Fc receptor IgG blocks antibody enhancement of viral replication in macrophages. *Nature*. 289, 189–191.

Perelygin, A.A., Scherbik, S.S., Zhulin, I.B., Stockman, B.M., Li, Y., and Briton, M.A. (2002). Positional cloning of the murine flavivirus resistance gene. *Proc. NAtl. Acad. Sci.* 99:9322-9327.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nuc. Acid. Res.* 29(9):2002-2007.

Phillpotts, R.J., Jones, L.D., Lukaszewski, R.A, Lawrie, C., and Brooks, T.J.G. (2003). Antibody and Interleukin-12 reatment in murine models of encephalitogenic flavivirus (st Louis Encephalitis, Tick Borne encephalitis) and Alphavirus (Venezuelan Equine encephalitis) infection. *J. Interferon Cytok. Research.* 23:47-50.

Pogodina, V.V., Frolova, M.P., Malenko, G.V., Fokina, G.I., Koreshkova, G.V., Kiseleva, L.L., Bochkova, N.G., and Ralph, N.M. (1983). Study on West Nile virus persisitencein monkeys. *Arch. Virol*. 75:71-86.

Prat, A., Biernacki, K., Wosik, K., and Antel, J.P. (2001). Glial cell influence on the human blood brain barrier. *Glia* 36:145-155.

Prince, H.E., Tobler, L.H., Lape-Nixon, M., Foster, G.A., Stramer, S.L., and Busch, M.P. (2005) Development and persistence of West Nile virus-specific immunoglobulin M (IgM), IgA, and IgG in viremic blood donors. *J Clin Microbiol*. 43(9):4316-20.

Pryor, M.J., Carr, J.M., Hocking, H., Davidson, A.D., Li, P., and Wrigth, P.J., (2001). Replication of dengue virus in human monocyte-derived macrophages: comparisons of isolates and recombinant viruses with substitutions at amino acid 390 in the envelope glycoprotein. *Am. J. Trop. Med. Hyg*. 65, 427–434.

Ponnuraj, E.M., Springer, J., Hayward, A.R., Wilson, H., and Simoes, E.A. (2003). Antibody-dependent enhancement, a possible mechanism in augmented pulmonary disease of respiratory syncytial virus in the Bonnet monkey model. *J Infect Dis.* 187(8):1257-1263. Epub 2003 Apr 2.

Quirin, R., Salas, M., Zientara, S., Zeller, H., Labie, J., Murri, S., Lefrancois, T., Petitclerc, M., and Martinez, D. (2004). West Nile virus, Guadeloupe. *Emerg. Infect. Dis.* 10 (4): 710-718.

Raabe, M.R., Issel C.J., and Montelaro R.C., (1998). Equine monocyte-derived macrophage cultures and their applications for infectivity and neutralization studies of equine infectious anemia virus. *J. Virol. Methods* 71, 87–104.

Ramakrishna, C., Ravi, V., Desai, A. Subbakrishna, D.K., Shankar, S.K., amd Chandramuki, A. (2003). T helper responses to Japanese encephalitis virus infection are dependent on the route of inoculation and the strain of mouse used. *J. Gen. Virol.* 84:1559-1567.

Ransohoff, R.M., Kivisakk, P., and Kidd, G., (2003). Three or more routes for leukocyte migration into the central nervous system. *Nat. Rev. Immunol*. 3, 569–581.

Rothman, A.L. (2004). Dengue: defining protective versus pathologic immunity. *J. Clin. Invest*. 113(7):946-951.

Rothman, A.L., and Ennis, F.A. (1999). Immunopathogenesis of dengue hemorrhagic fever. *Virology*. 257:1-6.

Samuel, C.E.,(2001). Antiviral actions of interferons. *Clin. Microbiol. Rev.* 14, 778-809.

Samuel, M.A., and Diamond, M.S. (2005). Alpha/Beta Interferon protects against lethal West Nile virus infection by restricting cellular tropism and enhancing neuronal survival. *J. Virol.* 79:13350-13361.

Santoro, M.G., Rossi, A., and Amici, C. (2003). NF-kappaB and virus infection: who controls whom. *EMBO J.* 22**,** 2552-2560.

Sarafi, M.N., Garcia-Zepeda, E.A., McLean, J.A., Charo, I.F., and Luster, A. (1997). Murine monocyte chemoattractant protein (MCP)-5: A novel CC chemokine that is a structural and functional homologue of human MCP-1. *J. Exp. Med.* 185(1):99-109.

Scholle, F., and Mason, P.W. (2005). West Nile virus replication interferes with both poly(I:C)-induced interferon gene transcription and response to interferon treatment. *Virology*. 342:77-87.

Sharma, S., Marthur, A., Prakash, V., Kulshrestha, R., Kumar, R., Caturvedi, U.C., (1991). Japanese encephalitis virus latency in peripheral blood lymphocytes and recurrence of infection in children. *Clin. Exp. Immunol*. 85, 85–89.

Shen, J., Devery, J.M., and King, N.J., (1995). Adherence status regulates the primary cellular activation responses to the flavivirus West Nile. *Immunol*. 84, 254–264.

Shen, J., T-To, S.S., Schrieber, L., and King, N.J.C. (1997). Early E-selectin, VCAM-1, ICAM, 1, and late major histocompatibility complex antigen induction on human

endothelial cells by flavivirus and comodulation of adhesion molecule expression by immune cytokines. *J.Virol*. 71:9323-9332.

Shrestha, B., and Diamond, M.S. (2004a). Role of CD8+ T cells in control of West Nile virus infection. *J. Virol*. 78:8312-8321.

Shrestha, B., Gottlieb, D., amd Diamond, M. (2003). Infection and injury of neurons by West Nile encephalitis virus. *J.Virol.* 77(24):13203-13213.

Shresta, S., Kyle, J.L., Snider, H.M., Basavapatna, M., Beatty, R.P., and Harris, E. (2004b). Interferon-dependent immunity is essential for resisitance to primary dengue virus infection in mice, whereas T- and B-cell dependent immunity are less critical. *J. Virol*. 78:2701-2710.

Silvia, O.J.,Shellman, G.R., and Urosevic, N. (2001). Innate resistance to flavivirus infection in mice controlled by Flv is nitric oxide-independent. *J. Gen. Virol.* 82:603-607.

Smithburn, J.S., Hughes, T.P., Burke, A.W., and Paul, J.H. (1970). A neurotropic virus isolated from the blood of a native in Uganda. *Am. J. Trop. Med. Hyg*. 20: 471-492.

Solomon, T.; Ooi, M.H.; Beasley, D.W.C.; Mallewa, M.; (2003). West Nile encephalitis. Clinical review. *BMJ* 326: 865-869

Steele, KE, Linn, M.J., Schoepp, R.J., Komar, N., Geisbert, T.W., Manduca, R.M., Calle, P.P., Raphael, B.L., Clipinger, P.L., Larsen, T., Smith, J., Lanciotti, R.S., Panella, N. A., and McNamara, T.S. (2000). Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, NY. *Vet. Pathol*. 37:208-224

Subbramanian, R.A., Xu, J., Toma, E., Morisset, R., Cohen, E.A., Menezes, J., and Ahmad, A. (2002). Comparison of human immunodeficiency virus (HIV)-specific infection-enhancing and -inhibiting antibodies in AIDS patients.*J Clin Microbiol*. 40(6):2141-2146.

Suchetana Mukhopadhyay, Bong-Suk Kim, Paul R. Chipman, Michael G. Rossmann, Richard J. Kuhn. (2003) Structure of West Nile Virus. Science 10 Vol. 302.

Suhrbier, A and Linn M.L (2003). Suppression of antiviral responses by antibody-dependent enhancement of macrophage infection. *Trends in immunology* 24: 165-168.

Sydow, F.F., Santiago, M.A., Neves-Souza, P.C., Cerqueira, D.I., Gouevea, A.S., Lavatori, M.F.H., Bertho, A.L., and Kubelka, C.F., (2000). Comparison of dengue infection in human mononuclear leukocytes with mosquito C6/36 and mammalian vero cells using flow cytometry to detect virus antigen. *Mem. Inst. Oswaldo Cruz*. 95, 483–489.

Takada, A., Feldmann, H., Ksiazek, T.G., and Kawaoka, Y. (2003). Antibody-dependent enhancement of Ebola virus infection. *J Virol.* 77(13):7539-7544.

Takada, A., and Kawaoka, Y. (2003). Antibody-dependent enhancement of viral infection: molecular mechanisms and in vivo implications. *Rev Med Virol.* 13(6):387-98. Review

Takada, A., Watanabe, S., Okazaki, K., Kida, H., and Kawaoka, Y. (2001). Infectivity-enhancing antibodies to Ebola virus glycoprotein. *J Virol*. 75(5):2324-2330

Takai, T.(2002) Roles of Fc receptors in autoimmunity.*Nat Rev Immunol.* 2(8):580-92. Review.

Tassaneetrithep, B., Burgess, T.H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., Eller, M.A., Pattanapanyasat, K., Sarasombath, S., Birx, D.L., Steinman, R.M., Schlesinger, S., and Marovich, M.A. (2003). DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J.Exp. Med*. 197 (7): 823-829.

Tirado, S.M., Yoon, K.J. (2003). Antibody-dependent enhancement of virus infection and disease.*Viral Immunol.* 16(1):69-86. Review

Tizard I.R. Antibodies: soluble forms of BCR. In: Veterinary Immunology, an introduction. pp. 152-153. 7th Edition Saunders. 2004

Tizard, I.R. 2004. Cytokines and the immune system in: Veterinary Immunology. An introduction. pp. 133-144. 7th Edition .Saunders. 2004.

Vilcek, J. and Sen, G.C., (1996). Interferons and other cytokines. In: Fields, B.N., Knipe, M.D.M., Howley, P.M., Chanock R. M. (Eds.), Fields Virology, 3rd edn, Lippincott Williams & Wilkins, pp. 375-399.

Wallace, M.J., Smith, D. W, Broom, A. K., Mackenzie, J. S., Hall, R. A., Shellam, G. R, and McMinn, P. C. (2003). Antibody-dependent enhancement of Murray Valley encephalitis virus virulence in mice. *J. Gen. Virol*. 84: 1723–1728.

Wang, Y., Lobigs, M., Lee, E., and Müllbacher, A. (2003). CD8+ T cells mediate recovery and immunpathology in West Nile virus encephalitis. *J. Virol*. 77:13323-13334.

Wang, Y., Lobigs, M., Lee, E., and Müllbacher, A. (2004). Exocytosis and Fas mediated cytolytic mechanisms exert protection from West Nile virus induced encephalitis in mice. *Immunol. and Cell Biol*. 82:170-173.

Wang, T., Scully, E., Yin, Z., Kim, J.H., Wang, S., Yan, J., Mamula, M., Anderson, J.F., Craft, J., and Fikrig, E. (2003). IFN-γ-producing γδ T cells help control murine West Nile virus infection. *J. Immunol*. 171:2524-2431.

Wang, W.K., Sung, T.Z., Tsai, Y.C., Kao, C.L., Chang, S.M., and King, C.C., (2002). Detection of dengue virus replication in peripheral blood mononuclear cells from dengue virus type 2-infected patients by a reverse transcription real-time PCR assay. *J. Clin. Microbiol*. 40, 4472-4478.

Wang T., Town, T., Alexopoulou, L., Anderson, J.F., Fikrig, E., and Flavell, R.A., (2004). Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat. Med*. 10, 1366-1373.

Ward, M.P., Levy, M., Thacker, H.L., Ash, M., Norman, S.K., Moore, G.E., and Webb, P.W. (2004). Investigation of an outbreak of encephalomyelitis caused by West Nile virus in 136 horses. *J Am Vet Med Assoc*. 225(1):84-9.

Weingartl, H.M., Neufeld, J.L., Copps, J., and Marszal, P. (2004). Experimental West Nile virus infection in blue jays (Cyanocitta cristata) and crows (Corvus brachyrhynchos). *Vet Pathol*. 41(4):362-70

Wu, S.J., Grouard-Vogel, G., Sun, W., Mascola, J.R., Brachtel, E., Putvatana, R., Louder, M., Filgueira, L., Marovich, M.A., Wong, H.K., Blauvelt, A., Murphy, G.S., Robb, M.L., Innis, B.L., Birx, D.L., Hayes, C.G., and Frankel, S.S., (2000). Human skin Langerhans cells are targets of dengue virus infections. *Nat. Med*. 6, 816-820.

Xiao, S.Y., Guzman, H., Zhang, H., Travassos, da Rosa, A.P., and Tesh, R.B. (2001). West Nile virus infection in the golden hamster (*Mesocricetus auratus*): A model for West Nile encephalitis. *Emerg. Infect. Dis.* 7:714-721

Yamshchikov V.F, Wengler, G., Perelygin, A.A., Brinton, M.A., and Compans, R.W. 2001. An infectious clone of the West Nile flavivirus. *Virology*. 281:294-304.

Yang, J.S., Ramanathan, M.T., Muthumani, K., Choo, A.Y., Jin, S.H., Yu, Q.C., Hwang, D.S., Choo, D.K., Lee, M.D., Dang, K., Tang, W., Kim, J.J., and Weiner, D.B. (2002). Induction of inflammation by West Nile virus capsid through the caspase-9 apoptotic pathway. *Emerg. Infect. Dis*. 8(12): 1379-1384.

Yang, D. K.; Yeh, W.T.; Yang, M.Y.; Chen R.F. and Shaio, M.F. (2001). Antibody-Dependent enhancement of Heterotypic Dengue Infections Involved in Suppression of IFNγ. Production. *J. Med. Virol*. 63:150 – 157.

VITA

David Garcia-Tapia was born August 26th, 1971 in Cuautla, Morelos, Mexico to Maria del Pilar Tapia Carrillo and David Garcia Torres. After attending public Schools in Cuautla, Morelos, Mexico, he received the following degrees: DVM from Universidad Nacional Autonoma de Mexico-UNAM- (National University of Mexico) (1993); M.Sc. in immuno-parasitology from the Escuela Nacional de Ciencias Biologicas –ENCB-IPN- (National School of Biological Sciences-IPN) (1998); Ph.D. in Veterinary Pathobiology from the University of Missouri-Columbia (2006). He is presently a resident in Veterinary Pathology at University of Missouri-Columbia, Missouri, and he is married to Giseel Prada Sosa of Cuautla, Morelos, Mexico, currently they have three children: Giselle (9), Daniela Alejandra (4) and David Ammon (3).